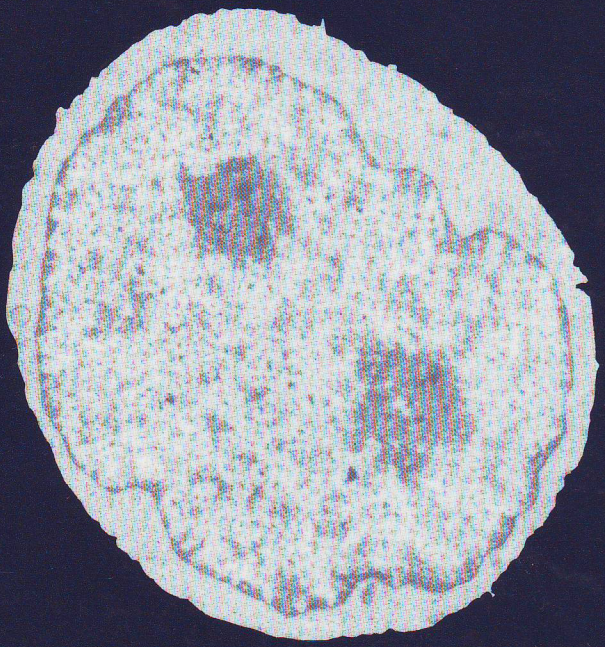
Autologous Marrow and Blood Transplantation

Proceedings of the Seventh International Symposium
Arlington, Texas



Edited by

Karel A. Dicke & Armand Keating

Autologous Marrow and Blood Transplantation

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Karel A. Dicke and Armand Keating

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AML	Alan K. Burnett
ALL	Daniel Weisdorf
CML	Angelo Carella
Lymphoma	Thierry Philip and Phil Bierman
Myeloma	Barthel Barlogie
Breast Cancer	Karen Antman
Solid Tumors	Roger Herzig
Peripheral Stem Cells	L. B. To
Radiolabeled Antibodies	Huibert Vriesendorp
New Avenues	Karel Dicke and Armand Keating

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PREFACE

During the ten years since the first Symposium in 1984, there has been considerable progress in the field of autologous stem cell transplantation. Results have been promising; this modality challenged established treatment and randomized studies are ongoing to determine the "truth". Such painstakingly laborious undertakings were already started several years ago in leukemia and lymphoma and are likely to be completed soon. In breast cancer, an explosion of activity to compare "new" against "old" has just started. In anticipation of a positive outcome, important steps to reduce costs of transplantation have been made and will definitely facilitate its use in a larger group of patients.

The format of this Symposium has contributed to communication among scientists from all over the world. Due to their efforts, as well as to the courage of our patients, this field will continue to develop and open many more frontiers of treatment which make the impossible of today possible in the near future. Such developments motivated us again to prepare proceedings, and was a stimulus to organize another symposium in 1996. The title of this Symposium will be, "Autologous Marrow and Blood Transplantation, the 8th International Symposium". Besides marrow, the use of peripheral blood as a source of stem cells has been established; therefore peripheral blood deserves to be incorporated into the title of this Symposium, as well as on the cover of the current Proceedings.

Karel A. Dicke
Armand Keating

ACKNOWLEDGEMENTS

This Symposium was generously supported by grants and contributions from the companies listed below. Without their support this meeting would not have been possible. Such educational and scientifically crucial events are instrumental for moving this field ahead and will be, therefore, of tremendous benefit for our patients whose lives are dependent on progress in medicine.

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The organizers sincerely thank Mrs. Kathy White and Mrs. Anita Arriaga. They were instrumental in the preparation of these Proceedings and also in the organization of the Symposium.

These Proceedings are dedicated to our friend and colleague,
Dr. Eva Lotzova, whose work and efforts established
the role of the NK cells in tumor control.

Eva Lotzova

1936 - 1994

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SESSION I: AML

BONE MARROW TRANSPLANTATION OR INTENSIVE CONSOLIDATION CHEMOTHERAPY IN ACUTE MYELOID LEUKEMIA. THE EORTC-LCG AND GIMEMA EXPERIENCE (1986-1993)

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INTRODUCTION

Most patients under the age of 50 years with acute myeloid leukemia in first remission undergo bone marrow transplantation or will receive several courses of intensive chemotherapy.[1] Both treatment modalities have been claimed to prolong remission and result in disease-free survival in about 25-50% of the patients.[2-7] The EORTC and GIMEMA Leukemia Cooperative Groups performed a randomized phase III study (AML-8A) to compare prospectively and according to intention to treat allogeneic bone marrow transplantation, autologous bone transplantation and short intensive chemotherapy during first complete remission of acute myeloid leukemia in a large number of patients and centers.[8] Since in this trial bone marrow transplantation resulted in a longer disease-free survival than intensive chemotherapy, subsequently they designed a study to be used as pilot study for a new randomized trial (AML-10). This AML-10 pilot study had the following aims: to extend the eligibility of patients until 60 years of age; to increase the remission percentage and to improve the feasibility to perform allogeneic or autologous marrow transplantation in **all** CR patients. Preliminary results showed indeed that these aims can well be reached and the randomized study was started in November 1993.

MATERIALS AND METHODS

All patients with previously untreated acute myeloid leukemia were eligible. In the AML-8A trial, most centers entered only the patients between 10 and 45 years. In a few centers, also patients up to 60 years

were included. In the AML-10 pilot study, ages from 10 to 60 years were allowed. Patients with AML secondary to other myeloproliferative diseases or myelodysplastic syndrome of more than 6 months duration were excluded. The median age of the patients was 33 years (range 11-59) for the AML-8A trial and 48 years (range 12-59) for the AML-10 pilot study.

Treatment protocol AML-8A: The induction treatment consisted of one or two courses of cytarabine (Ara-C) 200 mg/M^2 as continuous infusion on days 1-7 and daunorubicine 45 mg/M^2 IV on days 1, 2 and 3. All patients who achieved complete remission were scheduled to receive intensive consolidation with Ara-C 1000 mg/M^2 as 2-hour infusion every 12 hours on days 1-6 and m-AMSA 120 mg/M^2 IV on days 5, 6 and 7. After one year of study the dose of Ara-C was decreased to 500 mg/M^2 because of observed severe toxicity. Patients with a complete remission and an HLA-identical family donor were scheduled to undergo allogeneic bone marrow transplantation. The remaining patients were to be randomized to receive an autologous bone marrow transplantation or a second intensive consolidation course. Bone marrow transplantation was performed after a conditioning of cyclophosphamide (60 mg/kg per day on day 2 consecutive days), total body irradiation or busulfan (4 mg/kg/day on days -6 to -3), and cyclophosphamide (60 mg/kg/day on days -2 and -1). In case of allogeneic bone marrow transplantation graft versus host prophylaxis consisted of cyclosporine or cyclosporine and methotrexate. The second intensive consolidation course consisted of Ara-C 2000 mg/M^2 as 2-hour infusion every 12 hours on days 1-4 and daunorubicin 45 mg/M^2/day on days 5, 6 and 7.

Treatment protocol AML-10: Remission induction consisted of idarubicine 12 mg/M^2 (after the first 70 patients decreased to 10 mg/M^2) IV on days 1, 3, 5, cytarabine 100 mg/M^2 daily as continuous infusion for 10 days, and VP16-213 100 mg/M^2 daily on days 1 to 5. This regimen was followed by an intensive consolidation course consisting of cytarabine 500 mg/M^2 as a two-hour infusion twice daily for 5 days and mitoxantrone 12 mg/M^2 on days 4, 5 and 6. All patients were supposed to undergo a bone marrow transplantation: an allogeneic BMT for patients under the age of 50 in case an HLA-identical sibling was available, and an autologous BMT in all other patients. Conditioning regimen and post-BMT measures were similar as in the AML-8A trial.

Statistics. Duration of survival was calculated from the date of diagnosis until death. The disease-free survival was calculated from the

data of first complete remission until the date of first relapse, or the date of death in first complete remission. The duration of survival from complete remission corresponds to the time from first CR to the date of death. Actuarial curves were calculated according to the Kaplan-Meier technique, the differences between curves were tested for statistical significance using the two-tailed log-rank test.

RESULTS

Between November 1986 and April 1993, 941 patients were included in the AML-8A trial and 262 in the AML-10 pilot study. After one or two induction courses, complete remission was achieved in the AML-8A trial in 623 patients (66.2%) and in 211 patients (80.5%) in the AML-10 pilot study; 576 AML-8A patients and 192 AML-10 pilot patients received first consolidation; 168 AML-8A and 52 AML-10 pilot patients had an HLA-identical sibling and were planned to receive an allogeneic bone marrow transplantation but 22 and 7 patients, respectively, relapsed already before allogeneic BMT or refused BMT; 254 AML-8A patients were randomized to undergo an autologous bone marrow transplantation (128 patients), or receive a second intensive consolidation (126 patients). The reason that AML-8A patients without an HLA-identical donor were not randomized was mainly due to severe toxicity and patients refusal after the consolidation course. In the AML-10 pilot study, 136 patients were eligible for autologous BMT. Forty-seven of them could not undergo a BMT due to early relapse or inadequate bone marrow harvesting. Fifty-six randomized AML-8A patients did not receive the allocated arm mainly due to refusal, toxicity of previous chemotherapy, or early relapses. This means that 44% (416/941) of AML-8A patients and 51% (134/262) of the AML-10 pilot patients finally received one of the treatment options. The median time between the achievement of a complete remission and autologous bone marrow transplantation was 3 months for the bone marrow transplantation for AML-8A patients, compared to 5 months for AML-10 pilot patients. In the AML-8A trial, the distribution of relapses and of death in CR were the following: allo-BMT (41 and 29), autologous BMT (52 and 12) and second intensive consolidation (72 and 9).

The time to hematologic recovery was longer following autologous BMT than following allogeneic BMT or second consolidation.[8] In the AML-10 pilot study, the median time of neutrophil recovery (>0.5 x

10^9/L) after autologous BMT was 6 weeks, and of platelets (>50 x 10^9/L) was 15 weeks; similar figures were obtained in AML-8A study. Disease-free survival in the AML-8A trial for allogeneic bone marrow transplantation, autologous bone marrow transplantation, or second intensive consolidation was 50, 48 and 30% respectively, at 4 years (p=0.04). Probably due to achievement of a high number of second remissions in the second intensive consolidation group overall survival of the three groups was not different, approximately 50% at 4 years.[8] Follow-up in the AML-10 pilot study is still short. The disease-free survival at 18 months was 50%. Overall survival for the whole AML-8A group and in the AML-10 pilot study group was 38% at 4 years and 50% at 2 years, respectively.

DISCUSSION

The AML-8A trial, performed by the EORTC and GIMEMA Leukemia Cooperative Groups, shows that intensive consolidation therapy through bone marrow transplantation gives a better outlook with respect to DFS than through high-dose chemotherapy. Disadvantages of the trial are the limited age range, mainly patients between 15 and 45 years of age, and the relative low percentage of patients that finally received the appropriate treatment regimen. A new randomized trial needs to address these topics in addition to further improving the remission induction percentages and to further decreasing the relapse incidence after autologous bone marrow transplantation.

For this reason, the EORTC and GIMEMA Leukemia Cooperative Groups designed the AML-10 pilot study which included all patients between the ages of 15 and 60 years. A more intensive remission regimen was introduced and autologous BMT was to be given to all patients who did not meet the criteria for an allogeneic transplantation.

Compared to the AML-8A trial, age in the pilot study increased from a median of 33 to a median of 48 which is more comparable to the median age of the previous EORTC Leukemia Cooperative Group AML trials.

The percentage of patients that reached a complete remission was 75% after one course and 80% after two courses and this seems better than the percentages achieved in the AML-8A trial, also taken into account that the median age of the AML-10 pilot group was ± 15 years higher.

About 50% of all AML-10 pilot patients reached the designed treatment arm, i.e. bone marrow transplantation. In both trials, early relapses and treatment refusal played an important role in the final achievement of the designated treatment arm. Although the pattern of relapses does not seem to be altered among both trials, less refusals, possibly due to the absence of randomization, were seen in the AML-10 pilot patients.

Specific problems of the AML-10 pilot study are the delayed performance of the autologous BMT (5 months after CR) and the delayed platelet recovery after autologous BMT. These problems may be due to the intensity of the induction/consolidation regimen. Since the primary aims of the AML-10 pilot are fulfilled, the EORTC and GIMEMA Leukemia Cooperative Groups decided to start a prospectively randomized trial based on the AML-10 pilot study.

This trial which is eligible for all patients between 15 and 60 years of age includes a randomization at diagnosis comparing the tested intensive induction regimen with a similar regimen but where daunorubicin or mitoxantrone replaces idarubicin, a consolidation course containing intermediate dose Ara-C and the same anthracycline as in the induction course, followed by bone marrow transplantation.

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PROGRESS REPORT ON MRC AML 10

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For the MRC Leukemia Working Parties

The MRC AML-10 Trial has recruited 1820 patients and will close at the end of September, 1994. This trial compares ADE vs DAT induction (2 courses) followed by two intensification courses with MACE and MiDAC: Patients are randomized to receive an autoBMT as a 5th course versus no further treatment (STOP). All patients with sibling donors should receive allogeneic BMT as a 5th course. Patients with secondary leukemia can be admitted, and the age range is 0-55 years. Sixty-six percent of secondary cases entered CR (n=63) compared with an 81% remission rate overall. Children under 15 years had a CR rate of 91%.

The overall survival from diagnosis at 5 years is 39% with 50% of patients under 35 years alive at 5 years compared with 30% of 36-55 year olds. Survival from remission at 5 years is 58% in the <35 years and 38% in the 35-55 yrs. If BMT patients are censored at BMT, the remission durations at 5 years are 48% and 30% respectively in these two age cohorts.

Of 762 patients who entered remission and are evaluable, 296 had a matched donor and will be analyzed as having alloBMT (on an intention to treat basis) although only 174 have so far actually received the allograft.

Ninety-one patients relapsed or died in CR and therefore could not be randomized to the auto vs STOP option. Sixty-two chose autograft and 300 were not randomized because of patient or physician preference for STOP. Three hundred fifty-five have been randomized to autograft (n=178) or STOP (n=177), i.e., approximately 40% of patients who entered CR.

The overall survival from CR at 5 years of patients with a donor was 58% compared with 51% for those with no donor (p=0.7). The outcome of auto vs STOP is still coded but the risk of death in CR post BMT was 20% for allograft and 18% for autoBMT.

The overall outcome was dictated by two major risk factors: a) cytogenetics, b) % blasts in bone marrow after the first treatment course, no matter what treatment was given. Given the dominance of the biology

and response of the disease large numbers of patients will be needed to demonstrate a clear benefit of any of the post-CR treatment modalities.

Of 63 patients who relapsed in the STOP arm, 54% (n=34) achieved CR2 of whom 21 had an autograft. The survival from relapse in these patients is 18% at 2 years and the duration of second remission 19%. The strategy of reserving autograft for use upon relapse has therefore only been modestly effective.

COMPARISON OF INTENSIVE CONSOLIDATION CHEMOTHERAPY AND UNPURGED AUTOLOGOUS BONE MARROW TRANSPLANTATION AS POST REMISSION THERAPY IN ADULT ACUTE MYELOID LEUKEMIA

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INTRODUCTION

When this study was designed, the optimal consolidation therapy after remission achievement in acute myeloid leukemia (AML) was already controversial. Three different approaches were discussed: allogenic bone marrow transplantation (allo BMT), chemotherapy and, mainly in Europe, autologous bone marrow transplantation (ABMT). The indications of allo BMT were, at that time, rapidly increasing and the results were improving. Some investigators considered that allo BMT was the best option for patients under the age of 40 and with an HLA identical sibling. Others stated that allo BMT should be proposed only in second remission.[1,2] Conventional chemotherapy was still used but several pilot studies showed that a disease-free survival of 30 to 50% could be achieved after short-term intensive consolidation chemotherapy (ICC) without maintenance treatment.[3-7] ABMT after myeloablative treatment was another attractive approach. In pilot single center studies or in the annual survey of the European registry, disease-free survival rates round 50% were obtained.[8-10] However, in all these studies, the issue of patient selection was raised. Thus, randomized studies comparing ABMT and ICC were mandatory. In 1987, the French group GOELAM initiated such a study.

PATIENTS AND METHODS

Patients aged 15 to 50 years with de novo AML were included in a multicenter study involving 16 centers of the GOELAM group. Patients

with preexisting myelodysplastic syndromes or with blastic transformations of chronic myeloproliferative disorders were not included. Chemo/radio induced leukemias were also excluded. From November 1987 to April 1994, 470 patients were enrolled and 420 patients are currently evaluable.

Induction Treatment

For the remission induction treatment, patients were randomized between cytarabine (Ara-C) 200 mg/M^2/d (continuous infusion) for 7 days plus idarubicin (IDR) 8 mg/M^2/d IV for 5 days and Ara-C at the same dosage plus zorubicine (ZRB) 200 mg/M^2 for 4 days. A bone marrow aspiration was performed at day 17: if the marrow remained blastic (<50% blasts) a second course was administered with 3 days of Ara-C plus 2 days of either IDR 10 mg/M^2/d or ZRB 200 mg/M^2/d.

Post-Remission Treatment

Patients in complete remission (CR) were allografted if they were under the age of 40 years and had an HLA-identical sibling. The conditioning regimen and the graft-versus-host disease prophylaxis and treatment varied according to protocols used in the different transplant centers. Patients over 40 years or without a suitable donor received a first course of ICC (ICC1) Ara-C (3 g/M^2 in 3 hours infusion every 12 hours for eight doses, d1 to d4) and either IDR 10 mg/M^2/d [d5-6] or ZRB 200 mg/M^2/d [d5-6]. After hematopoietic recovery, marrow was collected. No *in vitro* manipulation was done. Patients were then randomized between a second course of ICC (ICC2) and ABMT. The second course was m AMSA 150 mg/M^2/d [d1 to d5] and VP-16 100 mg/M^2/d [d1 to d5]. The preparative regimen for ABMT was the Baltimore protocol (busulfan 4 mg/kg/d for 4 days and cyclophosphamide 50 mg/kg/d for 4 days).

RESULTS

Induction Treatment

The median age of the 420 evaluable patients was 35 years. There was no significant difference between the IDR arm (216 patients) and the ZRB arm (204 patients) regarding the following initial characteristics: sex, age, performance status, fever, white blood cell count, hemoglobin level, platelet count, FAB classification, incidence of karyotypic abnormalities.

Of the 420 patients, 326 (77.5%) achieved CR with no significant difference between the IDR arm (77%) and the ZRB arm (78%). CR was achieved in one course in 95% of the cases. There were 8 early deaths, 19 deaths in aplasia and 67 (16%) failures, with no significant difference between the two groups.

Of the 326 patients in CR, 70 were planned to undergo an allo BMT and 59 have actually been transplanted 40 to 220 days (median 64) after CR achievement. Sixty-five patients who should have received ICC1 were excluded for a variety of reasons (protocol violation, infection and other visceral complication after remission induction, refusal, relapse, data not yet available). Thus, 191 patients are evaluable for ICC1. The median time between CR achievement and ICC1 was 20 days (1-112). The median duration of neutropenia ($<0.5.10^9$/L neutrophils) after ICC1 was 18 days and 5 toxic deaths were recorded.

Second Intensification

Of the 186 patients still in CR after ICC1, only 171 have been randomized (87 ABMT, 84 ICC2). However, on the basis of the summary reports received so far, only 115 patients actually underwent the randomized treatment (58 ABMT, 57 ICC2).

Survival

The median follow-up time is 44 months. By July 1994, there were 16 relapses and 14 procedure-related deaths in the allo BMT group, 79 relapses and 10 toxic deaths in the group of 191 patients receiving ICC1. The actuarial risk of relapse at 5 years is 32% after allo BMT and 50% after ICC1. The 5-year event-free survival (EFS) (Fig 1) and 5-year overall survival (OS) are respectively 36% and 56% after BMT, 46% and 52% after ICC1. The comparison between allo BMT and ICC1 is statistically unvalid since all allo BMT patients did not receive the same post-remission therapy and the same conditioning regimen. However, the EFS and OS curves appear to be identical, even when considering the results according to the intention to trial. There is no significant difference between ABMT and ICC2. For the patients in CR who actually received the randomized consolidation, the actuarial risk of relapse at 5 years is the same (43%), the 5 years EFS and OS are respectively 52% and 56% for ABMT, 56% and 59% for ICC2 (Fig 2).

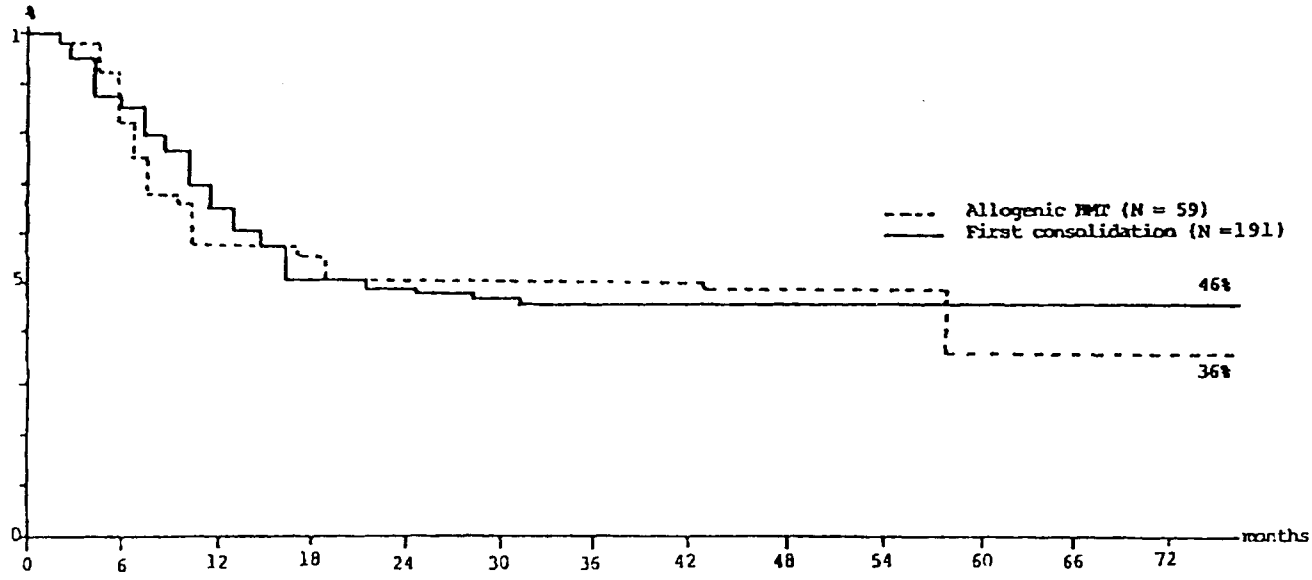

Figure 1. *EVENT FREE SURVIVAL (patients who actually received the planned treatment)*

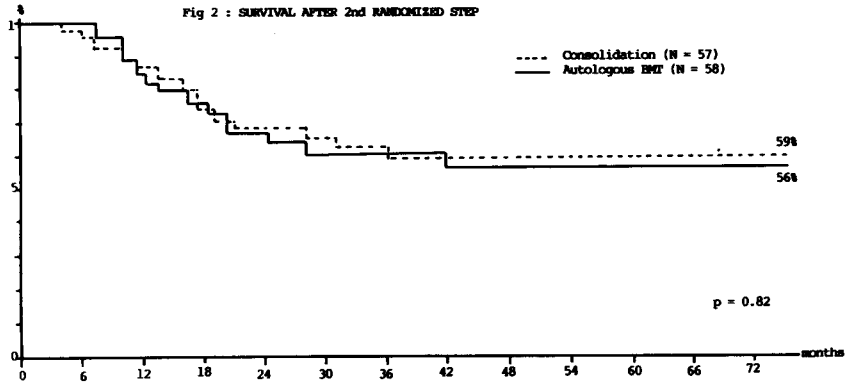

Figure 2.

DISCUSSION

This trial is presently closed but the final analysis has not yet been done. Definitive conclusions cannot be drawn. However, several points have to be emphasized.

1. The CR rate is high (77.5%) especially when considering that it is a multicenter study and that all patients having received the first day of treatment have been evaluated. This confirms the results we previously achieved with ZRB[7] and the good antileukemic activity of IDR recently shown by other groups.[11-12] However, only 5% of the CR were achieved with the second course of induction treatment and there were 16% failures. Strategies to increase the antileukemic activity of the remission treatment and of the salvage regimen in case of primary failure are still needed.

2. Regarding the post-remission therapy, we confirm the results of the previous analysis showing no significant difference between ICC2 and unpurged ABMT in terms of relapse rate, EFS, OS. To demonstrate a hypothetical advantage of one arm would require a very large cohort of patients. It should be noted that some patients who could not undergo ABMT because of poor or slow hematopoietic recovery after ICC1 were treated off protocol with ICC2 and they share the same prognosis as randomized patients.

Thus after ICC1, ICC2 appears to be as effective as ABMT and can be offered to a larger number of patients. These results differ from those of the EORTC study in which ABMT appeared to be superior to chemotherapy in terms of EFS.[13]

This discrepancy does not appear to be due to inferior results of ABMT in our group but actually to better results of ICC2. We have no explanation for this apparent superiority of our ICC regimen, since the designs of the two protocols were very similar and since in both studies, the interval between each phase of the treatment had to be the shortest possible. We can only underline several differences: the dose of m AMSA is much higher in the GOELAM protocol (600 mg/M^2 total dose versus 360 mg/M^2), VP-16 is used in the GOELAM protocol not in the EORTC protocol, the EORTC group use daunorubicin for induction and ICC whereas the GOELAM group use IDR or ZRB.

As in other studies on intensive post-remission therapy, the issue of tolerability must be raised. Only 53% of the patients in CR could complete the whole protocol treatment (allo BMT, ICC or ABMT). New strategies are needed to reduce the toxicity of these intensive regimens. The use of hematopoietic growth factors (G-CSF or GM-CSF) could be of value in this regard, since it appears that after intensive chemotherapy the risk of leukemic blast stimulation is reduced.

3. In our experience, allo BMT does not appear to give better results than ICC or ABMT. These results with allo BMT are inferior to those currently achieved for AML in first CR by other groups. This is due to a relatively high risk of relapse (32% at 5 years). Thus, new strategies are discussed in order to decrease the relapse rate after allo BMT (ICC prior to allo BMT or more intensive conditioning regimen).

CONCLUSION

These results confirm that a significant improvement of survival can be obtained in AML for patients up to 50 years of age, thanks to different modalities of intensive therapy. However, this study fails to demonstrate the superiority of any of the three approaches tested. In our next study, ICC will be considered as the reference arm and ABMT will be no longer used as consolidation of first remission.

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AUTOLOGOUS STEM CELL TRANSPLANTATION FOR ACUTE MYELOGENOUS LEUKEMIA IN ADULTS: THE BGMT EXPERIENCE

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INTRODUCTION

In adult patients with "de novo" acute myeloid leukemia (AML) under the age of 50-60 years who achieve a complete remission, controversy still persists regarding the choice of optimal subsequent treatment to prevent leukemia relapse and prolong survival.[1] Three different approaches are available: allogeneic bone marrow transplantation (AlloBMT) which is only suitable for young patients with an HLA-identical sibling donor; autologous marrow (purged or unpurged) marrow transplantation (AutoBMT); intensification with chemotherapy (high dose ara-C in most cases) eventually followed by maintenance chemotherapy. The decision to use chemotherapy or transplants is very difficult because the results of these different therapeutic strategies overlap.

To compare AlloBMT, AutoBMT and chemotherapy, prospective studies have been conducted over the last decade. They generally indicate that AlloBMT is superior to chemotherapy in preventing relapse and to a lesser extent in increasing the proportion of disease-free patients alive at 3-5 years.[2] The value of AutoBMT is more difficult to appreciate as very few prospective studies have compared either AlloBMT and AutoBMT or AutoBMT and chemotherapy.

In 1984, we started a prospective cooperative study (BGM 84 study) comparing AlloBMT, AutoBMT and intensive chemotherapy. We found that the results of AlloBMT were significantly better than those in the group of patients treated with AutoBMT or chemotherapy.[3] In 1987, a

new protocol (BGMT 87 study) was designed: 1) to compare AlloBMT with other therapeutic approaches (AutoBMT + chemotherapy); 2) to compare autologous stem cell transplantation (ASCT) and chemotherapy; 3) to compare ASCT using either unpurged bone marrow or peripheral blood stem cells.[3] Finally, in 1991 (ongoing BGMT 91 study) the study was designed to compare AlloBMT and AutoBMT. This report summarizes the results of these three protocols.

PATIENTS AND METHODS

Eligible patients had to have a diagnosis of AML according to the FAB classification. Patients with a prior history of myelodysplasia or preleukemia were excluded, but patients with previous neoplasia were included when there was no evidence of preleukemia or myelodysplasia before diagnosis of AML. All patients were more than 15 years old; the upper limit of age was 50 years (BGM 84 study) or 55 years (BGMT 87 and 91 studies).

STUDY DESIGN

In the three consecutive protocols, the induction and consolidation chemotherapy regimens were similar. The patients received ara-C (100 mg/M^2/day, continuous infusion) over a 10-day period and DNR (60 mg/M^2/day, bolus for three days). The patients who failed to enter complete remission (CR) after one course of chemotherapy were given a second course of induction chemotherapy identical to the first. When CR was achieved (after one or two courses of induction treatment) the patients received consolidation therapy 28-35 days after completion of induction treatment, consisting of ara-C (50 mg/M^2/12.h, subcutaneously, 7 days) and DNR (60 mg/M^2/day, intravenous bolus, 2 days). One month after consolidation (about 60-70 days after diagnosis), the patients who were still in CR were designated to receive either AlloBMT or to be given another treatment (chemotherapy or ASCT).

The patients were designated to receive AlloBMT when they fulfilled the following criteria: age $\leq$45 years; presence of an HLA-identical sibling donor with unreactive mixed lymphocyte reaction.

All other patients still in CR but not fulfilling the eligibility criteria for AlloBMT received different treatments.

BGM 84 Study

The patients were randomized to receive either four monthly courses of intensive chemotherapy (VP 16 + AMSA/ara-C alone/ara-C + DNR/POMP regimen) or AutoBMT. AutoBMT consisted of a double transplant of unpurged marrow with Melphalan (HDM = 140 mg/M^2) given intravenously before every transplant.[3]

BGM 87 Study

The patients received an intensification (high dose ara-C: 3g/M^2/12.h, 8 doses and DNR: 45 mg/M^2/day, 3 days), then they were randomly designated to receive either autologous stem cell transplantation (ASCT) or maintenance chemotherapy. The patients who were randomized for ASCT underwent either a bone marrow harvest (in Grenoble, Marseille and Toulouse), or peripheral blood stem cell collection (PBSC) (Bordeaux), then they were subsequently given a conditioning regimen consisting of Busulfan (4 mg/kg/day, 4 days) and Melphalan (140 mg/M^2) before the reinfusion of bone marrow or PBSC. No further treatment was administered after ASCT.[4] The patients who were randomly assigned to receive maintenance chemotherapy after intensification treatment were given five cycles of Ara-C (50 mg/M^2/12.h subcutaneously 5 days) and DNR (1 mg/kg/day, one day) which were adminstered 1, 3, 6, 9 and 12 months after intensification. Between these five courses of chemotherapy and after the fifth cycle, the patients received a continuous treatment consisting of altered courses of 6-mercaptopurine and methotrexate for a total period of two years.

BGMT 91 Study

In this protocol, all the patients not eligible for AlloBMT received an intensification with lower doses of Ara-C (500 mg/M^2/12.h) and DNR as in the BGMT 87 study. Then, they received the cyclophosphamide (120 mg/kg) + total body irradiation (12 Gy) regimen before the reinfusion of unpurged marrow collected before intensification. Some patients received interleukin-2 after AutoBMT.

Treatment cohorts were compared by t-test and Chi-square test. Survival curves were calculated by the method of Kaplan and Meier and the log rank test was used for comparison of these curves. Remission duration (disease-free survival, [DFS]) was calculated from the date of CR to the date of relapse or death.

RESULTS

In the description of results, we will focus on the results of ASCT in comparison with those of either AlloBMT or chemotherapy.

BGM 84 Study

The results were recently updated (Table 1). The actual number of relapses was significantly higher after chemotherapy than after either ASCT or AlloBMT. The actuarial DFS curves are shown on Figure 1. Due to the low number of patients included in this study, the difference was statistically significant only when AlloBMT and chemotherapy were compared (p=0.003). AutoBMT produced intermediate results (Figure 1). The actuarial risk of relapse was significantly lower after AlloBMT or AutoBMT than after ISC ($p<0.001$ and $p<0.05$).

Table 1. Actual Results

		AlloBMT	ASCT	Chemo
BGM 84	CCR*	11	5	2
	Deaths	5	1	1
	Relapses	4	6	17
BGMT 87	CCR*	23	16	--
	Deaths	5	2	15
	Relapses	5	15	23
BGMT 91	CCR*	16	40	--
	Deaths	10	2	--
	Relapses	1	22	--

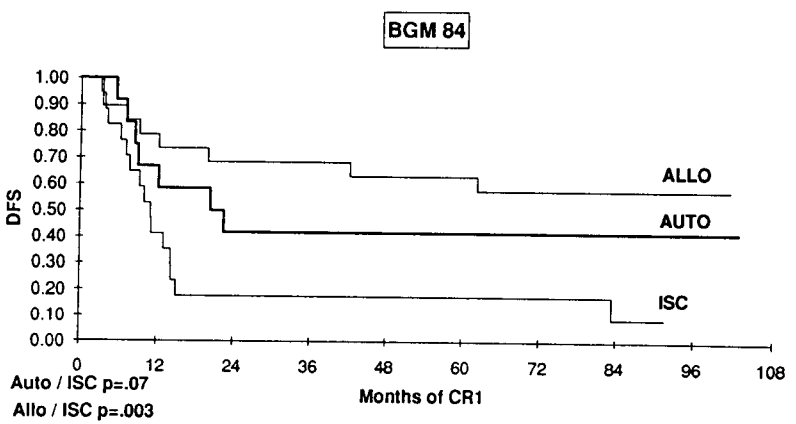

Figure 1. *Disease-free survival according to the type of post-induction treatment (BGM 84 study)*

BGMT 87 Study

Of the 99 patients who achieved CR and could not respectively undergo AlloBMT, 77 patients were randomized for either maintenance chemotherapy (n=38) or ASCT (n=39). Of the 77 patients, 42 patients relapsed 5 to 50 months after CR (median = 10.8 months); two patients died early after ASCT from transplant-related complications; 33 patients are still alive in CR 28 to 81 months after CR (median = 56 months). Of the 39 patients allocated to ASCT, 33 patients did undergo ASCT using either bone marrow (n=16) or blood stem cells (n=17) and six did not because of relapse (n=3), refusal (n=2), or poor hematopoietic progenitor content in the transplant product (n=1). As shown on Table 1, the number of patients who survived without relapse was not statistically different after ASCT and maintenance chemotherapy. The actuarial DFS curves (shown on Figure 2) of ASCT and chemotherapy did not differ significantly.

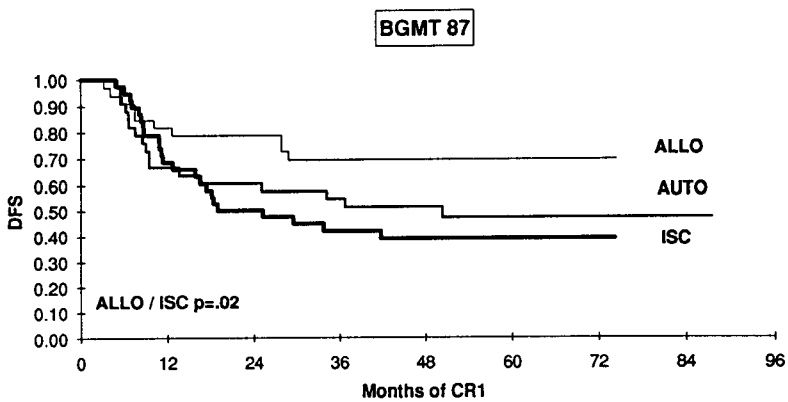

Figure 2. *Disease-free survival according to the type of post-induction treatment (BGMT 87 study)*

BGMT 91 Study

This study is still ongoing. By April 1, 1994, 64 patients were treated with AutoBMT. There were two transplant-related deaths, 22 patients had a relapse and finally 40 patients are still alive in complete remission (Table 1). That contrasts with that encountered after AlloBMT: of the 27 patients transplanted, there were: one relapse, 10 transplant-

related deaths and 16 patients alive in CCR. The actuarial proportion of patients who survived without recurrent disease at 2 years was 45.1 ±12.6% (95% CI) and did not differ significantly from that found for AlloBMT (58.1±18.8%; 95% CI) (p=NS) (Figure 3).

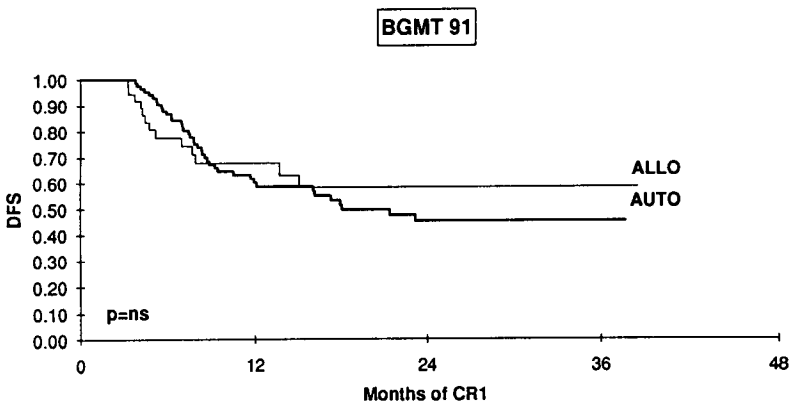

Figure 3. *Disease-free survival according to the type of post-induction treatment (BGMT 91 study)*

Meta-analysis. For the comparison of ASCT with either AlloBMT or chemotherapy, we performed a meta-analysis of the results of the three protocols. The comparison of ASCT with AlloBMT was possible in the three protocols, but ASCT could be compared with chemotherapy only in the BGM 84 and BGMT 87 studies. As shown on Figures 4 and 5, the 5-year DFS after ASCT was significantly lower than after AlloBMT (41.9±10.8% versus 58.3±12.6%; p = 0.04) and did not differ significantly from that found after chemotherapy (45.8±15.2% versus 32.3±12.7; p=0.14). The actual results also showed a higher proportion of long-term survivors after AlloBMT than after either ASCT or chemotherapy (Table 1).

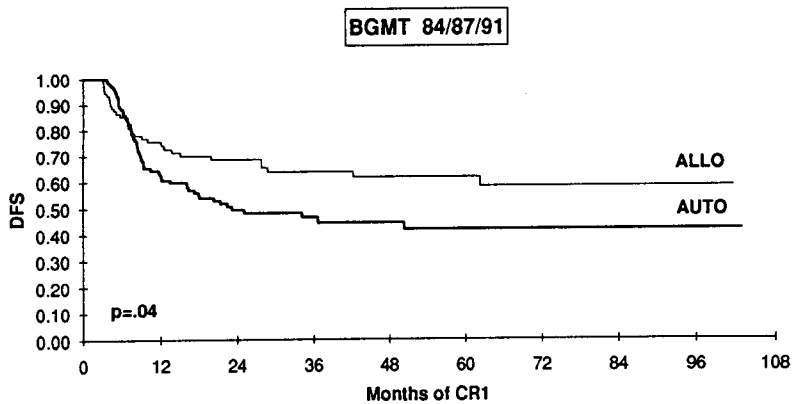

Figure 4. *Disease-free survival of patients undergoing AlloBMT or ASCT (meta-analysis of BGM 84, BGMT 87 and BGMT 91 studies)*

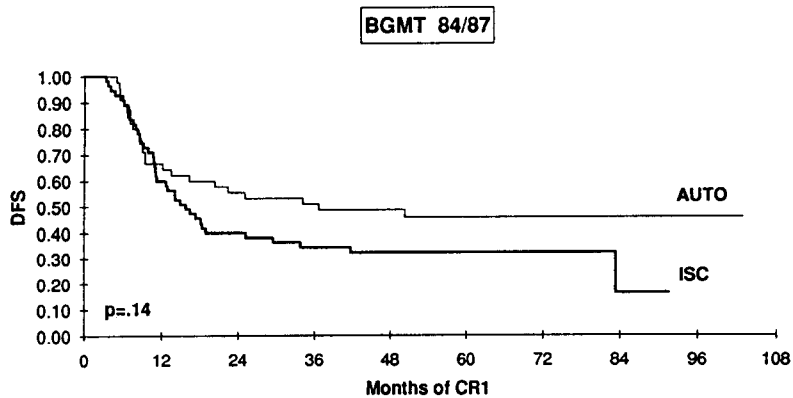

Figure 5. *Disease-free survival of patients undergoing ASCT or chemotherapy (meta-analysis of BGM 84 and BGMT 87 studies)*

DISCUSSION

The results of ASCT that we report in these consecutive studies did not differ significantly for those published elsewhere.[5] We did not find any significant difference in the DFS of patients treated with either ASCT or chemotherapy. Similar results have been found in some studies but were not confirmed in other prospective studies showing a significant advantage of ASCT over chemotherapy.[5,6] Thus, this question remains

controversial and further prospective studies are needed to better assess the place of ASCT in the treatment of adult AML. Prospective studies are also needed to explore the role of bone marrow purging.

Very few prospective studies have compared AlloBMT and ASCT. Our results suggest that AlloBMT is superior to ASCT to increase the proportion of long-term survivors. That was confirmed in other prospective studies.[7] Moreover, AlloBMT seems to be more cost-effective than ASCT in AML patients.[8] Thus, we feel that ASCT could be discussed when AlloBMT is not feasible.

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AUTOLOGOUS BONE MARROW TRANSPLANTATION FOR ACUTE LEUKEMIA. THE STATUS IN 1994

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INTRODUCTION

In the past 5 years, a considerable amount of information has become available in the field of autologous bone marrow transplantation (ABMT) for acute leukemia (AL). ABMT has been shown to be associated with a high rate of leukemia-free survival (LFS) in patients with acute myelocytic leukemia (AML) both in first (CR) and second (CR2) complete remission. Despite the absence of randomized studies, the amount of data in favor of marrow purging with cyclophosphamide derivatives (4 hydroperoxycyclophosphamide and mafosfamide) has been such that we, in Paris, now consider the infusion of unpurged marrow in AML, apart from a randomized study, as unethical. Results of ABMT in ALL presently do not reach the level achieved in AML and reports from various institutions remain controversial. Arguments in favor of purging in ALL do exist but they are less convincing than in AML. The suggestion however is that, while purging may be just as needed in ALL as in AML, present purging tools are not effective enough in ALL and tumor resistance in the body to the pretransplant regimen (which leads to a persisting high rate of relapse) precludes an accurate evaluation of its role, and possibly masks any potential efficacy.

Meanwhile, reports from the EBMT registry and results of the EORTC AML-8 study have given indications of the comparative roles of allogeneic BMT, ABMT and conventional chemotherapy (CT). Globally, allogeneic BMT, which remains restricted to patients with an HLA-identical sibling donor, is associated with a lower relapse rate, a higher transplant related mortality and a higher LFS than ABMT. Both allogeneic BMT and ABMT give superior LFS as compared to CT in AML. This information is summarized below.

Overall Review of ABMT in Acute Leukemia

We reviewed information published on ABMT in AL. Tables 1, 2, 3, and 4 summarize the LFS reported in AML CR1 with purged marrow (Table 1), with unpurged marrow for adults (Table 2) and children (Table 3) and the LFS reported in AML CR2 (Table 4). Table 5 lists the arguments in favor of marrow purging for AML and Table 6 details the role of mafosfamide when used *in vitro*. This role goes far beyond a mere tumor cytotoxic effect. Table 7 is a compendium of the results of ABMT in both AML and ALL, as estimated from individual centers and analyzed on the EBMT data base. Table 8 lists some data suggesting that purging may be effective, although not established in ALL.

A Ten-Year Single Institution Experience of ABMT with Marrow Purged by Mafosfamide. Report of the Paris St. Antoine Team

We analyzed the data from 125 consecutive AL adult patients we autografted with a marrow purged by mafosfamide (ASTA Z) during the period of January, 1983 to January, 1993. The median follow-up was 64 months (range: 3-126). There were 84 AML and 41 ALL. At time of ABMT, 64 AML were in CR1 and 20 in CR2; 35 ALL were in CR1 and 6 in CR2. The median age of the patients was 33 years (16-55). The median interval between achieving CR and autografting was 5 months (1.3-23). The pretransplant regimen consisted of cyclophosphamide (120 mg/kg) and total body irradiation (TBI). All patients were grafted with autologous marrow treated *in vitro* with mafosfamide used at levels individually adjusted (AD) in 95 patients and at a standard dose (SD) in 30 patients. The initial richness in granulomacrophagic progenitors (CFU-GM1) of the harvested marrows was 5.16 x 10^4 CFU-GM/kg (0.55-33). Following mafosfamide purging the residual CFU-GM number (CFU-GM2) was 0.021 x 10^4/kg (0-1.78). Thirty-three patients were grafted with marrows containing no detectable residual CFU-GM.

RESULTS

Outcome

For AML: leukemia-free survival (LFS): 58±7%, relapse incidence (RI): 25±6% at 8 years, in CR1. LFS: 34±11%, RI 48±12% in CR2 at 5 years. Transplant related mortality (TRM): 25±6%.

For ALL: LFS: 57±8%, RI: 37±8% and TRM: 10±5% at 8 years.

There was no statistical difference when comparing the survival curves for AML and ALL (LFS: p=0.63, RI: p=0.72, TRM: p=0.19). However, the incidence of late relapses (i.e. >1 year posttransplant) was significantly higher for ALL: 24±8% vs 4±3% (p=0.004).

Toxicity

Twenty patients died from toxicity: 16 out of 84 AML and 4 out of 41 ALL. Patients receiving marrow containing no detectable CFU-GM behaved as the global population.

Engraftment. the median days of engraftment were: for neutrophils (PN >0.5 x 10^9/l): 30 days (D) (12-153) for AML and 20 D (13-136) for ALL (p=0.0004); for platelets (plt >50x10^9/l): 90 D (19-850) for AML and 37 D (15-120) for ALL (p=0.003). The probability of engraftment was significantly higher in ALL ($p<10^{-3}$) for both neutrophils and platelets.

By multivariate analysis, 4 factors were found to favorably influence engraftment, in addition to a diagnosis of ALL: a younger age, ABMT performed in CR1, the AD technique of purging and a shorter interval from CR to ABMT (Table 9).

Figures 1 and 2 compare the kinetics of engraftment for neutrophils (Fig. 1) and platelets (Fig. 2), according to whether the marrow was purged with mafosfamide at doses individually adjusted or at a constant dose. The analysis shows that patients engrafted with marrow purged at the constant dose have a considerably higher probability to engraft more slowly than those receiving marrow purged at a variable dose. This however did not translate into a better outcome in terms of better LFS and lower TRM, probably due to good patient care. However, the slow kinetics of engraftment of AML patients is a matter of concern since it has direct implications on the total duration of hospitalization and the overall cost of the procedure. We consider that it is of considerable practical clinical interest to try to identify prognostic factors for rapid engraftment. In this respect, the present study favors younger patients, ABMT in CR1 and purging at levels individually adjusted. AML patients with these 3 characteristics would have an RR of 3.3 for quicker engraftment on PN and 5 for quicker engraftment on platelets. Two factors were correlated with a better outcome:

1. The LFS was higher (p=0.04) and the TRM was lower (p=0.003) in patients who received richer marrow (Fig. 3).

2. The RI was lower in patients autografted within 150 days from CR ($p=0.03$).

Our experience initiated in January, 1983, takes advantage of being one with the longest existing follow-up. We conclude first on clinical grounds that ABMT with marrow purged by mafosfamide indeed is feasible for both AML and ALL in a large fraction of patients and is a therapeutic approach associated with a high LFS. The results of multivariate analyses has led us to collect as much marrow as feasible, and do ABMT preferentially in CR1. Research programs in our institution are presently aiming at speeding up engraftment in AML either with the use of agents protecting normal stem cells *in vitro* during purging or with cytokines administered *in vivo* posttransplant (Table 10).

Comparative Evaluation of Allogeneic and Autologous BMT: EBMT Analyses

The EBMT has recently analyzed retrospectively the data from 1696 patients with AML transplanted in Europe from 1987 to 1992. One thousand one hundred fourteen adult patients were transplanted in CR1; 598 received an autograft and 516 an allograft. The TRM was significantly higher following allo-BMT (27% vs 13%, $p<10^{-4}$); the LFS was higher following ABMT (52% vs 42%, $p=0.006$). Two hundred eighty-eight adult patients were transplanted in CR2. In these patients, the LFS was 39% and 30% respectively for allo-BMT and ABMT, but this difference was not statistically significant. Finally, when comparing 113 children autografted to 129 allografted in CR1, the LFS was better following allogeneic BMT: 68% vs 47% ($p=0.002$). Results are detailed in Tables 11 (adult AML CR1), 12 (adult AML CR2) and 13 (childhood AML CR1).

The EBMT database for the same analyses in ALL consisted of 1336 patients: 850 adult and 486 children. The group of adult patients contained 326 allografts in CR1 vs 300 ABMT, 108 allografts in CR2 vs 116 ABMT. Seventy-nine children were allografted and 52 autografted in CR1, 174 were allografted and 181 autografted in CR2. The analyses focused on those who received TBI as a pretransplant regimen since the use of TBI in ALL was consistently associated with better outcome. Results are shown in Tables 14 (adult ALL CR1), 15 (adult ALL CR2) and 16 (childhood ALL CR2). In adults, the TRM was significantly lower after ABMT in CR1 and CR2 and the relapse incidence significantly higher after ABMT in CR1. The LFS was not significantly different after

ABMT or allogeneic BMT although the trend was in favor of allogeneic BMT in CR1 (47±3% vs 40±4%, p=0.06). In children in CR2, the TRM was small following either ABMT or allogeneic BMT; the relapse incidence was significantly higher after ABMT (60±5% vs 32±5%,$p<10^{-4}$) and as a consequence, the LFS was significantly higher after allogeneic BMT (54±4% vs 36±4%, p=0.003).

Finally, we investigated the occurrence of late events (beyond 2 years) in patients with acute leukemia who received an allogeneic BMT (n=1059) or an ABMT (n=656) in Europe during the period from January 1979 to December 1990. Patients with no recurrence of leukemia at 2 years had an overall 82% chance of being alive in complete remission at 9 years following transplantation regardless of the nature of the leukemia, the status at transplant and the type of transplant. The incidence of late relapses continuously decreased with time. The latest relapses in AML were observed following BMT at 6.6 years in a patient transplanted in CR1 and at 3.7 years in a patient transplanted in CR2, and following ABMT at 6 years and 5.1 years respectively. The latest relapses in ALL were observed following BMT at 4 years in a patient transplanted in CR1 and 6.8 years in a patient transplanted in CR2, and following ABMT at 5.3 years and 4.5 years respectively.

Several factors predictive for late relapses or deaths were identified. Patients allografted experienced a lower frequency of late relapses than patients autografted. Consequently, LFS and survival were better after allogeneic BMT for ALL, but a similar effect was not detectable in AML due to a parallel increase in the procedure related mortality. Of the numberous other prognostic factors studied, the female sex in AML, the use of TBI in ALL and the status in CR1 rather than CR2-3 for both ALL and AML allografted were correlated with a lower relapse incidence. The use of TBI in ALL was also associated with a better LFS and survival. The absence of acute GVHD in allografted AML correlated with a better LFS and a better survival, but had no influence on the relapse incidence. This study indicates that patients alive and well at 2 years posttransplant have a very high probability of being cured, but the possibility of a late relapse still remains. Fig. 4 (AML) and Fig. 5 (ALL) indicate the LFS and relapse incidence in patients autografted who are alive and well at 2 years posttransplant.

CONCLUSION

ABMT now is part of the routine strategy for treatment of acute leukemias, however, several problems remain: the need for better purging, essentially in ALL, and the need for an improved engraftment, essentially in AML. Marrow protection against mafosfamide such as provided by etiofos is one direction of research we follow. The use of cytokines post-ABMT, including long-term G-CSF or GM-CSF in an effort to boost the normal myeloid compartment and possibly depress the leukemic clone is one other direction of interest, especially in ALL. Other ways to better control minimal residual disease consist of the use of interleukin-2 or linomide posttransplant. Interesting results have been obtained with maintenance chemotherapy post-ABMT for ALL CR1.

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Table 1. Results of ABMT with Purged Marrow in AML CR1

Group	No. of Patients	Pretransplant Regimen	LFS %
St. Antoine, Paris	64	CY-TBI	58
Heidelberg	35	CY-TBI	58
Parma	20	CY-TBI	69
Manchester	30	CY-TBI	78
Atlanta	27	various	63
Baltimore	48	BU-CY	38
ECOG	39	BU-CY	54
Stanford	34	BU-E	57
San Francisco	35	BU-E	79
TOTAL/MEDIAN	332		~58
	NO LATE RELAPSE		

Table 2. Results of ABMT with Unpurged Marrow in Adult AML CR1

Group	No. of Patients	Pretransplant Regimen	LFS %
UCH-London	82	CT	48
Glasgow	73	CY-TBI	44
Barcelona	24	CY-TBI	48
Genoa	55	CY-TBI	49
Belgium	33	+ ARA-C	31
Roma	101	CY-TBI/BAVC	41
Stanford	34	BU-E	32
TOTAL/MEDIAN	402		~45
EBMT	598	various	42

Table 3. Results of ABMT with Unpurged Marrow in Childhood AML CR1

Group	No. of Patients	Pretransplant Regimen	LFS %
Parkville-Australia	25	HDM	68
EBMT	49	CY-TBI	63
EBMT	37	various	42
TOTAL/MEDIAN	111		~63

Table 4. Results of ABMT in AML CR2

	PURGED MARROW		
Group	No. of Patients	Pretransplant Regimen	LFS %
St. Antoine, Paris	20	CY-TBI	34
Heidelberg	30	CY-TBI	34
Atlanta	28	various	39
Baltimore	82	BU-CY	38
Pittsburgh	27	BU-CY	48
San Francisco	21	BU-E	52
TOTAL/MEDIAN	208		~35
	UNPURGED MARROW		
Roma	31	BAVC	56
Barcelona	18	CY-TBI	28
EBMT	190 adults	various	30
	35 children	various	40

Table 5. Evidence in Favor of Purging in ABMT for AML.

- BNML rat model (Sharkis 1980, Wiley 1991).
- The characteristics of the purged graft predict outcome
  Better LFS for:
  residual CFU-GM <1% (Rowley 1989)
  CR LPC sensitive to 4 HC (Miller 1991)
- EBMT surveys (Gorin 1990, 1991)
  Better LFS with purged marrow
  slow remitters
  early transplants
- Gene marking in: AML (Brenner 1993)
  CML (Deisseroth 1994)

Table 6. The Role of Mafosfamide in Marrow Treatment.

Direct cytotoxic effect on committed progenitor cells

Induction of apoptosis in human AML, enhancement by IL3, IL6 (Bullock 1993)

Increase of NK cell population and activity in mice following mafosfamide ABMT (Skorski, 1988)

In lymphocytes of normal volunteers cultured *in vitro* with 4HC (Sharma, 1984)

In patients autografted for:
AML
NHL
but not ALL (Carlo-Stella, 1994)

Table 7. ABMT for Acute Leukemia.
Compendium of results from Individual Teams and EBMT

Disease/Status	LFS #	RI #
AML CR1		
purged	58	25
unpurged	45	45
AML CR2	35	55
ALL CR1 (adults)	40	55
ALL CR2		
children	40	60
adults	30	65

Table 8. Data Suggesting an Efficacy of Purging in ABMT for ALL.

- BCL 1 mouse model.
- Correlation of the number of LPC infused to the outcome
  Simmonson 1989
  Uckun, Kersey 1990, 1992
- EBMT survey
  possible efficacy in early remitters, i.e. chemosensitive disease
  unpublished 1992
- Occasional observations
  Grande, Gorin 1994
- Effective marrow purging in NHL (BCL2-PCR) predicts favorable outcome
  Gribben, Nadler 1991

Table 9. Factors Influencing Engraftment Multivariate Analyses* 125 adult patients with primary acute leukemia autografted with marrow purged by mafosfamide at hôpital St. Antoine

Factor	Neutrophils		Platelets	
	RR**	p	RR**	p
Patient age >33 y	0.4	0.0001	0.38	0.003
Purging with mafosfamide at adjusted levels	2.16	0.002	2.23	0.012
Transplant in CR2	0.53	0.02	0.44	0.014
Interval from CR to ABMT >5 months	0.65	0.048	0.61	0.044

* *Stratified at diagnosis.*

***Relative risk for probability of engraftment.*

Higher doses of marrow in CFU-GM/kg pre-purging tended to be associated with faster neutrophil engraftment (p=0.06).

Table 10. ABMT in Acute Leukemia: Future Programs. Hopital Saint-Antoine, Paris

- Protection of normal stem cells during *in vitro* purging
  - Etiofos
  - Acetyl S-DKP
- Positive selection of normal progenitors
  CD34 + Lin - Thy 1 + 10
- Cytokines postABMT
  To accelerate engraftment
  To control MRD

Table 11. Allogeneic Versus Autologous BMT in Adults with AML CR1: 4 Years Results

	LFS	RI	TRM
Autologous BMT N = 998	42 ± 3%	52 ± 3%	13 ± 2%
Allogeneic BMT N = 516	55 ± 3%	25 ± 3%	27 ± 2%
p. Univariate	0.006	$<10^{-4}$	$<10^{-4}$
p. Multivariate	0.006	$<10^{-4}$	$<10^{-4}$

Table 12. Allogeneic Versus Autologous BMT in Adults with AML CR2: 4 Years Results

	LFS	RI	TRM
Autologous BMT N = 190	30 ± 4%	63 ± 5%	20 ± 3%
Allogeneic BMT N = 98	39 ± 7%	42 ± 8%	32 ± 5%
p. Univariate	0.22	0.001	0.02
p. Multivariate	NS	0.0002	0.004

Table 13. Allogeneic Versus Autologous BMT in Childhood AML CR1: 4 Years Results

	LFS	RI	TRM
Autologous BMT	47 ± 6%	48 ± 6%	8 ± 4%
Allogeneic BMT	68 ± 5%	25 ± 5%	9 ± 3%
p. Univariate	0.0014	10^{-4}	0.56
p. Multivariate	0.005	0.0008	NS

Table 14. Allogeneic Versus Autologous BMT Following TBI in Adults with ALL CR1: 4 Years Results

	LFS	RI	TRM
Autologous BMT	40 ± 4%	55 ± 4%	11 ± 3%
Allogeneic BMT	47 ± 3%	35 ± 4%	27 ± 3%
p. Univariate	0.19	$<10^{-4}$	$<10^{-4}$
p. Multivariate	NS (0.06)	$<10^{-4}$	$<10^{-4}$

Table 15. Allogeneic Versus Autologous BMT Following TBI in Adult Patients with ALL CR2: 4 Years Results

	LFS	RI	TRM
Autologous BMT	30 ± 6%	64 ± 7%	16 ± 7%
Allogeneic BMT	25 ± 6%	52 ± 8%	48 ± 8%
p. Univariate	0.30	0.17	0.0002
p. Multivariate	NS	NS	0.0002

Table 16. Allogeneic Versus Autologous BMT Following TBI in Childhood ALL CR2: 4 Years Results

	LFS	RI	TRM
Autologous BMT	36 ± 4%	60 ± 5%	11 ± 3%
Allogeneic BMT	54 ± 4%	32 ± 5%	21 ± 4%
p. Univariate	0.003	$<10^{-4}$	0.09
p. Multivariate	0.003	$= 10^{-4}$	NS

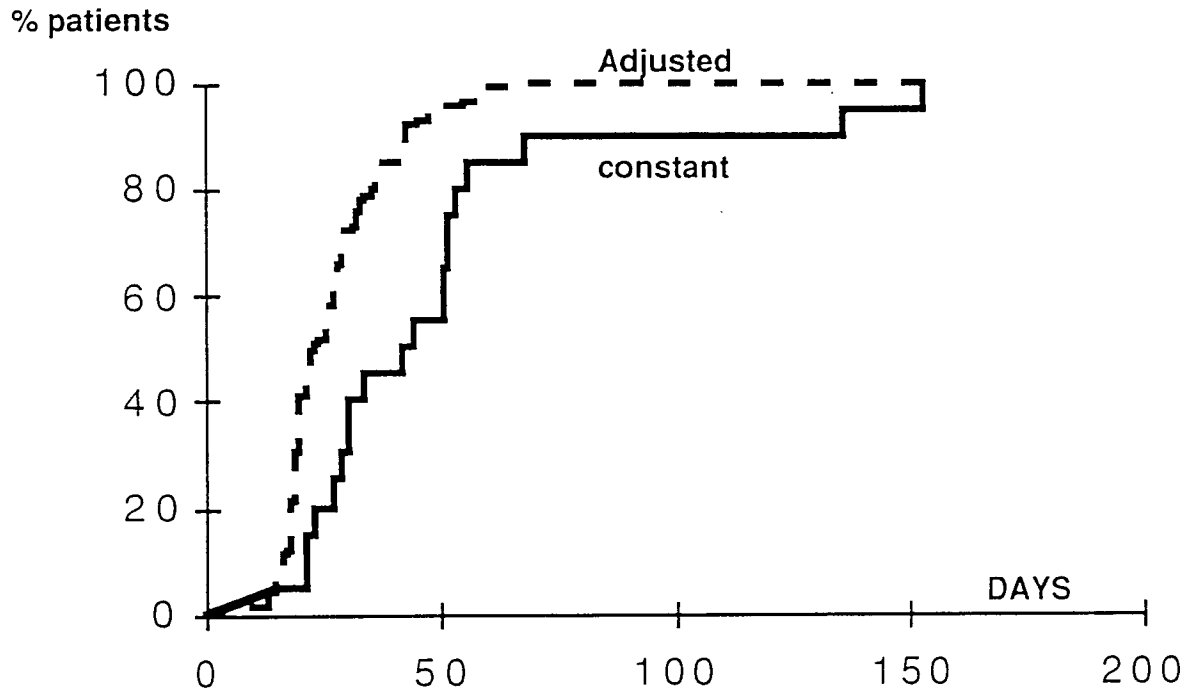

Figure 1. Probability of patients to engraft with neutrophils in relation to the technique of marrow purging with mafosfamide used
Adjusted : dose of mafosfamide adjusted to the individual sensitivity of the patients (CFU-GM LD95).
Constant: dose of mafosfamide constant

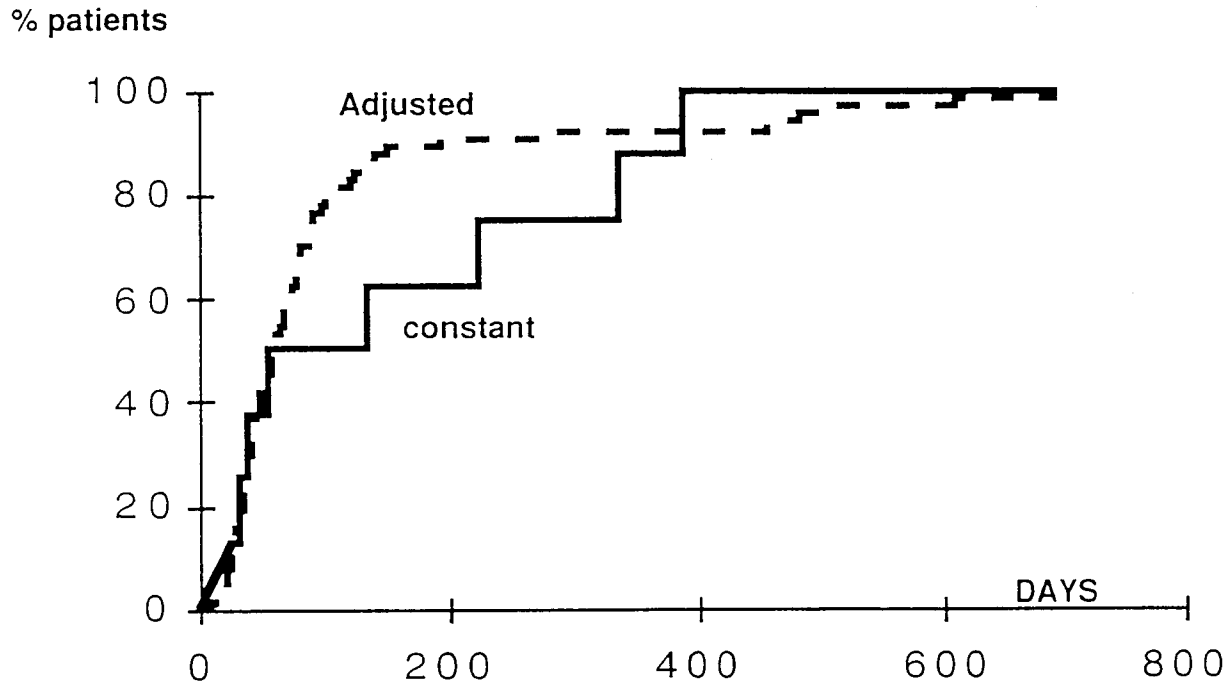

Figure 2. Probability of patients to engraft with platelets in relation to the technique of marrow purging with mafosfamide used.
Adjusted: dose of mafosfamide adjusted to the individual sensitivity of the patients (CFU-GM LD95)
Constant: dose of mafosfamide constant

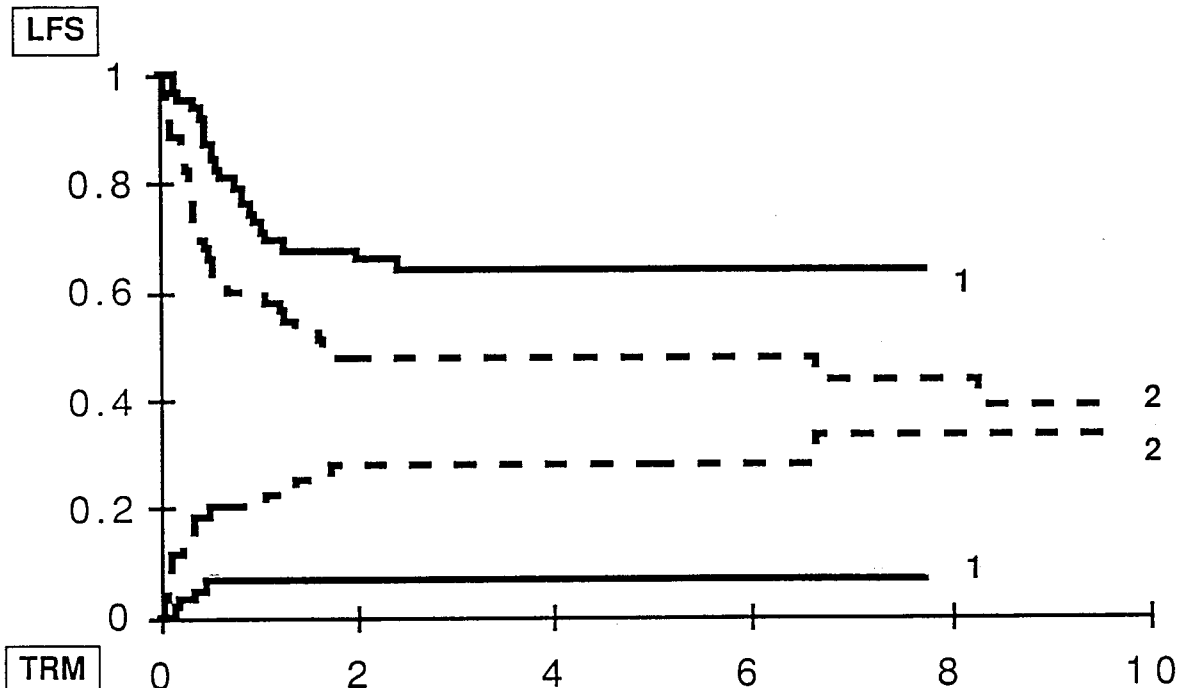

Figure 3: Leukemia-free survival (LFS) and transplant-related mortality (TRM) in relation to the dose of marrow infused (evaluated before purging)

1. CFU-GM/kg $>5.15x10^4$ (n=62)

2. CFU-GM/kg $\leq 5.15x10^4$ (n=63)

Multivariate analysis: p = 0.045 for LFS, p = 0.0003 for TRM.

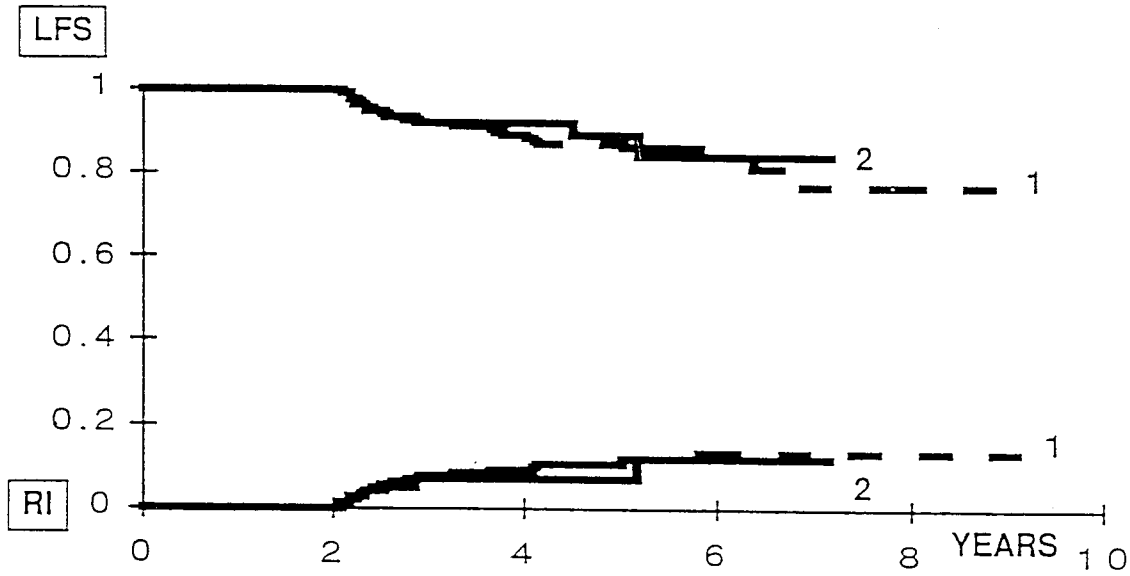

Figure 4. Leukemia-free survival and relapse incidence in patients autografted for acute myelocytic leukemia, who are alive and well at 2 years posttransplant.

1. *Patients transplanted in CR1.*
2. *Patients transplanted in CR2 and CR3.*

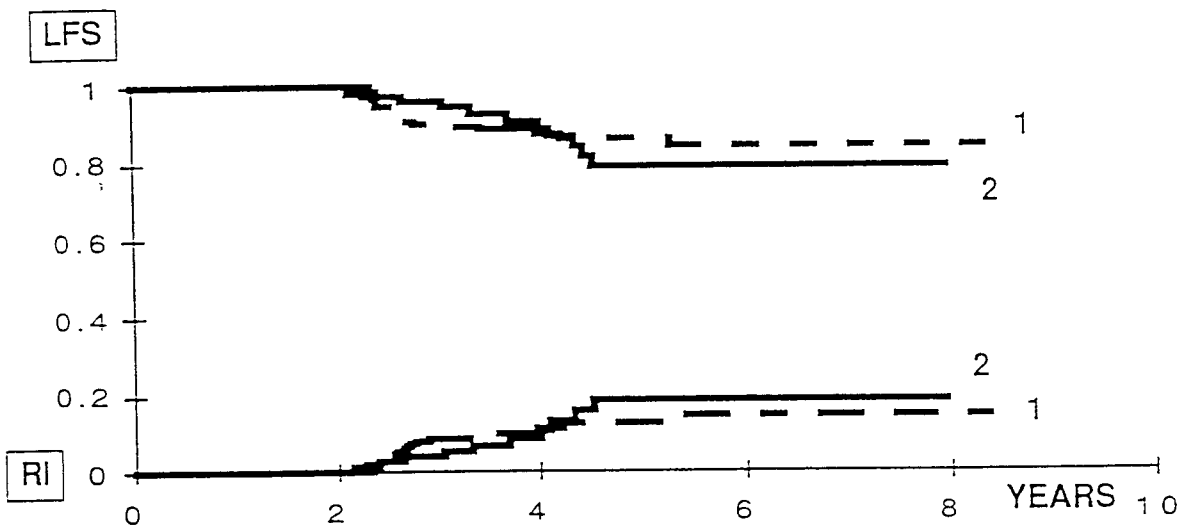

Figure 5. Leukemia-free survival and relapse incidence in patients autografted for acute lymphocytic leukemia, who are alive and well at 2 years posttransplant.

1. *Patients transplanted in CR1.*
2. *Patients transplanted in CR2 and CR3.*

THE RECIPE FOR SUCCESSFUL ABMT IN ACUTE LEUKEMIA

1. Optimal *in vivo* purging
   - Several consolidation courses
   - Boost radiotherapy

2. Optimal *in vitro* purging

3. Optimal pretransplant regimen
   eg. High dose TBI for ALL

4. Optimal timing: in CR

AND ...

BELIEVE IN IT !!!

AUTOTRANSPLANTS FOR ACUTE MYELOGENOUS LEUKEMIA IN NORTH AMERICA

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For the Acute Myeloid Leukemia Working Committee of the North American Autologous Bone Marrow Transplant Registry

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High-dose chemotherapy and/or radiotherapy followed by an allogeneic bone marrow transplant, usually from an HLA-identical sibling, is an effective and established treatment for acute myelogenous leukemia. However, only one-third of otherwise eligible patients have such an HLA-identical sibling donor. Therefore, there is considerable interest in autologous transplants, where patients' own stem cells are used for hematopoietic support after high-dose therapy.[1-3]

Controversies exist regarding who is most likely to benefit, the optimal timing and the most effective preparative regimens for autotransplants. Although the randomized clinical trial is generally accepted as the best way of assessing new treatments[4,5], there are few randomized studies in bone marrow transplantation. Most published data

on the use of autotransplants comes from phase I and II trials in small series of patients and the medical literature gives conflicting information. The cost and logistic difficulties of conducting randomized clinical trials and the limitations of small single center studies provide an impetus to explore alternative methods of evaluating new treatments, especially for preliminary stages of investigation.[6-10] Data from consecutive series of patients treated in multiple centers, accumulated in an observational database, as has been developed and maintained by the International Bone Marrow Transplant Registry (IBMTR) and the Autologous Blood and Marrow Transplant Registry of North America (ABMTR) are ideal for this purpose. Participation of many centers in an observational database is easier to achieve than multicenter trials since it does not require adherence to a common protocol, only agreement to report all patients.

The ABMTR was founded in 1990 by a group of clinical investigators in response to the rapid increase in autotransplants in North America. It was modeled after the IBMTR which has successfully collected and analyzed data on allogeneic bone marrow transplants since 1972. One hundred and twenty institutions in North America have contributed data for over 11,000 patients receiving autotransplants since 1989. The ABMTR is governed by an Advisory Board of 31 distinguished scientists. Data collection by the ABMTR occurs at two levels. First, participating institutions register each consecutive autotransplant, providing core disease, treatment and outcome information. Registration allows the ABMTR to monitor trends in autotransplant use and outcomes, and select patients for clinical studies. The second level of data collection is submission of detailed report forms for patients in specific studies. Comprehensive reports are currently required for transplants for breast cancer, lymphoma and acute leukemia. The data for the preliminary analyses of autotransplants for acute myelogenous leukemia (AML) (described below) was provided by 98 institutions, predominantly from North America but also South America. Core data were analyzed for 1288 patients; more detailed data were available and analyzed for 697 patients who received autotransplants between 1989 and 1993.

Over the past five years there was a dramatic increase in the number of autotransplants performed in North America. This resulted from increasing use of autotransplants to treat breast cancer and non-Hodgkin's lymphoma. The annual number of autotransplants for AML, reported to the ABMTR, remained relatively constant during this time, about 250 per year. Most autotransplants for AML were done in first

remission. The median age of autotransplant recipients was 32 years (range 1 to 75).

Transplant-related mortality (TRM) was about 15% and did not change between 1989 to 1992. The risk of TRM was approximately 15% after transplants in first remission (N=432), 17% in second remission (N=175), and 20% in other disease states (N=90). These differences were not statistically significant. The probability of relapse at three years was about 35% for patients receiving autotransplants in first remission, 60% in second remission, and 75% in other disease states. The three-year probability of leukemia-free survival (LFS) was about 55% after transplants in first remission, 40% in second remission, and 20% in other disease states. These differences in relapse and LFS are highly statistically significant.

CONCLUSION

Despite widespread increase in application of autotransplant technology for other diseases, numbers performed for AML have remained relatively constant over the last five years. Data collected by the ABMTR will allow investigation of potentially important prognostic and treatment variables affecting outcome of autotransplants. Comparisons will be made with other therapies for AML such as allogeneic bone marrow transplantation. This will be achieved by comparing patients reported to the ABMTR with those from the IBMTR database transplanted over concurrent time periods and adjusting for significant prognostic factors. Although randomized controlled trials remain the gold standard for assessing therapies, registries such as the ABMTR and IBMTR are being increasingly recognized as essential resources in evaluating transplant strategies.[11]

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APPENDICES

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Cleveland Clinic Foundation	Cleveland	U.S.
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Ohio State University Hospital	Columbus	U.S.
Hospital Privade de Cordoba	Cordoba	Argentina
Hospital de Clinicas	Curitiba	Brazil
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Baylor University Medical Center	Dallas	U.S.
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Wayne State University	Detroit	U.S.
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Vancouver General Hospital	Vancouver	Canada
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St. Francis Hospital	Wichita	U.S.
Manitoba Cancer Treatment Center	Winnipeg	Canada
Wake Forest University	Winston-Salem	U.S.

RESULTS OF CHEMOTHERAPY WITHOUT TRANSPLANT IN AML IN SECOND CR AT M.D. ANDERSON CANCER CENTER

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The purpose of this manuscript is to describe M.D. Anderson's experience in AML patients who in second remission (CR) received chemotherapy without bone marrow transplantation. This describes the great majority of AML second CR patients at M.D. Anderson. Thus, between 1980 and 1993 chemotherapy without transplant produced second CRs in 195 AML patients. One hundred sixty-five of these (85%) continued to receive chemotherapy only, 15 received an autologous transplant, and 15 received an allogeneic transplant. The chemotherapies used in the 165 patients were variable, as was the duration of continued therapy in second CR. It would be fair to say however that the great majority of the 165 received ara-C in either high- or intermediate-dose and that the duration of therapy was generally between 6-12 months.

Characteristics of the 165 patients are shown in Table 1. Their low median age and long first CR duration (relative to that of all first relapse AML patients at our hospital) reflects the association of age or duration of first CR and probability of achieving a second CR.[1] Similarly, the relatively high frequency of inv(16), t(8;21), or t(15;17), and the relatively low frequency of -5 and/or -7 or +8 is a result of the association between initial cytogenetic pattern and probability of second CR.

Table 1. Characteristics of chemotherapy patients

Patients	165
Median age	45
Median weeks first CR	60
Inv(16) or t(8;21), or t(15;17)	27%
Normal	42%
-5/-7	1%
+8	2%
Other	17%
Insufficient for analysis	10%

One hundred twenty-nine of the 165 patients given chemotherapy without a transplant in second, chemotherapy induced CR have had recurrence of AML. Kaplan-Meier analysis indicates that disease will recur in 50% of the patients by 26 weeks. At one year the probability of remaining in CR is .25±.04(SEM) and at two years the probability is .15±.03. When results are plotted on a logarithmic rather than a linear scale, it appears that there is a constant relapse rate such that by 2-1/2 years the probability of continued CR is essentially zero. This constant relapse rate is very different from what one sees in AML patients in first CR.[2] Among 692 of the latter, the relapse rate appeared to slow substantially after two years in CR.

Are there second CR patients who have a better prognosis than the average for all 165 patients described above? We looked at two possible prognostic characteristics: Cytogenetics and duration of first CR. Patients were divided into 3 cytogenetic groups, using the karyotype found at initial presentation. In one group were 45 patients with either inv(16), t(8;21), or t(15;17), in the second were 69 patients with a normal karyotype, and in the third were 51 patients with other karyotypes. While there was some tendency for the first group to do better than the second or third, this was not pronounced and there was no difference between the second or third, (log rank observed/expected values were 0.79, 1.17, and 1.04 respectively) and the log rank p-value for the comparison between the three groups was 0.19. The prognostic insignificance of cytogenetics in second CR patients given chemotherapy of course contrasts starkly with what one sees in first CR.[3] This clearly indicates not only the heterogeneity to be found within any general prognostic group (e.g., patients with a given karyotype) but illustrates the need both to determine over what time period any prognostic characteristic is operative and to identify new posttreatment prognostic characteristics. It is possible that cytogenetic pattern might have been prognostically significant if we had available cytogenetic data at time of the first relapse. However, to date, analysis suggests that cytogenetic pattern at first relapse adds little information to that obtained from initial cytogenetics in predicting probability of second CR.[4]

In contrast to cytogenetics, duration of first CR was strongly correlated with duration of second CR. Patients with first remissions under 27 weeks had shorter second CRs than patients with first remissions 27-52 weeks who in turn had shorter second CRs than patients with first remissions 53-104 weeks who had shorter second CRs than patients with first remissions in excess of 2 years. It is possible that the relationship between probability of second CR and duration of first CR is monotonic.

Certainly it is more than binary [>1 year vs <1 year] and this is important because, for example, the IBMTR is considering duration of first CR only as a binary variable in their comparison of chemotherapy vs transplant in second CR. Plotting on a log scale indicates that only in patients in second CR after a first CR lasting over 2 years does the rate of relapse decrease with time. In this respect, these second CR patients are similar to the average first CR patient. Frequently, a second CR that lasts longer than a first CR is taken as evidence of the superiority of transplantation over chemotherapy alone ("inversion"). It was thus interesting to note that in seven patients, duration of second CR after chemotherapy was longer than duration of first CR after chemotherapy. Clearly, then an "inversion" can be a result of a "poor" treatment applied in first CR as well as a "good" treatment applied in second CR.

Because only 8% of our 195 second CR patients received an allogeneic transplantation and only 8% received an autologous transplant, formal regression analysis comparing these modalities with chemotherapy in second CR would be difficult. Rather, we attempted to match each patient who received an allogeneic transplant in second CR with two patients who only received chemotherapy in second CR. Two criteria were used for matching. First, the chemotherapy patient had to be alive in CR when his/her match was transplanted. It should be pointed out that no attempt was made however to determine how close the chemotherapy patient was to relapse at that time although we considered the possibility of requiring the chemotherapy patient to be in CR for X + weeks where X is the time from second CR when the transplant patient was transplanted. Second, the chemotherapy patients and the transplant patient had to have the same or very close to the same first CR duration since this was clearly related, perhaps as noted above, in monotonic fashion to duration of second CR. Results of the analysis are shown in Table 2 and summarized in Table 3. Note the endpoint was survival in CR. Table 3 indicates that when one compares results in the transplant patients with the first set of chemotherapy matches, the transplanted patients had longer DFS in 11 cases, the chemotherapy patients had longer DFS in 1 patient, there were 2 cases in which DFS was the same, and in 1 case the results are as yet indeterminate (25 + weeks DFS for the chemotherapy patient, 46 weeks for the transplant patient). Using McNemer's test, the results were superior in the transplanted patients at p=.006. However, when the transplanted patients were compared with the second set of chemotherapy matches there was no statistical differences (p=.77). As might be expected then, when the results in the two sets of chemotherapy patients were

compared, the first chemotherapy group had longer DFS than the second in 11 of 15 cases (p=.12 with however a power of only 0.42)! This suggests that the variation in outcome in chemotherapy patients matched for duration of first CR was so great as to confound our chemotherapy-transplant comparison.

Table 2. DFS in Second CR for Fifteen Patients Given Allografts in Second CR With Each Matched With Two Chemotherapy Patients

Weeks 1st CR		Weeks Survival in 2nd CR		
Trans Pt	Trans Pt	Chemo Matches	Trans Pt	Chemo Matches
1	62	63,63	19	6,30
2	121	120,120	8	6,32
3	64	65,65	9	18,44
4	30	30,30	11	11,10
5	43	43,44	14	4,121
6	33	33,34	16	8,16
7	4	4,4	17	8,21
8	36	35,36	18	18,5
9	28	28,27	19	7,10
10	6	5,8	25+	46,14
11	30	30,30	30	26,18
12	203	206,218	45+	34,252
13	30	30,30	70+	11,234+
14	73	72,75	471+	24,49
15	31	34,39	500+	20,36

Table 3. Summary of Analysis in Table 2.

Trans Pt vs 1st Chemo Pt		Trans Pt vs 2nd Chemo Pt	1st Chemo Pt vs 2nd Chemo Pt
Chemo wins	1	5	4 (chemo 1 wins)
Trans wins	11	7	11 (chemo 2 wins)
TIE	2	1	0
Indeterminate	1	2	0
	p=.006[a]	p=.77[a]	p=.12[a,b]

[a] McNemer's test
[b] Power = .42

In summary, our analysis of 195 patients in second CR at M.D. Anderson showed: 1) that the median remission duration in the 165 of 195 patients given only chemotherapy is 26 weeks; 2) that only in second CR patients whose first CR lasted over 2 years may be a decreasing relapse rate with time; these constituted 20% of the 165 patients; 3) that duration of first CR may be monotonically related to duration of second CR confounding analyses that regard duration of first CR only as a binary variable, and 4) that pretreatment cytogenetics is not related to duration of second CR.

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BAVC REGIMEN FOR AUTOGRAFT IN ACUTE MYELOGENOUS LEUKEMIA IN SECOND COMPLETE REMISSION: UPDATED EXPERIENCE ON 60 CASES

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SUMMARY

Since 1984, BAVC (BCNU, Amsacrine, Vepesid, Citosine-Arabinoside) has been utilized as a conditioning regimen for AML patients autografted in second CR. We have previously reported our results on 21 cases, showing a DFS probability of 52% at 63 months. So far, a total of 60 patients (31 males, median age 29 years, ranging from 1 to 54) have been treated and DFS probability is 41% projected at ~10 years.

Concerning characteristics of the 60 patients, the median duration of first CR was 14 months (range 1-43) and median time from second CR to graft was 2 months. Marrow was collected from most of the patients in second CR, at the time of transplant. No *in vitro* purging was utilized. Second remission was generally reinduced with a schema including intermediate- or high-dose Ara-C. From 1990, all-*trans* retinoic acid was utilized as induction treatment in 13 patients with promyelocytic leukemia.

The BAVC regimen was well tolerated: 3 patients (5%) died during aplasia (1 from myocardial infarction, 2 from fungal infection). In 57 evaluable cases, the median time to recovery was 24 days for neutrophils (range 11-64) and 38 days for platelets (range 13-225).

Relapse occurred in 29 patients after a median of 6 months from transplant (range 2-28) and 28 patients are in CCR after a median follow up of 53 months (range 1-116); CR duration was >3 years in 18 cases and in 21 patients the second CR is longer than the first one.

These results confirm the antileukemic efficacy and the low toxicity of the BAVC regimen, demonstrating the possibility of obtaining long- term second remissions in AML patients after autografting with unpurged marrow.

INTRODUCTION

Despite advances in the treatment of acute leukemias obtained by adding new drugs and utilizing a better supportive therapy, the prognosis of Acute Myelogenous Leukemia (AML) after first relapse remains unsatisfactory; chemotherapy does not offer a real probability of long-term remissions and all eligible patients are considered for myelosuppressive therapy with allogeneic or autologous bone marrow transplantation (ABMT).[1] Conditioning regimens utilized are generally the same as for allogeneic BMT.[1-2] These aggressive preparative regimens are responsible for considerable transplant-related toxicity mainly in heavily pretreated patients.

This prompted us to evaluate the efficacy of an original conditioning regimen, BAVC (BCNU, AMSA, VP-16, Ara-C), that seems to have lower hematological and extra-medullary toxicity, but antileukemic activity comparable with other conditioning regimens. We report an update of our experience concerning a total of 60 patients autografted in second CR utilizing this regimen.[2]

PATIENTS AND METHODS

Patients

From October 1984 to June 1994, 60 eligible consecutive patients with AML in second CR without HLA compatible sibling entered this study. Median age was 29 years (range 1-54), 31 were males. Median duration of first CR was 14 months (range 1-43). All FAB subtypes have been enrolled in the study. At relapse, the majority of patients received at relapse high- or intermediate-dose Ara-C plus Mitoxantrone with or without VP-16;[3] as of 1990, all-*trans* retinoic acid has been utilized as reinduction treatment in 13 patients with promyelocytic leukemia. Bone marrow was harvested in all but 6 cases during second CR, immediately before autograft, at a median of 2 months from achievement of second CR; no purging techniques were employed. A median of $2x10^8$ (range 0.4-4.5) mononuclear cells (MNC) were collected. The techniques of marrow collection, cryopreservation and reinfusion have been previously described.[4]

The BAVC regimen consisted of BCNU 800 mg/m^2 given on day -6, Amsacrine 150 mg/m^2, Etoposide 150 mg/m^2, Ara-C 300 mg/m^{2}given on days -5, -4, -3. Colony stimulating factors were not employed during aplasia. The results were analyzed as of June 30, 1994.

Supportive Care

From 1984 to 1988, patients were nursed in a reverse isolation single room; from 1989 onwards, patients were managed in a double room. A central venous catheter (CVC) was placed in all patients for chemotherapy, fluids and blood product administration. Cotrimoxazole, norfloxacine, and recently, ciprofloxacin have been utilized as prophylactic treatment. All patients started acyclovir IV 15 mg/kg/day from day +1 for prevention of herpes virus infection. Broad spectrum antibiotic therapy was instituted for fever >38° C when neutrophils <500 μL, IV amphotericin B was added after 72-96 hours in case of persistent fever without documented bacterial etiology or suspected fungal infection. All blood products were irradiated before infusion.

RESULTS

Recovery

Recovery is evaluable in 57/60 patients. The remaining 3 patients died early during aplasia. The median time to obtain a neutrophil count >500/μl was 24 days (range 11-64); a platelet count >50,000/μl was reached after a median of 38 days (range 13-225).

All patients with promyelocytic leukemia treated in relapse with all-*trans* retinoic acid reached recovery in intervals not different from the others.

Toxicity

The conditioning regimen was well tolerated; nausea and vomiting were common, particularly during BCNU administration. Various degrees of mucositis occurred in the majority of cases and in 7 patients it was greater than grade 2 according to the WHO classification.

Fever occurred in 43 patients and in all, broad spectrum antibiotic treatment was started. In 27 cases a bacterial infection was documented. Amphotericin B was given in 6 cases; in 4 of them, a fungal infection was subsequently documented. Three patients died during aplasia, 2 from fungal infection and 1 from myocardial infarction. Late complications occurred in 2 patients who developed bilateral femoral head necrosis, requiring surgery. Both are alive and well at 115 and 53 months after transplant.

Clinical Results

As of June 30, 1994, 28/57 evaluable patients are in continuous CR after a median of 53 months from ABMT (range 1-116) and 29 patients relapsed after a median of 6 months (range 2-28). The second remission is longer than the previous one in 21/28 cases and 18 patients have been long-term survivors for over 36 months. The overall disease-free survival is 41% projected at more than 9 years.

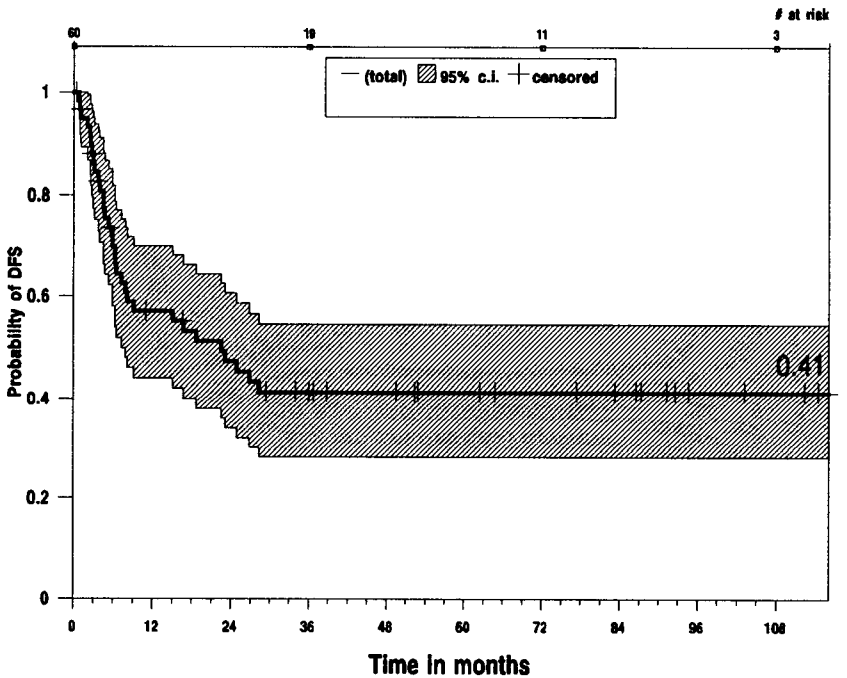

Figure 1. *Disease-free survival (DFS) of AML patients autografted in 2nd CR-BAVC regimen.*

Statistical Analysis

Univariate analysis did not show any influence on DFS of age (≤15 versus >15 years), sex, time to achieve first CR (≤40 versus >40 days), first CR duration (≤12 versus >12 months), time from second CR to ABMT (≤2 versus >2 months), number of reinfused cells or FAB subtype. The influence of age, first CR duration, time from second CR to ABMT and number of reinfused cells were also analyzed in a multivariate proportional hazard model; the length of first CR showed a positive,

statistically significant, influence on DFS (p=0.01), while a trend was present in favor of pediatric patients (p=0.08).

DISCUSSION

We previously reported our results in 21 AML patients autografted with unpurged marrow after conditioning with the BAVC regimen. The probability of DFS was 52% projected at 60 months. The relatively small number of patients and the short follow-up allowed only preliminary conclusions concerning the antileukemic efficacy of our regimen. Nevertheless, the protocol was associated with low toxicity: less than 5% of transplant-related deaths in a heavily pretreated group of patients.[5]

Up to now, a total of 60 AML cases received autografts in second CR after BAVC. The clinical results of our previous report are confirmed: 1) probability of DFS is 41% projected at ~10 years, with 18 patients who are long-term survivors at >3 years and are potentially cured; 2) the transplant-related toxicity is low, with 5% of early deaths; no life-threatening side effects are observed in the long-term follow-up of these patients.

Our BAVC regimen gives similar results in terms of antileukemic efficacy as compared to other conditioning regimens but has fewer toxic effects and, above all, allows for a better quality of life. Furthermore, these results do not seem to be inferior to those obtained after allogeneic BMT. Prospective randomized trials should be performed to verify the efficacy of our BAVC regimen versus the more established regimens, but may not be feasible because of potential difficulties in accruing sufficient numbers of patients.

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AUTOLOGOUS BONE MARROW TRANSPLANT WITH 4-HYDROPEROXYCYCLOPHOSPHAMIDE PURGING IN PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA IN SECOND COMPLETE REMISSION

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While chemotherapy offers the potential for cure in patients with acute myelogenous leukemia (AML) in first complete remission,[1,2] cure is rare with chemotherapy in patients who have relapsed. Bone marrow transplant offers the potential for cure in patients in first relapse or greater. Allogeneic bone marrow transplant from a matched sibling has been the treatment of choice in relapsed patients with AML in second remission if a suitable donor is available.[3,4] Allogeneic bone marrow transplant offers the advantage that a second remission need not be obtained[5] as well as the graft-versus-leukemia effect.[6] Many patients do not have a suitable sibling donor or are too old to undergo allogeneic bone marrow transplant. Autologous bone marrow transplant is an option for patients without a suitable donor.[7-13] Disadvantages of autologous bone marrow transplant is the need for adequate marrow that is not contaminated with leukemia cells. Different strategies have been used to decrease leukemia burden of marrow graft.[14-18] We have used 4-hydroperoxycyclophosphamide (4HC), a cyclophosphamide cogener, to purge marrows in patients with acute myelogenous leukemia in second remission since completion of a phase one trial.[19] Since 1983, 90 patients with AML in second complete remission have been treated with autologous bone marrow transplant with 4HC purged marrow. Between 1983 and 1988, mononuclear cells collected from a buffy coat preparation of marrow were treated with 100 micrograms/ml of 4HC. After 1988, in order to decrease variability of 4HC purging based on red blood cell contamination,[20] bone marrow cells were ficolled to remove red blood cells and granulocytes prior to treatment with 4HC at 60 micrograms/ml of nucleated marrow cells.

Patient characteristics of the 90 patients are in Table 1. Thirty percent of the patients were considered high risk as defined by chromosomal abnormalities other than t(8;21), t(15;17), or inverted 16, white blood count greater than 100,000/mm at presentation, presence of

extramedullary disease, secondary AML or preexisting myelodysplastic syndrome, or failure to achieve a complete remission with initial chemotherapy. The remaining patients were standard or good risk patients. Median age was 22 years. Forty percent of the patients were consolidated prior to harvest. In all cases, marrow was harvested during second remission and myeloid growth factors were not used routinely. All patients were prepared with busulfan (4 mg/kg/day orally for four days followed by cyclophosphamide 50 mg/kg IV daily for 4 days).

Table 1. Patient Characteristics

Age	
Median	22 years
Range	1-54 months
High risk at presentation	
Yes	30%
No	70%
CR to BMT	
Median	3 months
Range	1-9 months
Consolidation	
Yes	40%
No	60%

Actuarial disease-free survival and risk of relapse for all patients at 10 years are shown in Figures 1 and 2. Median follow-up is 70 months (range 6 to 135). Actuarial disease-free survival at 10 years is 30% with an estimated relapse risk of 62%. Median time to relapse was 10 months with only 1 patient relapsing after 2 years. There was a trend to improve disease-free survival and risk of relapse in the patients who were treated with ficolled marrow compared to buffy coat marrow with a 40% actuarial disease-free survival (Figures 3 and 4, $p=0.08$). This improved survival is possibly related to a more standard and slightly more potent actual concentration of 4HC in the ficolled patients as the patient groups are similar.

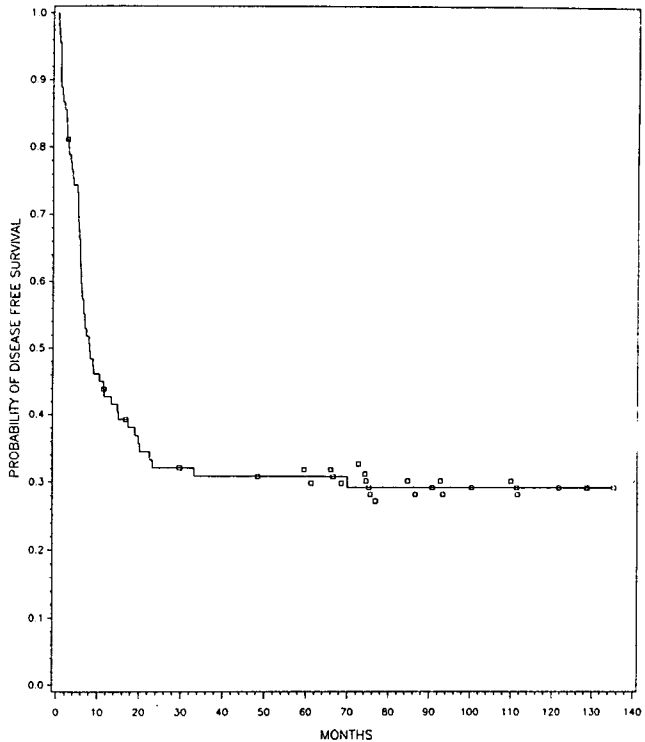

Figure 1. *Disease-free survival in 90 patients with AML in CR2 autotransplanted with 4HC purged marrow.*

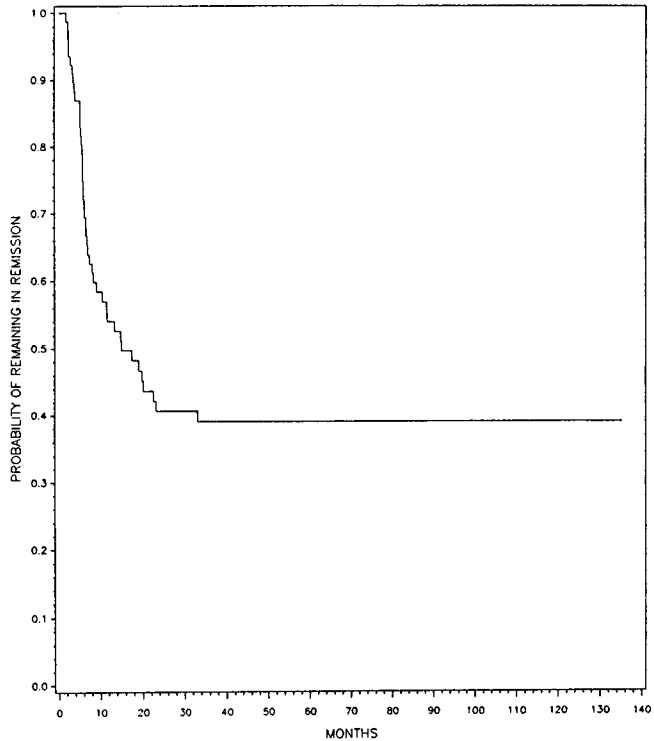

Figure 2. *Relapse-free survival in 90 patients with AML in CR2 autotransplanted with 4HC purged marrow.*

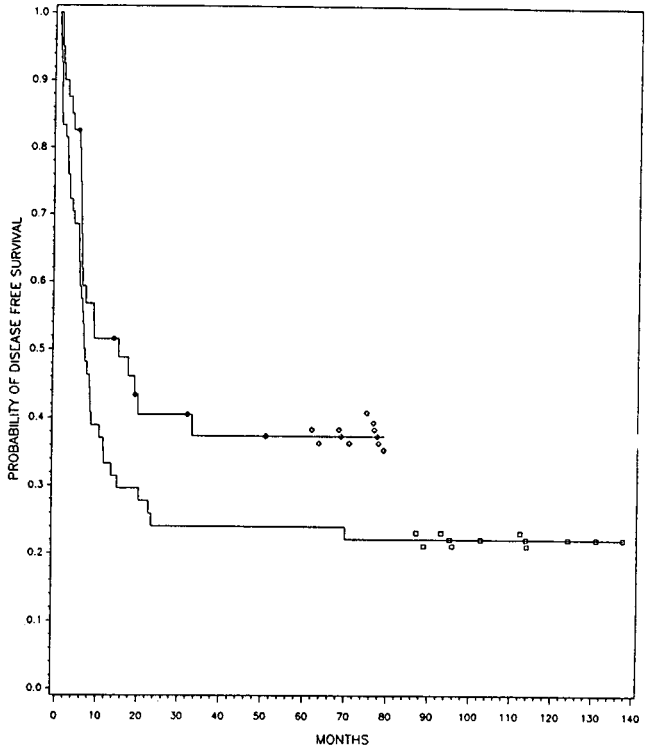

Figure 3. *Disease-free survival in patients transplanted with buffy coat marrow (◊) versus ficolled marrows (▫).*

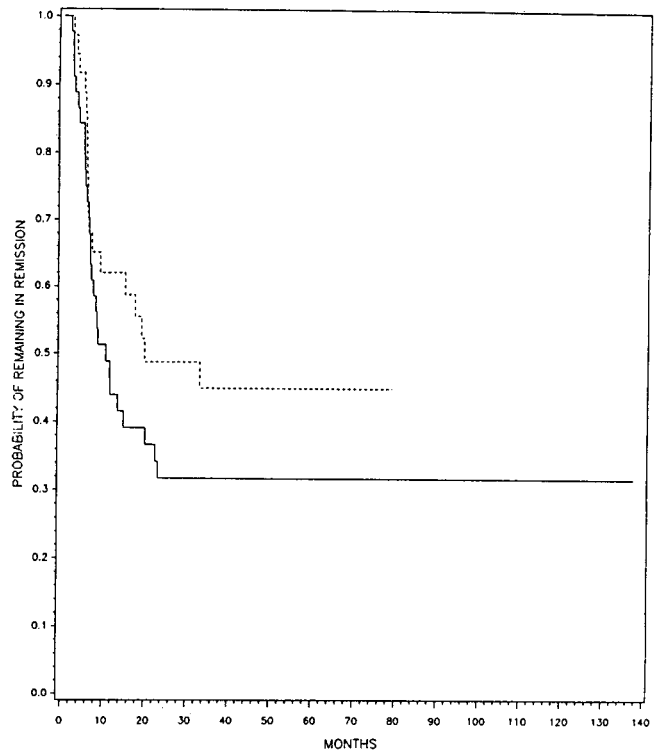

Figure 4. *Relapse-free survival in patients transplanted with buffy coat marrows (____) versus ficolled marrows (---).*

When disease-free survival in the patient group treated with autologous transplants was compared to patients treated with allogeneic transplant from an HLA identical sibling during the same period, no differences were seen (Figure 5). Risk of relapse was significantly higher in the autologous patients compared to the allogeneic patients (Figure 6), however, transplant related mortality (mainly graft-versus-host disease) was higher with allogeneic transplant recipients.

Non-relapse mortality was 16% in the autologous transplant patients. Cause of death in all autografted patients is shown in Table 2. Median day of transplant related death was 25 days (range 8 to 272). Sepsis early after transplant and venooclusive disease of the liver were the most common causes of death.

Table 2. Cause of Death

BMT Day	Cause of Death
24	CNS bleed
155	Sepsis, failure to engraft
13	Sepsis
17	Sepsis
9	Sepsis
24	Venooclusive disease of liver
24	Aspergillus
8	Sepsis
37	Sepsis
27	Venooclusive disease of liver
43	Venooclusive disease of liver
165	Interstitial pneumonia
25	Venooclusive disease of liver
272	Sepsis, failure to engraft

Day to neutrophil count greater than 500/mm^{3}was 45 days (range 20 to greater than 272 days). While period of neutropenia was long, it did not contribute to excess mortality as only two of the patients with neutropenia lasting more than 50 days died of non-relapse mortality (sepsis days 155 and 272). Platelet recovery was prolonged with only 30% of the patients recovering platelet independence before day 50. Due to excellent platelet support, despite prolonged thrombocytopenia, only one patient died of hemorrhage.

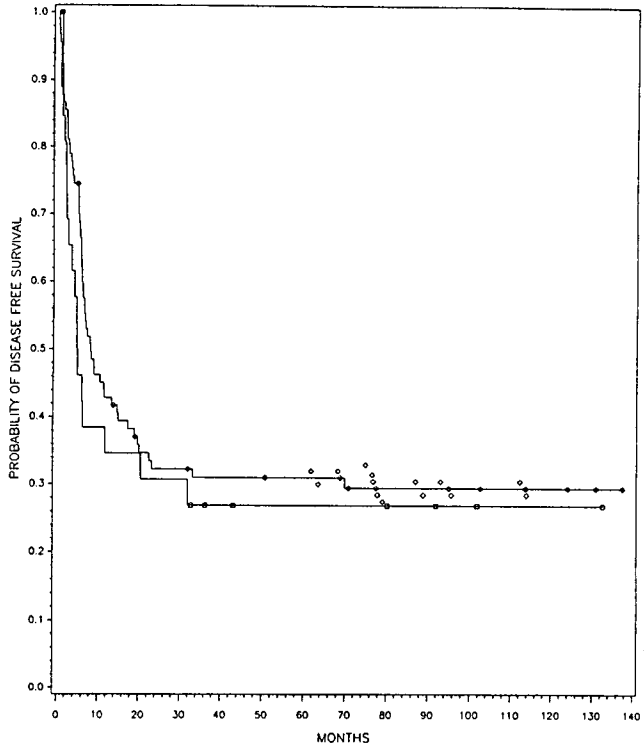

Figure 5. *Disease-free survival in patients receiving autologous transplants for AML in CR2 (◊) compared with allogeneic transplants for AML in CR2 (▫).*

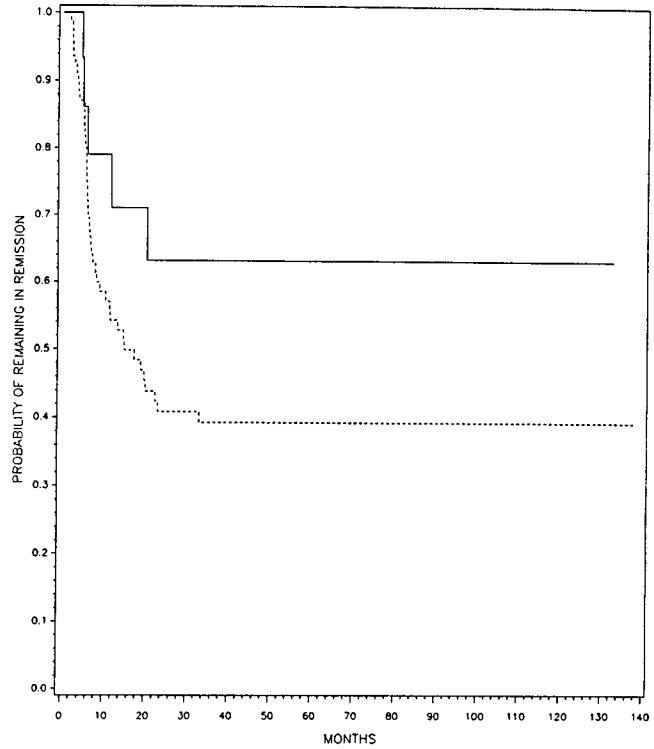

Figure 6. *Relapse-free survival in patients receiving autologous transplants for AML in CR2 (____) compared with allogeneic transplants for AML in CR2 (---).*

In summary, autologous bone marrow transplant with 4HC purged marrow cures approximately 40% of patients autografted in CR2 using our current protocol. While this is comparable to disease-free survival in allografts, autologous bone marrow transplant requires marrow that is harvested in remission (data not shown). Therefore, we are recommending that patients with AML who are not candidates or who refuse bone marrow transplant in first complete remission have marrow harvested and purged with 4HC in first remission for use as salvage with subsequent relapse.

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MONOCLONAL ANTIBODY-MEDIATED APPROACHES TO THERAPEUTICS IN ACUTE MYELOID LEUKEMIA: MARROW PURGING FOR AUTOLOGOUS BONE MARROW TRANSPLANTATION AND *IN VIVO* SEROTHERAPY

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ABSTRACT

We are exploring several approaches to improving outcomes in acute myeloid leukemia (AML) using monoclonal antibodies (mAb) directed to myeloid differentiation antigens. We have conducted a trial of ABMT for AML using mAb (PM-81, AML-2-23) and complement (C')-mediated purging targeting myeloid antigens CD15 and CD14 since 1984. The preparative regimen was initially Cy/TBI (60 mg/kg/day x 2, fractionated total body irradiation (TBI)-total dose, 1,200 cGy) but was changed to Bu/Cy (busulfan, 4 mg/kg/day x 4, Cy, 60 mg/kg/day x 2) in 1988. The Bu/Cy regimen has been associated with an improved outcome. In our last formal data analysis we studied 7 first CR (CR1), 45 2nd or 3rd CR (CR2/3), and 11 1st relapse (R1) patients who were treated with Cy/TBI or Bu/Cy followed by ABMT with mAb-purged bone marrow (BM). Median age at ABMT for the 56 CR2/3 and R1 patients was 36 years. Patients in CR2/3 or R1 treated with Bu/Cy had an actuarial 3-year disease-free survival (DFS) of 48% (95% confidence interval [C.I.], 29-67%). In contrast, patients treated with Cy/TBI had an actuarial 3-year DFS of 31% (95% [C.I.], 16-46%). We conclude that long-term DFS can be achieved in slightly less than 50% of patients with advanced remissions and relapsed AML using Bu/Cy with mAb-purged BM. There was no difference in outcome comparing patients transplanted in first relapse or CR2/3. This suggests that harvesting marrow in first CR with the intention of transplantation at first relapse represents an attractive strategy for managing patients in first remission. We are planning a trial of ABMT with purged vs unpurged marrow in patients in CR2/3 to address the question of the clinical value of purging.

To develop *in vivo* therapeutics, we are exploring two approaches. In one, we are using the C'-activating properties of PM-81 (an IgM) and

have instituted a phase I/II trial of *in vivo* therapy with PM-81 mAb in patients with relapsed AML in conjunction with chemotherapy. After determining the maximally tolerated dose, a Phase II trial of *in vivo* mAb therapy following ABMT is planned with the hypothesis that serotherapy will lower relapse rates by eliminating minimal residual disease. In another approach, we are developing bispecific antibodies (BsAb) comprised of an anti-CD33 mAb (251) and an anti-CD16 or CD64 (Fcγ Receptor III and I) that are capable of activating normal leukocyte effector cells and redirecting their cytotoxicity to myeloid leukemia cell targets. Significant degrees of natural killer cell and monocyte-mediated killing have been achieved with these bifunctional molecules. Clinical trials of BsAb appear to be warranted.

INTRODUCTION

For over ten years we have used two monoclonal antibodies (mAb) (PM-81 and AML-2-23) to treat bone marrow from patients with acute myeloid leukemia (AML) whose cells manifest one or both of these antigens and who are in first or greater remission for subsequent reinfusion after ablative chemoradiotherapy.[1,2] We have pioneered a novel approach to *in vitro* manipulation of marrow cells with mAb and complement using an automated cell processor to expose cells to a constant infusion of complement.[3] The majority of patients have been in second or third complete remission (CR) at the time of transplant. Another group of patients have been transplanted in early first relapse with marrow harvested and purged in first CR. A smaller number of patients have been transplanted in first CR.

Our studies and others [1,2,4-7] have shown that long-term disease-free survival (DFS) can be achieved in patients with AML after first relapse with ABMT using both purged and unpurged marrow. The issues that need to be addressed are: 1) the role of purging, 2) the optimal timing of ABMT in the management of AML, and 3) the role of posttransplant immunotherapy. This paper will discuss our ideas and approaches to these questions.

The Role of Purging

Reports from our ongoing study and others have confirmed that long-term DFS can be achieved in 30-50% of patients with AML in second or third CR when ABMT is performed.[1,2,4-7] While these results are influenced by patient selection bias and other factors, the consistency of

reports from centers around the world is convincing that ABMT results in superior outcomes compared to conventional chemotherapy only-induced remissions. However, the role that marrow purging plays in the outcomes of ABMT is not entirely clear. Good results have been reported from groups using unpurged marrow and from those using purged marrow. The issue is complicated by the multitude of preparative regimens employed, the previous treatments that the patients received, and other factors including selection bias. Thus, it is difficult to draw definite conclusions from these unrandomized and disparate trials.

The probable efficacy of marrow purging has been shown in several studies.[5] Importantly, recent reports of Neomycin-resistance gene-marked autologous bone marrow cells from patients with AML undergoing ABMT have shown that leukemia cells sampled at relapse contained the transferred gene demonstrating that occult marrow leukemia cells can at least contribute to relapse.[8] However, the ideal demonstration of purging efficacy would be a prospective randomized study using a common preparative regimen and stratifying for prognostic factors.

The two most common purging methods have been chemical and immunological. The former approach has been widely tested using one of two cyclophosphamide derivatives, 4-hydroperoxycyclophosphamide and mafosfamide. Immunologic approaches have included mAb-mediated cytotoxicity (complement or immunotoxin) and culture of marrow in interleukin-2.

We reported recently the results from the first 63 patients treated on our study of mAb-purged marrow.[2] Forty-six second/third CR patients were transplanted using PM-81 and AML-2-23-treated bone marrow. We noted that the long-term DFS of these patients has been improved through implementation of the Bu/Cy preparative regimen (Bu, 16 mg/kg and Cy, 120 mg/kg) supplanting Cy/TBI. Actuarial 3-year DFS of the patients in CR2/3 treated with Bu/Cy was 48%. Seven CR1 patients have undergone ABMT using PM-81 and AML-2-23-treated bone marrow. Although this small sample size makes it difficult to obtain accurate estimates of survival and remission duration, we were encouraged that the 3-year DFS in this group is 56%.

We are encouraged by these results. Future plans are to perform a randomized trial comparing purged to unpurged marrow ABMT for patients in second complete remission to better define the contribution of purging to disease-free survival following ABMT.

Table 1. Disease-free Survival of 63 Patients by Remission Status at BMT and by Regimen

CR	2-year (% + S.D.)	3-year (% + S.D.)	Relapse (3-year) (%)
1 (n=7)	71 ± 15	71 ± 20	29 ± 29
2/3 (Bu/Cy, n=18)	48 ± 20	48 ± 13	38 ± 25
2/3 (Cy/TBI, n=28)	26 ± 17	22 ± 8	55 ± 26
1st relapse (n=11)*	36 ± 35	36 ± 35	43 ± 40

**Includes two patients prepared with Cy/TBI and nine treated with Bu/Cy (see below for discussion of ABMT in first relapse).*

Optimal Timing of ABMT

Several studies have indicated that the optimal timing of ABMT is not clear. Performed in first CR, ABMT results appear comparable to allogeneic BMT and is slightly superior to standard chemotherapy. However, patients who relapse from chemotherapy may be salvaged by ABMT at first relapse or after achieving second remission.

We are conducting a prospective study of ABMT at the time of first relapse using the Bu/Cy ablative regimen[2] as the remission inducing therapy followed by transplantation of previously harvested bone marrow that was purged with mAb plus C'. We have found this approach to be feasible and attractive to patients. To date, we have transplanted marrow into 18 patients. The disease-free and overall survival of these patients is 43% and 49% respectively, at three years (Figure 1). Follow-up ranges from two to 62 months (median 20 months) in survivors. Six patients survive greater than two years and two patients are surviving greater than 5 years. Median survival is 15 months. We are not aware of any non-myeloablative remission-inducing chemotherapy programs capable of achieving these promising results. We will continue to accrue to this ongoing study to gain more confidence in the outcomes observed thus far.

This approach is attractive in several ways. One is that only patients who truly need a high-dose regimen with stem cell rescue are subjected to the morbidity and mortality of this intense procedure, another is that salvage of relapsed patients is possible with the result that the number of long-term disease-free survivors is similar to what is reported

with BMT performed in first CR. We have found that patients who relapse can be swiftly transplanted provided that the follow-up after marrow harvest is close thus insuring that relapse is diagnosed early. In our practice, this is accomplished by monthly bone marrow examinations and CBC in the first year after harvest.

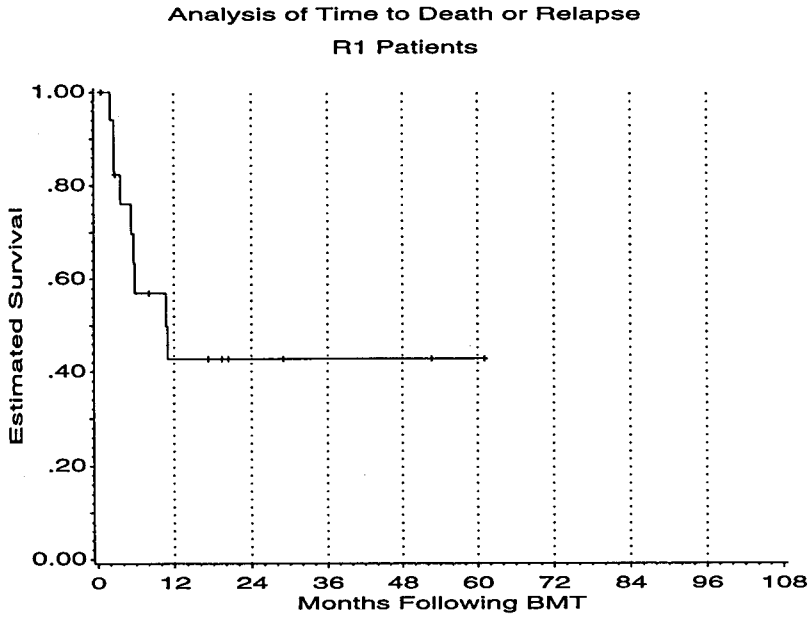

Figure 1. *Disease-free survival of 18 patients treated with ABMT using Bu/Cy and mAb-purged marrow in first relapse.*[2]

POST-TRANSPLANT IMMUNOTHERAPY

PM-81 Serotherapy in Patients with AML in first relapse and Second CR

Despite the controversy over which ABMT approach is best, it is sobering to note that up to 50% of transplanted patients eventually relapse. The failure to cure these patients deserves more attention and innovation. Post-ABMT immunotherapy is one approach under active study. Studies of mAb therapy, interleukin-2 infusion, natural killer cell augmenting pharmaceuticals such as linomide, and adoptive cellular therapy are underway. We are using mAb-based approaches to address this issue.

One of our approaches that is presently in phase I/II trials is discussed below.

PM-81 is an IgM mAb directed against the cellular differentiation antigen CD15, which is expressed on blast cells in 95% of AML patients.[9] PM-81 reacts with both normal granulocytes and monocytes and leukemic myeloid cells, and is capable of mediating complement (C')-dependent cytotoxicity in the presence of both rabbit and human C'.

We recently completed a study of PM-81 mAb serotherapy in patients with relapsed AML.[10] The study was a phase I design with the intention of establishing safety, feasibility and optimal biological dose of mAb. Patients were treated with escalating doses of purified PM-81 (MDX-11, Medarex, Inc., Annandale, N.J.) prepared under an IND approved by the United States Food and Drug Administration (FDA) using a continuous intravenous infusion over 24 hours followed by conventional dose chemotherapy.

Monoclonal Antibody Administration. Patients were hydrated with normal saline for 4 h prior to administration of PM-81, and premedicated with diphenhydramine and acetaminophen. PM-81 was administered intravenously through either a peripheral line or central line. Three patients received doses of 0.5 mg PM-81/kg, 8 patients received 1.0 mg/kg and 5 patients received 1.5 mg/kg all at an infusion rate of 10ml/hour.

Toxicities. Of the 16 patients entered onto the study, 13 received complete infusions of PM-81; the infusions of the remaining 3 patients were stopped within 30 minutes due to severe allergic reactions. In the 13 patients receiving the 0.5 or the 1.0 mg/kg dose, none suffered toxicities greater than grade II (ECOG). The majority of toxicities were minor allergic reactions that resolved with antihistamine therapy and/or decreasing the rate of infusion. One patient developed human anti-mouse antibodies noted 4 weeks post-infusion. This patient was treated with the 0.5 mg/kg dose of PM-81.

Effects of Treatment. Upon completion of the PM-81 infusion, 9 of 12 (75%) patients showed a decrease in WBC. Seven of the 9 patients with decreased WBC had a 50% or greater transient decrease in WBC. Blast counts at the end of infusion decreased for 7 of the 12 patients (58%), and 8 of 12 (73%) patients experienced a 50% or greater transient decrease in their blast counts. Significant transient decreases of WBC and blast counts were seen at all three PM-81 doses.

Pharmacokinetics. The dose of PM-81 received, along with the initial reservoir of CD15, whether cell-bound (on normal or leukemic cells) or soluble, affected the serum levels of PM-81. Patients receiving

the lowest dose of PM-81 (0.5 mg/kg) had no detectable serum mAb, while all patients receiving the highest dose (1.5 mg/kg) had quantifiable serum PM-81. Patients receiving the median dose (1.0 mg/kg) had detectable serum PM-81 levels. Serum PM-81 levels typically peaked between 12-24 h and returned to zero within 24 h after the infusion ended.

Patients with the lowest levels of serum CD15 at T=0 for their dosage achieved the highest serum levels of PM-81. As expected, patients had either free antibody or free antigen present in their serum at T=0 and T=24; in no case did we find both serum PM-81 and serum CD15 present simultaneously. Serum levels of CD15 were unrelated to the patient's initial WBC.

The percentage of peripheral leukemic blasts with cell-bound PM-81 was also an indicator of cell-bound PM-81 in marrow leukemic blasts. Those patients who had both quantifiable serum levels of PM-81 and >50% circulating blasts with cell-bound PM-81 had≥60% leukemic blasts in their marrow with cell-bound PM-81. Patients with lower percentages of circulating blasts with cell-bound PM-81 or serum levels of PM-81 that could not be quantified had a significantly lower percentage of cell-bound PM-81 positive blasts in their marrow (≤16%).

Conclusions. This phase I study shows that the infusion of mAb PM-81 was successful in decreasing the WBC of 75% of the patients treated, with more than half the patients experiencing a significant (>50%) transient decrease in their WBC. PM-81 was also successful in reducing the leukemic blast counts of over half the patients, with 67% experiencing significant transient decreases. These reductions were not dose-dependent or related to serum PM-81 levels.

The percent of CD15 positive leukemic blast cells remained fairly stable during the PM-81 infusion indicating no internalization or shift in antigen expression during PM-81 treatment. Shedding of surface antigen was also not apparent as serum CD15 levels generally decreased to 0 after 24h of mAb treatment, thus predicting that patients could be treated over a longer time period in future studies. Also, with only one patient developing HAMA, it is conceivable that some patients could be given subsequent mAb therapies without affecting pharmacokinetics or causing allergic reactions. It is likely that the study design of administering mAb prior to chemotherapy may have abrogated an immune response to mAb.

While mAb infusions are significantly less toxic than conventional chemotherapies, some side effects have been documented. Most common are fever, chills, and transaminasemia. Generally, allergic reactions to the

murine antibody occurs in less than 10% of patients, with 1% or less experiencing anaphylaxis.[11]

The results of this study indicate the need for patients to achieve serum levels of PM-81 to allow for access to the bone marrow compartment. While 1.0 mg/kg/24h of PM-81 was sufficient to achieve serum levels in some patients, those with high initial WBC, high tumor burden (unless the patient had a high percentage of blasts with low CD15 positivity), or high levels of serum CD15, required higher doses. Patients in the successor study may be able to achieve higher levels of serum PM-81 without significantly higher doses since their reservoir of CD15 will most likely be lower. In this phase I/II study that is underway, patients first receive conventional chemotherapies to achieve remission and lower the cell burden prior to PM-81 infusion. A bone marrow examination at day 14 must reveal a hypocellular marrow to go ahead with the mAb infusion. Although patients in our phase I/II study have lower initial WBCs and tumor burden, it is possible that these patients may still have high initial serum CD15 levels. Thus far, this study has shown that mAb administered during aplasia is better tolerated compared to the outcomes in the phase I study with no Grade 2 or greater allergic reactions in six patients. In the phase I/II study, patients will receive 1.0 mg/kg over a 24, 48 or 72 h period.

Bispecific Antibodies composed of anti-CD33 and anti-FcgRI and III (mAb 251 x mAb 22 and 3G8.

Another exciting approach to mAb-mediated antileukemia therapy is the use of bifunctional or bispecific antibodies that target both effector leukocytes and leukemia cells through cell surface antigens. We have prepared BsAb comprised of either the 3G8 mAb (anti-FcγRIII, expressed on NK cells) or the 22 mAb (anti-FcγRI) and mAb 251 (anti-CD33, expressed on myeloid leukemia cells, developed by EDB) using chemical linkage techniques. Briefly, the component mAb (3G8 or 22 and 251) were reacted with either of the bifunctional linkers, SATA or sulf-SMCC, and then co-incubated under conditions that allowed the creation of a disulfide bond thus creating a covalent link between the two mAb.

These two BsAb were capable of mediating ADCC of several NK-insensitive leukemia cell line targets (HL-60, NB4, and U937) using peripheral blood lymphocytes (PBL), adherent natural killer cells (A-NK), PMNs, and monocytes from normal volunteers as effector cells. Up to 78% cell lysis was achieved at effector:target ratios of 50:1 using monocytes and NK cells as effector cells (Figures 2 and 3). Somewhat

less, but significant, activity was seen with INFγ-incubated PMNs. Notably, resting NK cells were quite active with the BsAb 251x3G8 against cell lines without the addition of any exogenous IL-2 (Figure 4). Marked augmentation of killing by adherent-NK cells was observed.

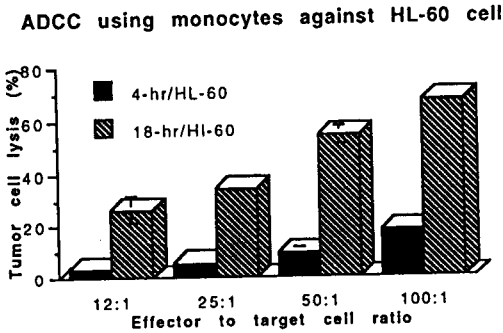

Figure 2. Lysis of HL-60 leukemia cells with BsAb (251x22) and IFNγ-activated monocytes.

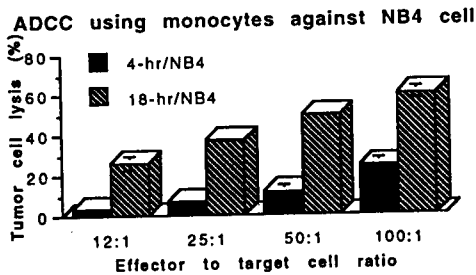

Figure 3. Lysis of NB-4 leukemia cells with BsAb (251x22) and IFNγ-activated monocytes.

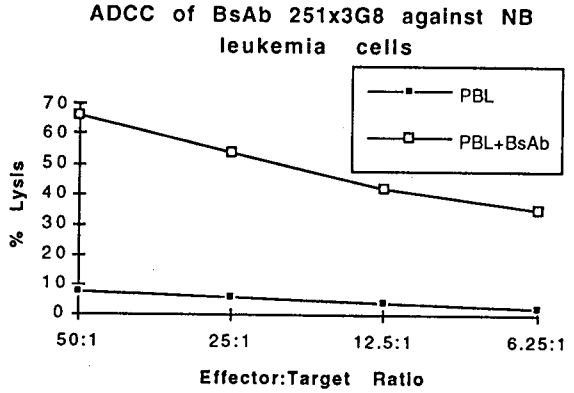

Figure 4. Lysis of NB-4 leukemia cells with BsAb (251x3G8) and Peripheral Blood Lymphocytes (no IL-2).

Peak activity with PBL effectors was observed with concentrations of BsAb of 0.1-0.5 μg/ml (Figure 5). In the absence of BsAb, only 5-10% lysis was observed with each of these effector cell populations. BsAb activity was inhibited by co-incubation with Fab fragments of each of the parental mAb, 3G8, 22 or 251, present in 10-fold excess.

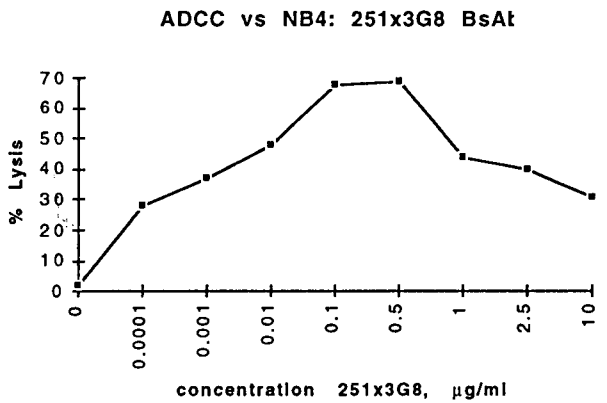

Figure 5. Concentration-dependence of ADCC of NB-4 leukemia cells with BsAb (251x3G8) and Peripheral Blood Lymphocytes.

An important feature of the mAb to FcγRI (22) is that its epitope is outside of the Fc binding site and thus not inhibited by immunoglobulin (Ig). We have shown the absence of blocking of the BsAb by the addition of Ig in this system under conditions where an IgG2a mAb directed to transferrin receptor (SCCL-1) was blocked from mediating ADCC. The implications of these findings are that *in vivo* binding of the BsAb will not be inhibited by endogenous Ig in contrast to a mAb engaging an FcR through its Fc domains.

We intend to develop these immunoconjugates for *in vivo* use in patients with CD33-positive AML and CML. An attractive strategy is to use the BsAb following ABMT during the engraftment period when augmented NK cell activity has been observed. The addition of IL-2 or G-CSF therapy may potentiate the effects of the BsAb and augment ADCC.

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PURGED AUTOLOGOUS BONE MARROW TRANSPLANTATION FOR ACUTE NONLYMPHOBLASTIC LEUKEMIA

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INTRODUCTION

Current combination chemotherapeutic protocols are able to achieve remission in 60 to 80% of patients with acute nonlymphoblastic leukemia (AML). Usual standard chemotherapies includes combination of continuous infusion of cytosine arabinoside; and daunomycin or idarubicin or mitoxantrone, often with other drugs. A multitude of modifications in dosage and scheduling over the last decade have failed to improve the long-term survival beyond 15 to 35%.[1-5] Use of various consolidation and maintenance schedules have also shown no significant benefit. One option is to explore the use of high dose intensification with either allogeneic or autologous bone marrow transplantation (BMT). Autologous BMT (ABMT) has been shown to be beneficial especially when combined with purging of harvested marrow using 4 hydroperoxycyclophosphamide (4-HC) and/or etoposide (VP-16). VP-16 has also been shown to be useful for treating patients with refractory or relapsed AML. We therefore used total body irradiation (TBI), VP-16 and cyclophosphamide (CY) as conditioning protocol; complete hematoablation was rescued with 4-HC and VP-16 purged marrow.[1,4]

MATERIALS AND METHODS

All patients with a confirmed diagnosis of AML in first or subsequent remission and without a HLA matched sibling were eligible for this study. An informed consent was obtained prior to commencing the therapy. A total of 38 patients have been included between February 1985 and January 1992. There were equal number of males and females (19 each) with a median (range) age of 36 (20-54) years. At the time of ABMT 21 were in first remission, 15 in second and 2 in third remission.

Bone marrow was harvested under general anesthesia in remission at a time convenient to the patient and physician (harvest was preferred in first remission). Of the harvested marrow, usually 2 x 10^8 mononuclear

cells per kg were purged with VP-16 (8.5 microM) and 4-HC (100 microM) for 60 minutes at 37°C as previously described and the remaining cells cryopreserved untreated as back up (if needed a second harvest was done to have at least 1 x 10^8 cells as back up). All patients except one had successful engraftment without requiring reinfusion of the back-up marrow.

Conditioning included 125 cGy hyperfractionated TBI thrice a day (day -10 to day -7), 250 mg/M^2 of intravenous VP-16 (day -6 to day -4) and 60 mg/kg of intravenous CY (day -3 and day -2). The purged and cryopreserved marrow was reinfused on day 0. Patients were followed up till death or relapse.[1] Statistical analysis was done using the method of Kaplan and Meier to plot disease-free survival (DFS).

RESULTS AND DISCUSSION

The actuarial DFS for all 38 patients is 45%. DFS showed no statistical difference between patients autografted in first versus subsequent remissions (Figure 1; p value=0.16). Survival was also not statistically different between the various age groups (Figure 2). A total of 17 patients are alive disease-free at present and the overall median DFS is 27 months. Of the 21 patients with adverse events, 15 relapsed and six (15.8%) had toxic deaths. These included infection (3), bleeding (2) and hepatic venoocclusive disease (VOD; 1). The median interval to the adverse event was 6.5 months (range 0.4 to 34.3 months).

Several studies have clearly shown the benefit of dose intensity in treating patients with AML. It is however not clear which therapeutic option gives best results.[6] One randomized study of 43 adults with AML given post consolidation therapy in first remission showed a 4-year event-free survival of 34% with allogeneic BMT, 45% with busulfan and 47% with ABMT. It is therefore clear that irrespective of the intensification chosen, the most important obstacle still remaining is the eradication of residual disease. Viable AML cells may persist in the host or could be reinfused with the autograft. There is evidence to show that both may be important. Our strategy was to devise a protocol that would address both these issues.

Currently, there is concern that newer BMT conditioning regimens are not truly myeloablative (hence, have the potential to leave behind viable tumor cells). However, the dose of TBI used by us is well established to lead to permanent aplasia. In order to enhance the tumoricidal effect, especially in resistant myeloblasts, we added VP-16 to

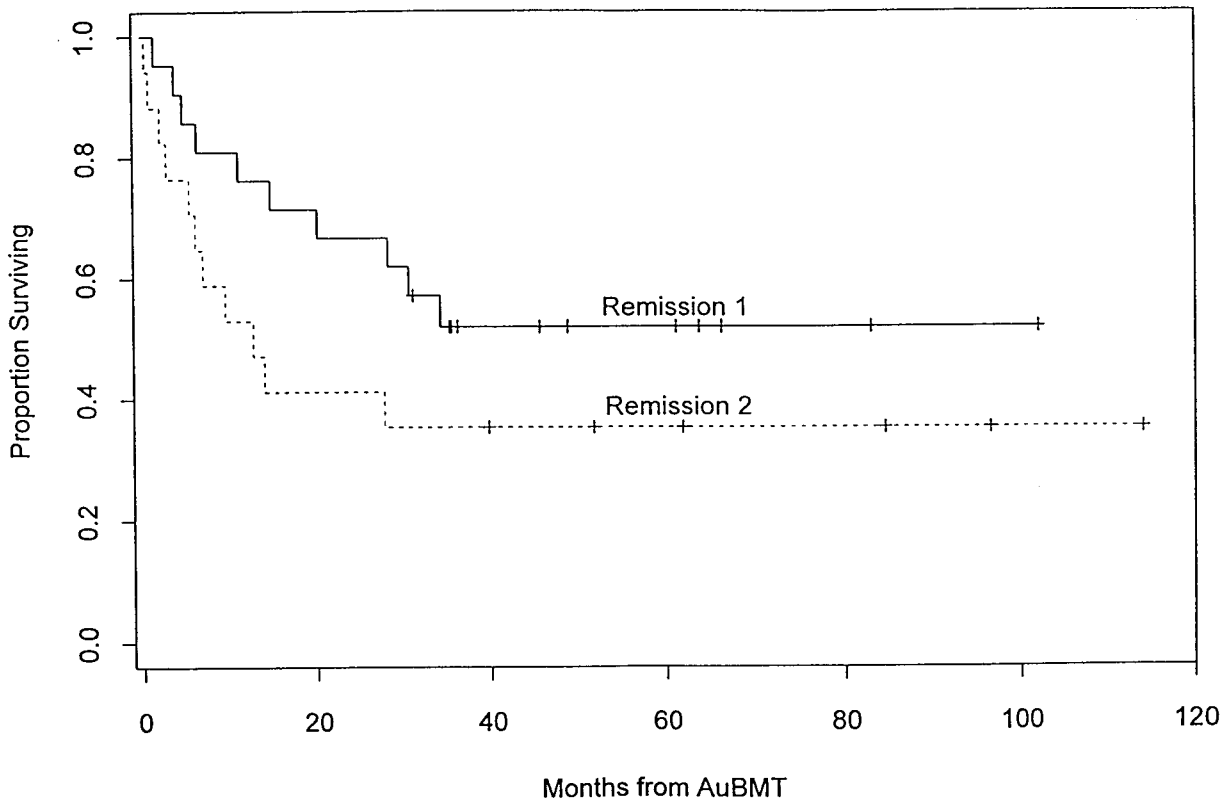

Figure 1. *Disease-free survival of patients autografted in first and subsequent remissions*

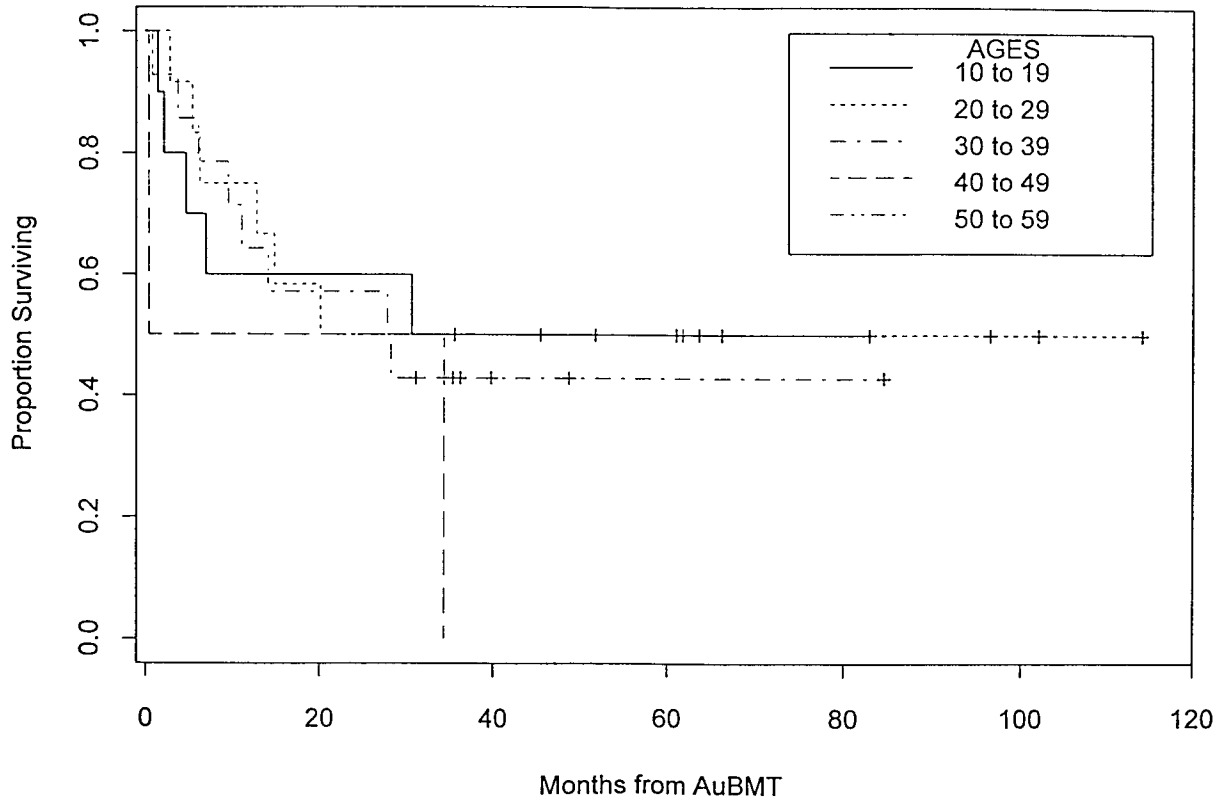

Figure 2. *Disease-free survival of patients autografted for AML by age distribution.*

the CY-TBI conditioning. In fact, VP-16 inhibits topoisomerase II and acts synergistically with alkylating agents.

Recent data show the benefit of purging in patients with bone marrow involvement in relapsed lymphomas. Persistence of bcl-2 marker by polymerase chain reaction (PCR) at the end of purging correlated strongly with a higher clinical relapse. Retrospective analysis in patients with AML also showed advantage in patients autografted within six months of the diagnosis and in first remission. This observation suggests that viable residual cells in the harvested marrow can lead to a relapse.[4,7] Furthermore, the current purging techniques (usually with 4-HC alone) were inadequate. Laboratory data has shown that 4-HC: VP-16 in the ratio of 1:0.342 is the best combination for purging AML marrow. We therefore decided to use this chemical method of negative selection. Other investigators have utilized methods of positive selection especially for normal CD34 stem cells enrichment. There are no consistent disease specific genetic markers to target for such detection. And even if such methods were available, the highly sensitive PCR methods of detecting minimal residual disease can still not detect as much as 15,000 contaminant cells in a usual bone marrow harvest of 2×10^8 mononuclear cells.

With an overall DFS of 45% and 60% for those autografted in first CR, our results are similar to or slightly better than those reported by Ranson et al.[2] It is difficult to separate the effects of TBI, CY, VP-16 conditioning versus 4-HC, VP-16 purging in our study.[4] Most of the toxic deaths were related to myelosuppression, only one being due to hepatic VOD. Our data suggests that use of VP-16 in conditioning and purging of ABMT (in combination with other modalities/drugs) is possible with acceptable toxicity. Our disease-free survival in first and second remission is not statistically different probably because of the small number of patients.

The role of ABMT is less controversial for AML in second remission. In this subset, most currently available purging have not been shown to be of significant benefit - probably because they have drug-resistant residual disease. Mafosfamide purging also has no significant benefit in patients achieving remission in less than 40 days. Presumably these patients had responsive disease which would be eradicated by induction chemotherapy and/or conditioning.[3,4] There is some data to show that the benefit of ABMT in AML is proven only for a small subset who take more than 40 days to achieve remission or are harvested within six months of diagnosis.[3] We are currently investigating the combination

of 4-HC with VP-16 as used in our purging method to decide on beneficial effects on specific subsets. Thus, our approach provides a useful new avenue in the treatment of patients with AML.

ACKNOWLEDGEMENTS

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ROQUINIMEX (LINOMIDE) IN AML AFTER ABMT - AN UPDATE OF TWO ONGOING RANDOMIZED STUDIES

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An unacceptably high rate of relapse continues to be a major problem in AML in remission after chemotherapy as well as after ABMT. A recent update of European registry data on 1282 ABMT recipients with AML in remission showed a relapse incidence of 52% in first complete remission (CR1) and 63% in second CR (CR2). The corresponding figures for allogeneic transplantation were 25% and 42% respectively.[1]

The allogeneic graft versus leukemia reaction (GVLR) appears to be the most important reason for the difference in relapse rates between allogeneic BMT and ABMT. Cytotoxic T cells and NK cells have demonstrated ability to kill leukemic cells in minimal residual disease, and may play important roles in the GVLR.[2-6]

Roquinimex (Linomide) is an orally active immunomodulatory compound that has shown therapeutic activity in preclinical models of autoimmune and malignant disorders. Roquinimex has been shown to enhance NK and T cell number and activity in patients with myeloid leukemia after ABMT.[7]

Aiming at determining if roquinimex can prolong the time to relapse or decrease the relapse rate in AML after ABMT, two multinational randomized double-blind placebo-controlled studies have been initiated (Figure 1).

CURRENT LINOMIDE STUDIES

Europe	CR	12-16D	***LINOMIDE***
AML →	***ABMT*** →		1 or 2 years treatment
USA Canada Australia		ANC >100	***Placebo***

Evaluation of:
Time to relapse
Relapse rate
Survival
Toxicity
Immune parameters

Figure 1. *Design of the studies of roquinimex (Linomide) in AML after autografting.*

EUROPEAN STUDY

The patients should have AML in first or subsequent remission obtained after any kind of induction and consolidation chemotherapy, be 10-65 years of age, and meet all institutional eligibility criteria for ABMT. Performance status should be 0-3, and the patients should not have any other disease which might substantially decrease the chance of survival, or have bilirubin, SGOT, SGPT, or creatinine levels of more than twice the upper reference value. All patients gave informed consent, and the study was approved at all the participating institutions' ethics committees, and by all health authorities in the participating countries.

Randomization between roquinimex and placebo was made prior to the ABMT, and study drug treatment started 12-16 days post ABMT. The patients were stratified by institution and between CR1 and CR>1. Remission was defined as <5% blast cells in a normocellular marrow, and relapse was defined as >10% blast cells, also that in a normocellular marrow. The initial dose was 0.05 mg/kg body weight twice during the first week, 0.1 mg/kg twice during the second week, and 0.2 mg/kg twice weekly thereafter for two years or until relapse, death or intolerable toxicity. Dose modifications were made in case of toxicity. Demographic data, patient history, and clinical and laboratory parameters were recorded. The study is still blinded, no information on the separate treatment arms is available.

Two hundred seventy-eight (278) patients have been randomized at 39 centers in 10 countries between February, 1991 and May, 1994. The numbers of males and females were similar, the median age was 39 years (range 11-64). Eighty percent of the patients were in their first remission, 2 patients were in CR3 and one in CR4. Sixty-four percent had FAB-class M1-M3, and 36% had M4-M7.

The most prominent toxicities have been nausea/vomiting, edema, musculoskeletal discomfort, headache, skin rash, diarrhea, fatigue, and pericarditis/serositis. Forty-five serious adverse events have been reported: 11 intracranial, 8 cytopenias, 8 with fever including hemorrhagic cystitis, 6 veno-occlusive disease, 4 cardiac including pericarditis, 3 gastrointestinal and hepatic, 2 mental, and one each of thrombosis, hypertriglyceridemia, and skin rash, respectively.

Of the first 255 patients, 91 are still ongoing, 76 have relapsed, 74 have died, and 17 have completed two years of treatment. Seventy-two (72) patients have been withdrawn; 43 due to adverse events, 15 due to patient refusal, and 14 for other reasons. The number of relapsed plus the

number of dead patients is 104 of 255, and the number of relapsed plus dead plus withdrawn is 147 of 255 patients. The median follow-up time is 18 months (range 4-40).

NORTH AMERICAN-AUSTRALIAN STUDY

This study is still accruing patients. Eligibility criteria are the same as for the European study, and the patients are being randomized to receive either roquinimex or placebo. The patients are registered and give their consent to participate prior to autografting, which may be with bone marrow or peripheral blood progenitor cells. Remission is defined as in the European study, but relapse is defined as >5% blast cells in the marrow. Extramedullary leukemia is a relapse criterion in both the European and the American-Australian studies.

In both studies, effective contraception is a strict requirement, since roquinimex may be teratogenic, as indicated by preclinical findings. Concommitant treatment with other immunomodulating agents, such as interferons, interleukin-2, cyclosporine, pentoxyfylline or GM-CSF or chemotherapy is not allowed. Corticosteroids, non-steroidal anti-inflammatory drugs, and ciprofloxacin should be avoided.

The patients are randomized when eligible for start of treatment, which is defined as when the absolute neutrophil count reaches 100 cells/μl post autografting. The dosage is the same as for the European study, with the difference that treatment is continued for one year only or until relapse, death, or untolerable toxicity. The same demographic, clinical and laboratory parameters as in the European study are recorded. Informed consent is obtained from all patients, and the study has been approved at all the IRBs/Ethics Committees at participating institutions, as well as by the health regulatory authorities in the US, Canada, and Australia. The study is blinded, and no information is available regarding the separate treatment arms.

Two hundred (200) patients have been registered, and 183 have been autografted. Twenty-seven (27) have relapsed, died or been withdrawn prior to randomization. One hundred fifty-seven (157) patients have been randomized at 20 centers in the US, 5 centers in Canada, and 9 in Australia. The patient population is similar to that in Europe, except that slightly more patients have received purged marrow or have been in a later stage of their disease. Further, a minority of American and Australian patients have been autografted with blood derived progenitor cells. Adverse events have been similar to what has been reported for the

European study. Forty-three (43) patients have relapsed, including 25 of 73 who have been followed for more than one year.

SUMMARY

In order to assess the potential role for immunotherapy of AML with the immunomodulator roquinimex in the autologous setting, randomized studies have been designed. Patients who have undergone autografting for AML in remission are being treated with roquinimex (Linomide) or placebo during one or two years in order to determine if this compound can increase the time to relapse or decrease the rate of relapse. Since observation time is too short and the studies are blinded, no conclusions can be made regarding efficacy in the entire group or in the actively treated patients.

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AUTOLOGOUS TRANSPLANTATION IN PATIENTS WITH ACUTE MYELOID LEUKEMIA IN FIRST REMISSION WITH IL-2 CULTURED MARROW OR PERIPHERAL BLOOD STEM CELLS FOLLOWED BY *IN VIVO* IL-2

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INTRODUCTION

Two factors are believed to contribute to the higher relapse rates seen after autologous BMT as compared to allogeneic BMT. First, autologous marrow, even when harvested in complete remission (CR), may contain enough residual leukemic stem cells to reestablish disease. Studies using genetically marked marrow autografts to determine the origin of relapse after autologous BMT have now shown unequivocally that relapse can originate from such residual leukemic cells present in the autograft.[1] This result strongly suggests that some form of marrow manipulation (i.e., purging) is essential. Second, after allogeneic BMT, T-lymphocytes from the donor can induce a cascade of antileukemic effects that have collectively been termed graft-versus-leukemia (GVL) effect.[2-4] Autologous BMT is not associated with a GVL effect. The fact that the probability of relapse after autologous BMT is similar to that seen after BMT from a twin, where a known normal marrow is transplanted, further supports the contention that residual leukemia remaining in the patient contributes in a significant way to relapse after autologous BMT. One way to try to compensate for the loss of a GVL effect after autologous BMT might be to increase the dose of radiation and chemotherapy given in preparation for BMT. However, this also causes an increase in regimen related toxicity to other tissues with the result that overall survival is not improved.[5] This has triggered considerable interest in the potential of immunotherapeutic approaches as these should be non-crossreactive and hence additive with the effects of chemotherapy and radiation and are thought to be particularly effective against residual disease.[6]

Although favorable results with respect to disease-free survival and relapse rate after autologous BMT have been reported in some studies,[7-13] such data need to be carefully analyzed with attention given to whether results reflect data from patients with 'standard risk' as well as others with 'high risk' features. Generally, relapse rates are high (50-70% within the first two years after BMT) in patients receiving a marrow transplant in first remission when 'high risk' features are present at diagnosis.[8-10] Analyses from chemotherapy and BMT trials have shown that 'high risk' can be defined as having: 1) a high number of peripheral blasts at diagnosis; 2) chromosomal abnormalities (except t(8;21), inv16, t(16;16), t(15;17)); 3) morphological features compatible with FAB M5 (monocytic); and 4) older age.[14-16] Conversely, patients with certain 'good risk' features (particularly FAB M3, t(15;17) generally have a lower relapse rate after autologous BMT.[17]

Autografts of peripheral blood progenitor cells (PBPC) obtained after mobilization with hematopoietic growth factors offer the additional advantage of giving a more rapid neutrophil and platelet recovery. However, in AML their use has also been associated with high relapse rates. The two studies in patients with AML in first remission have reported relapse rates of 60%[18] and 70%[19] at 2 years, respectively. The cause of these high relapse rates is not known but might be related to the higher nucleated cell numbers given with PBPC transplants and a possibly higher number of contaminating leukemic cells. Further purging (either pharmacological or with antibodies) of PBPC autografts has generally not been undertaken, as it is technically difficult to process the large number of cells involved. However, if the problem of high relapse could be overcome, the faster engraftment with PBPC transplants would clearly make them a preferred source of stem cell for transplantation.

Several years ago we showed that human AML cells maintained in culture without growth factors showed a selective decline in clonogenic leukemic cells with persistence of their normal counterparts.[20] More recently, studies in a murine model suggested that the infusion of marrow which has been activated by IL-2 for a few days prior to infusion could improve the survival of mice previously inoculated with the myeloid leukemic cell line C1498.[21] In order to achieve a durable antileukemic effect, it was also necessary to administer IL-2 to these mice immediately after infusion of the IL-2 activated bone marrow. Such a strategy thus builds on earlier studies from our own center[20] as well as those by Forman et al[22] and Long et al[23] by combining *in vitro* purging and an *in vivo* adoptive immunotherapy component after marrow infusion.

RESULTS AND DISCUSSION

Based on the above, we undertook a series of studies to develop a procedure that would incorporate the beneficial features of each of the above findings and also be feasible for an AML autografting protocol. Instead of culturing marrow with IL-2 for 48 hours,[21] we investigated the effect of culturing it for 8 days since this would allow the conditioning regimen to be given without requiring cryopreservation of the autograft. We already had previous positive experience with autografts of marrow that had been cultured for 10 days without IL-2.[24] In addition, it was anticipated that a greater purging effect might be achieved with a more prolonged culture period and also that a better activation of immune effector cells would occur. The preclinical experiments performed indicated that the cytotoxic activity of marrow/PBPC from patients with AML in first remission after *ex vivo* culture for 8 days in IL-2 is equivalent to that seen with normal human marrow[25] and that the number and functional performance of committed and more primitive hematopoietic cells is not affected by culture for 8 days in IL-2. Finally, the culture conditions lead to both the activation of effector cells with antileukemic activity and the release of antileukemic cytokines into the culture supernatant.[26]

On the basis of these findings, we initiated a phase I study using marrow autografts from patients with AML in first remission with 'high risk' features (as defined above) in which the marrow cells were cultured for 8 days in the presence of IL-2.[27] Escalating doses of IL-2 were also administered to the patient for the first week. Ten patients were treated and the results can be summarized as follows: 1) posttransplant IL-2 doses of up to 6 x 10^5 BRPM units/M^2/day (administered subcutaneously) were tolerated by patients in the first week posttransplant; 2) although this trial was not designed to provide efficacy data, the low relapse rate to date of 22% (median follow-up of 26 months) compares favorably with results published by other centers (Figure 1) considering that these were patients who had poor prognostic features, had not received any consolidation treatment and had received a transplant (with one exception) within 6 weeks of entering first remission; 3) neutrophil and platelet recovery was delayed with a median of 49 days to achieve an absolute neutrophil count of 0.5 x 10^9/L and 98 days to achieve a self-sustained platelet count of 20 x 10^9/L.

Although the recovery of neutrophils in this study was not different from what had been reported after transplantation of AML

patients with autologous marrow purged with 4-HC or even unpurged marrow,[28] it was felt that the protocol would not be generally useful unless it could be modified to achieve a faster hematologic recovery. Since earlier studies had shown that patients with AML could be engrafted using PBPC autografts collected after G-CSF mobilization,[18,19] we autografted the next 6 patients with G-CSF mobilized PBPC as a supplement to the IL-2 cultured marrow. Recovery of neutrophils (median 15 days) and platelets (median 22 days) in these was faster than in the initial 10 patients who had received marrow only (Table 1).

Table 1. Hematologic Recovery Parameters in Patients Given IL-2 Cultured Bone Marrow plus G-CSF Primed PBPC (not cultured)

UPN	ANC ≥0.5 x 10^9/L	Platelets ≥20 x 10^9/L	Discharge (Day)	Outcome
845	16	16	16	Rem, 15mos
873	n/e#	n/a	n/a	Died, Day +1
883	15	12	16	Rel, Day +77 (Died D + 177)
890	12	77*	82	Rem, 9mos
919	25	--@	n/a	Died, Day 70@
936	10	33	22	Rem, 5mos

 # - patient died on day 1 during the blood stem cell infusion of heart block that was felt to be related to DMSO toxicity;

** - platelet study;*

@ - died of gastrointestinal bleed and multiorgan failure, received platelet transfusions despite having achieved selfsustained platelet count ≥20 x 10^9/L;

Abbreviations:

ANC=absolute neutrophil count; Rem=remission; Rel=relapse; UPN=unique patient number.

Hematopoietic assays confirmed that the anticipated significant reduction in the number of hematopoietic progenitor cells in the bone marrow of leukemic patients in first remission by comparison to normal values suggesting why recovery times post-BMT might have been delayed in the first 10 patients who were given marrow only. Moreover, the numbers of these cells present in the G-CSF mobilized PBPC harvests obtained by leukapheresis were found consistently to be several times higher than in the marrow collections from the same patients (Table 2). Furthermore, cytotoxicity and growth inhibition studies suggested that the ability to purge leukemic cells is about 8 times higher in blood than in marrow.[29]

Table 2. Progenitor Cell Content of Bone Marrow vs PBPC for AML in First Remission

UPN	CFC ($x10^3$/kg)		LTC-IC ($x10^3$/kg)	
	BM	PBPC	BM	PBPC
845	0.8	5.5	0.12	10.3
873	1.3	6.9	1.50	1.7
883	0.7	21.6	0.04	5.3
890	1.0	28.3	0.07	8.2

Abbreviations:
UPN=unique patient number; BM=bone marrow;
BPC=blood progenitor cells; CFC=colony forming cells;
LTC-IC=long-term culture-initiating cells;
Results represent the number of CFC and LTC-IC of 3 consecutive leukaphereses performed on day 5-7 of G-CSF treatment

On the basis of these data, we chose to eliminate the use of marrow altogether and substitute in its place an equivalent number of IL-2 activated, G-CSF mobilized PBPC. Thus, part of a 3-day PBPC harvest was cultured in IL-2, and the remainder not treated with IL-2 but cryopreserved and infused on day +1. Results obtained in 3 patients treated in this way (Table 3) suggested that relatively swift and stable engraftment could be achieved with the infusion of such PBPC autografts (without marrow).

Table 3. Hematologic Recovery Parameters in Patients Given G-CSF Primed PBPC Cultured in IL-2 Plus Additional Cryopreserved PBPC (not cultured)

UPN	ANC ($\geq 0.5 \times 10^9$/L)	Platelets ($\geq 20 \times 10^9$/L)	Discharge (Day)	Outcome (Months)
915	32	50	47	Rem, 7mos
929	49	56	54	Rem, 5mos
951	12	51	33	Rem, 3mos

Parallel *in vitro* studies demonstrated that the concentration at which the PBPCs are cultured *ex vivo* in IL-2 could be increased from 1 x 10^6/mL to 3 x 10^6/mL without compromising either hematopoietic progenitor maintenance or immune effector cell activation but, of course, this triples the number of cells that can be cultured in a given volume. As

a result, most of the cells obtained in 2 days of leukapheresis can be cultured in IL-2 in a maximum volume of 10 L, with enough cells leftover for 1 x 10^8 cells/kg non IL-2 treated PBPC to be cryopreserved as a backup in case of primary graft failure (defined as ANC <0.1 x 10^9/L by day 28 post-PBPC infusion). Two patients have now been treated according to this modified protocol and have shown stable neutrophil recovery on day 23 and 44 after infusion respectively.

Thus, the initial feasibility of using PBPC cultured in IL-2 for 8 days as an exclusive source of cells for the hematologic rescue of AML patients given myeloablative chemotherapy has been established. The important modification compared with our previous study lies in the fact that patients can now be given only PBPC that have been subjected to the *ex vivo* purging and activation procedure and will not receive any untreated/purged cells.

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AUTOLOGOUS TRANSPLANT FOR ACUTE MYELOGENOUS LEUKEMIA IN FIRST REMISSION USING MARROW PURGED WITH MAFOSFAMIDE

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INTRODUCTION

Autologous bone marrow transplantation (ABMT) is a therapeutic approach that permits the administration of high-dose chemoradiotherapy followed by the infusion of patient's own marrow, previously collected during remission and cryopreserved.[1,2] Following ABMT, a variable relapse rate has been documented due to either the inability of conditioning regimens to eradicate "*in vivo*" minimal residual disease, or the persistence of malignant clonogenic cells in the reinfused marrow cells, or the lack of a graft-versus-leukemia effect.[3-7] Recently reported data obtained by marking autografted cells with the neomycin-resistance gene have shown that, at least in acute myelogenous leukemia (AML), relapse is due to reinfused leukemic cells.[8] This experimental evidence strongly supports a role for marrow purging to achieve elimination of residual malignant cells from the graft. The rationale for purging is based on the concept of different sensitivity, different specificity, or different behaviour between leukemic and normal stem cells when exposed either to chemical as well as immunological agents or cultured *in vitro*.[9] Various *in vitro* purging methods available result in a 4-6 log cell kill *in vitro* and in 1 log reduction in tumor burden.[4]

The most widely used agents for pharmacological purging are the active metabolites of cyclophosphamide, such as 4-Hydroperoxycyclophosphamide (4-HC), and the more stable compound mafosfamide. By using clonogenic assays, it has been demonstrated that mafosfamide inhibits in a similar dose-dependent manner the *in vitro* growth of normal CFU-Blast, CFU-GEMM, BFU-E, CFU-GM, as well as the leukemic progenitors CFU-AML.[10-12] It is now well established that normal CFU-Blast as well as CFU-GM express wide variation in sensitivity to mafosfamide.[11,12] In a group of AML patients studied at our institution, the dose of mafosfamide inducing 50% CFU-Blast growth

inhibition ranged from 61 to 146 μg/ml.[12] This demonstration has been used to establish procedures of individual adjustment of the dose of mafosfamide used for marrow purging in order to decontaminate the marrow with the maximum tolerated dose of mafosfamide. Recently, 4-HC has been reported to cause internucleosomal DNA fragmentation of leukemic cells, a typical feature of apoptosis.[13] Moreover, in a murine model, substantial data suggest that chemical purging may activate immune effector cells important for the control of hematologic malignancies after bone marrow transplantation.[14] Natural Killer (NK) cells constitute an immunocompetent cellular subpopulation with a regulatory function on hemopoiesis and a capability to exert immune surveillance on neoplastic cell growth.[15] In the allogeneic BMT setting, it is strongly suggested that a graft-versus-leukemia effect operates via the activation of a nonspecific effector system.[7] NK cells have been considered as effectors of an immune response that could play a possible role in preventing relapse after BMT.[16] Studies aimed at evaluating the recovery of immunocompetent cells following BMT demonstrated a rapid recovery in the number and function of NK cells after allogeneic BMT, whereas the findings have been controversial after ABMT, especially in relation to the different types of purging procedures employed (pharmacological or immunological).[18,19]

In this paper, we report our clinical results obtained in AML patients autografted with marrow purged either at standard- (100 μg/ml/20x10^6 buffy-coat cells) or adjusted-dose (concentration of mafosfamide inducing 50% inhibition of CFU-Blast growth mafosfamide). In addition, NK cell regeneration was investigated in a group of patients receiving marrow purged with mafosfamide at adjusted-dose.

PATIENTS AND METHODS

Patients

Forty-five patients with AML undergoing ABMT with marrow purged at standard- (n=21) or adjusted-dose (n=24) were included in this study. Patient characteristics are shown in Table 1. Patients (males = 23; females = 22) ranged in age from 9 to 56 years (median 35 years). All were transplanted in first complete remission defined as <5% bone marrow blasts on marrow aspirate.

Table 1. Patients Characteristics

	Standard-Dose (N=21)	Adjusted-Dose (N+24)
Age (years)		
Median	31	36
Range	9 - 48	19 - 56
Sex (Male/Female)	11/10	12/12
FAB Classification		
M1 (%)	15	21
M2 (%)	35	28
M3 (%)	12	15
M4 (%)	25	21
M5 (%)	13	15
Interval remission to harvest (months)		
Median	3	5*
Range	1 - 10	1 - 14
Interval remission to ABMT (months)		
Median	3	6*
Range	1 - 12	1 - 18
Pretransplant regimens		
TBI-CY	7	3
BU-CY	14	21

* $P \leq .025$

Conditioning Regimens

Thirty-five patients were conditioned with busulfan (BU) 4 mg/kg x 4 days followed by cyclophosphamide (CY) 50 mg/kg x 4 days and ten patients with CY 50 mg/kg x 4 days or 60 mg/kg x 2 days, plus total body irradiation (10 Gy single dose or 12 Gy fractionated dose).

Marrow Purging

Harvested marrow was centrifuged (2,500 rpm, 20 min) and the buffy-coat cells collected and resuspended ($20x10^6$ cells/ml) in TC199 medium (80%, v/v) supplemented with autologous plasma (20%, v/v). The hematocrit of the cell suspension was always <5%. Mafosfamide (ASTA Werke, Bielefeld, FRG) was provided as a lyophilized powder to be reconstituted with saline. Marrow cells were incubated with the drug (30 min, 37°C) with gentle agitation every 5 min, then the reaction was stopped by immersion in ice-cold water (4°C). When the standard purging

technique was used, mafosfamide concentration was 100 μg/ml/20x10^6 buffy-coat cells. The method of adjusted-dose purging has been described in detail elsewhere[12] and was performed on an individual basis aimed at evaluating mafosfamide-induced growth inhibition of primitive adherent blast colong-forming unit (CFU-Blast). Marrow was purged with the dose of mafosfamide resulting in 50% inhibition of CFU-Blast growth (ID_{50}). With this technique the value of CFU-Blast ID_{50} has been shown to be significantly higher ($P \leq 0.05$) than CFU-GM ID_{95} value.[12] After purging, marrow cells were resuspended (40x10^6/ml) in irradiated autologous plasma (55%, v/v), TC 199 (35%, v/v) and DMSO (10%, v/v). After cryopreservation performed at a controlled rate, marrow cells were stored in the liquid phase of liquid nitrogen.

Clinical Monitoring

After marrow infusion all patients were hospitalized in laminar air flow rooms and supported with irradiated platelets and packed red cells. The patients were routinely given transfusions when hemoglobin values were less than 8.0 g/dl, and received platelet transfusions when platelet counts were less than 20 x 10^9/L. Patients were regularly started on broad-spectrum intravenous antibiotics when the absolute neutrophil count fell below 500 x 10^6/L. They were monitored with daily physical examinations, daily blood counts, electrolyte measurements, kidney and liver function tests. Additional laboratory tests of serum were performed three times a week.

Immunological Monitoring

In order to investigate the immune effects induced *in vivo* by mafosfamide purging, NK cell number and activity were evaluated before ABMT and monthly thereafter. The surface antigen phenotype was evaluated with the monoclonal antibody CD16/Leu-11b (Becton Dickinson, Mountain View, CA, USA). Phenotypic analysis was performed with a FACSort flow cytometer (Becton Dickinson). Data were processed with a Hewlett-Packard personal computer using Lysis II software. Cytotoxic activity was measured in standard 4 hour 51Cr release assays at effector (E) to target (T) cell ratios of between 100:1 and 1:1. The K562 cell line was used as target for fresh NK cell activity.

Statistical Analysis

Disease-free survival (DFS) and probability of relapse (PR) were calculated by the Kaplan-Meier method. Differences between groups were determined by means of the log-rank test.

RESULTS

The main characteristics of the patients receiving standard- or adjusted-dose purged autografts are summarized in Table 1. There were no significant differences between the two groups in terms of sex, disease phase, FAB classification or pretransplant regimens. In the group of patients grafted with adjusted-dose purged marrow, the intervals from achieving remission to marrow collection (5 vs 3 months) and the interval from achieving remission to ABMT (6 vs 3 months) were significantly ($P \leq .025$) longer as compared to patients grafted with standard-dose purged marrow.

Twenty-four of 45 patients survive, with a median follow-up duration of 19 months (range 1 to 114). Nine of 21 patients transplanted with standard-dose purged marrow and fifteen of 24 patients transplanted with adjusted-dose purged marrow survive without evidence of recurrent leukemia. Two patients grafted with standard-dose purged marrow and three patients grafted with adjusted-dose purged marrow died of transplant-related toxicity. The DFS for patients grafted with standard- or adjusted-dose purged marrow were 47% (median follow-up duration 18 months, range: 2 to 114) and 68% (median follow-up duration 23 months, range: 1 to 96), respectively (Fig. 1). The difference between purge adjusted vs purge standard was statistically significant ($P < .05$). The probability of relapse for patients grafted with standard-dose and adjusted-dose purged marrow were 48% and 29% ($P < .05$), respectively (Fig. 2).

To test the possible immune-enhancing effect of mafosfamide, pre- and post-transplant modifications of NK cells were monitored in 8 AML patients by evaluating the NK functional activity and the percentages of circulating CD16+ cells (Fig 3). The mean (±SE) pre-transplant value of NK activity was 32.5±6%. Cytotoxic activity reached pre-transplant values two months after ABMT (34±4.5%, $p \leq 0.4$) and showed a significant and persistent increase at 4 (42.5±3%, $p \leq 0.025$), 6 (48±3%, $p \leq 0.025$) and 12 months (54±6%, $p \leq 0.05$). The mean (±SE) pre-transplant value of CD16+ cells was 5±1.3%. This value increased progressively up to 6.7±2% ($p \leq 0.375$) at 2 months, 8.4±2.2% ($p \leq 0.05$) at

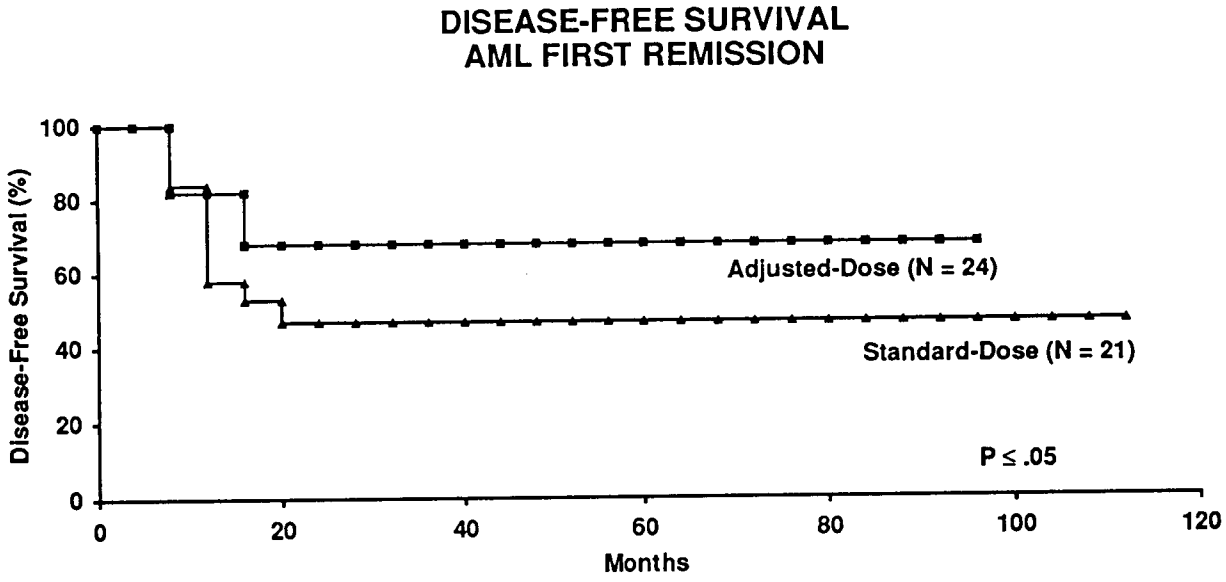

Figure 1. *Disease-free survival of patients autografted in first complete remission with marrow purged with mafosfamide at standard- or adjusted-dose.*

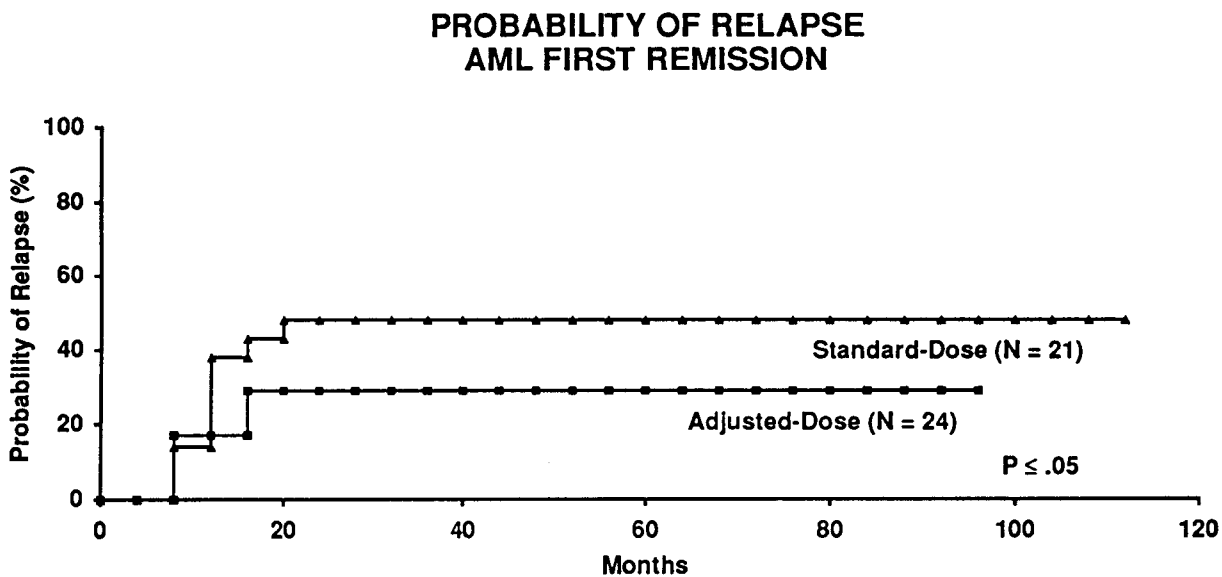

Figure 2. *Probability of relapse of patients autografted in first complete remission with marrow purged with mafosfamide at standard- or adjusted-dose.*

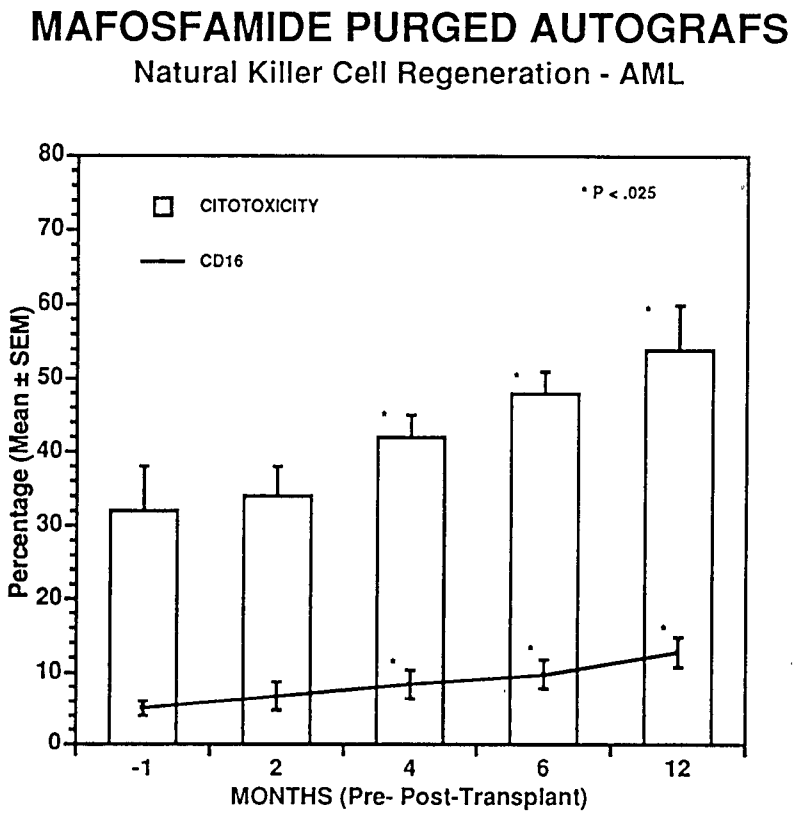

Figure 3. *Values of mean (±SEM) cytotoxic activity and CD16 cells before and after autologous bone marrow transplantation purged with mafosfamide at adjusted-dose.*

4 months, 9.8±2.4% ($p \leq 0.005$) at 6 months and reached 12.8±2.4% ($p \leq 0.005$) at 12 months.

DISCUSSION

High-dose radio-chemotherapy followed by ABMT is increasingly used to consolidate remission in hematological malignancies.[1-3] To prevent the risk of reinfusing tumor cells that might contribute to relapse, several strategies have been proposed to purge the graft of residual malignant cells. The strong requirement for efficient marrow purging approaches is now supported by studies employing gene marking techniques.[8] These studies have demonstrated that autologous marrow harvested from leukemia patients in complete remission contains malignant cells capable of inducing relapse.[8]

This paper reports the results updated to June 1994 of 45 AML patients autografted with either standard- or adjusted-dose purged marrow. The main aim was to give general figures on the value of marrow purging at standard or adjusted dose for ABMT in AML in first remission, both in terms of DFS and probability of relapse. In keeping with our previous analysis,[19] individual mafosfamide dose adjustment significantly increases DFS and decreases the probability of relapse as compared to standard-dose purge. These data are also in agreement with results reported by Gorin et al.[20,21]

Cyclophosphamide has been reported to modulate a number of immune responses *in vitro* and *in vivo*.[14,22] When cultured with cyclophosphamide, normal lymphocytes had an increase in NK cell activity in comparison to lymphocytes cultued in the absence of cyclophosphamide.[22] In a mouse model, an immunologic activity of mafosfamide has been demonstrated.[14] Since an enhanced regeneration of NK cells posttransplant could play a relevant role in the control or eradication of minimal residual disease, we evaluated the immune effects exerted by mafosfamide purging in patients undergoing ABMT. AML patients grafted with purged marrow showed a significant and long-lasting increase in NK function and number, still persisting after twelve months posttransplant. In keeping with findings reported herein, eight AML patients transplanted with unpurged marrow have been shown to lack any increase in K562 killing.[17] From these data, and considering that none of our patients showed any evidence of viral reactivation that could explain earlier and faster activation of the immune system,[23] it seems reasonable to ascribe the documented increase in the NK activity to the purging

procedure with mafosfamide.[24] Therefore, mafosfamide acts not only through a potent killing effect on clonogenic cells but also by enhancing NK cell regeneration. The NK cells, in fact, were fully functional as measured by lytic activity to K562 targets, and moreover normal to high cytotoxicity to target cells was maintained throughout the posttransplant period. The long-lasting increase in NK number and function observed post-ABMT could imply a graft-versus-leukemia effect able to play a major role in the control of minimal residual disease.

In conclusion, our data demonstrate that: a) as compared to standard-dose purge, adjusted-dose purge significantly affects DFS and PR; b) mafosfamide enhances posttransplant NK regeneration; c) collection and *ex vivo* expansion of NK cells by using immunostimulatory cytokines, such as IL-7 and IL-12, could be proposed as a posttransplant therapy in high-risk AML patients; d) randomized trial testing the efficacy of marrow purging are now ongoing.

ACKNOWLEDGEMENTS

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SESSION II: ALL

CHEMOTHERAPY IN ADULT ALL - STATE OF THE ART

D. Hoelzer

In adult acute lymphoblastic leukemia the complete remission (CR) rates are 65% - 85%. They can be achieved with cycling combination chemotherapy, usually including vincristine (VCR), prednisolone (PRED) and an anthracycline antibiotic with or without L-asparaginase, cytosine arabinoside (Ara-C) and cyclophosphamide (CPA). These high CR rates are not only beneficial for further chemotherapy but also for bone marrow transplantation (BMT) where a CR is still considered to be a prerequisite.

Prognostic factors known to influence the outcome of ALL, such as initial white blood cell count, organ involvement (e.g., CNS or mediastinal tumor, immunophenotype, cytogenetic aberrations and other variables), no longer have an impact on the CR rate. The only exception is age, which shows a strong inverse correlation. In adolescents the CR rate approaches 85% - 90% whereas it decreases to 40% or less in ALL patients over 60 years.

Leukemia-free survival (LFS) rates in adult ALL at 5 or more years in a large series of nearly 5000 patients range from 20% - 35%. There is no substantial improvement of the overall cure rate in the recent decade, but significant changes are observed in ALL subgroups. The outcome for adult ALL should therefore be considered within these specific groups (Table 1).

Table 1. Recent Chemotherapy Results in Adult ALL

Subgroup	Incidence	No. Pts	CR Rate	MRD	LFS
Overall		4802	76% (64-85)	23 m. (11-32)	32% (18-40)
Risk group					
High	70%			8-13 m.	20-25
Low	30%			72-NR	50 or >
Age					
15-20			82-95%		32-65%
20-50			80%		35%
50-60			40-70%		0-20%
>60			37-63%		0-2%
Subtype					
T-cell ALL	24%	367	83% (61-90)	27 m. (6-NR)	46% (26-60)

Table 1. (cont'd)

Subgroup	Incidence	No. Pts	CR Rate	MRD	LFS
common ALL	52%	702	78% (64-90)	26 m. (16-36)	34% (30-43)
B-cell ALL	3%	73	72% (63-83)		51% (20-62)
My+ ALL	19%	208	77% (29-80)		0-37%
Cytogenetics					
Ph[1]/BCR-ABL+	24%	264	64% (44-76)	8 m. (5-11)	0-16%

* *Pooled data from published literature*

Low Risk versus High Risk Patients

Low risk is defined in several adult ALL series by the following characteristics: younger age (at least below 50 years), time to CR within 4/5 weeks, lower leukocyte count, T-ALL subtype, and Philadelphia chromosome/*bcr-abl* rearrangement (Ph/*bcr-abl*) negativity. Such patients constitute a selected series of about 30% of the patients and their survival rate is 50% or even higher in some studies.[1,2]

High risk patients are defined by increased age, late achievement of CR (>4/5 weeks), high white blood cell count, and Ph/*bcr-abl* positivity detected by cytogenetic or molecular analysis. These patients constitute about 70% and their survival is 20% - 25%.

The relativeness of such risk definition is evident. Mature B-ALL, a rare but aggressive subtype with high risk of relapse, has changed its outcome from a survival of less than 10% to approximately 50% following treatment with short, intensive, high dose chemotherapy cycles. Are these patients still high risk patients?

Age

As for achievement of CR, age has the greatest impact on the outcome of adult ALL. Survival decreases from up to 60% in adolescents to a survival rate of nearly 0% in patients above 70 years. This has an implication for the outcome after chemotherapy with regard to overall survival, depending on what age groups are included in a reported series.

Immunophenotype

There have been several reports on the role of immunophenotype as an independent prognostic variable. However, with change in treatment and improved results, e.g., for T-ALL and mature B-cell ALL, the immune

phenotype is now more often used for subtype adjusted treatment strategies.

T-ALL. This subtype, usually associated with predominance of male sex, high WBC count, CNS infiltration, and mediastinal tumor, formerly had a poor outcome in children as well as in adults. The MRD was 10 months or less. Results for adult T-ALL have improved with CR rates of up to 95% and LFS has increased to 45 - 60%.[3] Sufficient *in vivo* and *in vitro* evidence has accumulated that CPA and Ara-C are mainly responsible for this improvement. The inclusion of Ara-C and CPA pulses during continuation therapy was beneficial in childhood T-ALL.[4] Also, in adult ALL the combination of Ara-C and CPA added to the conventional drugs improved CR rate and LFS in T-ALL.[5,6]

In recent studies the latest relapses for T-ALL occur at approximately 3 years. The optimal duration of treatment for T-ALL is as yet undefined as is also the value of a conventional maintenance therapy with MP and MTX, with or without additional treatment.

B-lineage ALL. This comprises, according to the stage of differentiation, the subtypes pre- pre-B-ALL, common ALL, pre-B-ALL and mature B-ALL.

Common ALL is the most frequent subtype in adults (52%) as well as in children (65%). The outcome has not changed very much in recent years and the survival remains 25 - 35%. This may be partly explained by the fact that about 45% of adult patients with c-ALL are Ph/*bcr-abl* positive ALL's[7] whose prognosis remains poor. However, for adults with Ph/*bcr-abl* negative, c-ALL results have not greatly improved, at least not in the German multicentre ALL (GMALL) studies (>700 c-ALL patients). In particular, most studies indicate that these patients relapse over a period of up to 5-6 years. It seems that some of the intensified regimens, particularly with short intensive treatment cycles such as high dose Ara-C, may not have had a substantial benefit for c-ALL.

Pre-B-ALL also includes Ph/*bcr-abl* positive cases; the outcome is similar to that for common ALL but has not been analyzed separately in most adult ALL series.

Pre-pre-B-ALL. The majority of these patients (60-70%) have the translocation t(4;11). Pre-pre-B-ALL was associated with a poor outcome in children, especially in infants and in adults. In recent adult ALL studies, it seems that the prognosis can be improved by the use of intensive treatment regimens to a continuous CR (CCR) rate of 48% at 3-1/2 years.[8]

Mature B-ALL is associated with male predominance, often lymphadenopathy, abdominal tumor masses, renal, bone and CNS involvement.

In earlier trials, remission rates for adult B-ALL were low and remission duration and survival was poor. In nine studies with a total of 44 patients, the weighted mean CR rate was 44% and most patients relapsed rapidly, reflected by the MRD of 11 months and the very low survival rate (<10%).

There is a change in the outcome of B-ALL and Burkitt's lymphoma in several childhood and adult studies. In childhood B-ALL, the outcome has significantly improved, with CR rates of 81-96% and LFS rates of up to 76%.[9-11]

The drugs responsible for this improvement are fractionated high dose CPA,[9] high dose methotrexate (HdM) (0.5 to 8 g/M^2), and high dose Ara-C (HdAC); these have resulted in an 81% CR rate in the Pediatric Oncology Group study and an 88% CR rate in the French Pediatric Oncology Society study. In the Berlin-Frankfurt-Münster (BFM) protocols, CR rates of 91-93% were obtained with moderate doses of CPA or ifosfamide and HdM (0.5 to 5 g/M^2) but without HdAC. In addition to CPA, HdM, or HdAC, the regimens contained the cytostatic drugs adriamycin (ADM), Ara-C, teniposide, VCR, PRED, daunorubicin, and etoposide.

When these childhood B-ALL protocols were applied in adult patients with B-ALL, results were also improved.[12-15] The CR rates now approach 70% and CCR rates range between 20 and 63% with a weighted mean value of 51%. In the childhood as well as in the adult B-ALL regimens above, relapses occur almost exclusively within the first year and thereafter the patients can be considered to be cured. Thus maintenance therapy is no longer indicated.

Myeloid antigen positive ALL. With current more detailed immunological analyses, increasing numbers of patients with myeloid antigen positive (My+) ALL (approximately 20%) can be detected. Common to these leukemias is that in addition to the markers specific for ALL, the myeloid markers CD13, CD14, CD15, CD33 and CDw65 may be expressed on 20% or more of the cells.

Whether My+ ALL has an adverse impact on outcome is controversial.[16] Among childhood ALL studies, there are some which show a lower LFS for My+ patients of 38% and 39% compared to 75% and 78% for patients without myeloid markers. Other studies, including those of the BFM group, have found no disadvantage for My+ patients in

CR rate or LFS. The few adult studies available indicate an inferior outcome for My+ ALL patients, both for CR rate (77%) and LFS, but in a recent GMALL analysis the LFS rate is 37%. Larger patient numbers and longer follow-ups are required before it can be definitely stated that, even with intensified treatment schedules, My+ ALL has still an adverse prognosis.

Philadelphia chromosome/bcr-abl positive ALL. Ph[1]/*bcr-abl* positive ALL is the subgroup of ALL having the worst prognosis in children as well as in adults. A new diagnostic approach for Ph[1] positive ALL is the detection of *bcr-abl* rearrangements by molecular analysis.[7,17,18] In eight cooperative trials, the incidence of Ph[1]/*bcr-abl* positive ALL ranged from 9% to 30%.

In 12 studies with a total of 264 patients, the weighted mean CR rate is 64%. The MRD in all series is short (5 to 11 months) and the survival rate, from 0 to 16% at 3 to 5 years, is extremely poor in all reports. There is no difference in outcome between patient groups when the Philadelphia chromosome is detected cytogenetically nor between those with molecular detection of the *bcr-abl* rearrangement, neither within the latter between the p190 or the p210 groups.[17,19]

The treatment of Ph[1]/*bcr-abl* positive ALL patients remains an unsolved problem. With intensified induction regimens, the CR rate can be increased to 75%. Thus, obtaining CR is not as difficult as maintaining it. HdAC (3 g/M^2 x 6-8) with VCR, ADM and dexamethasone as consolidation treatment and HdAC (1 or 3 g/M^{2}x 8) with mitoxantrone in the GMALL trials seems to have a beneficial effect.[14]

A new approach to the treatment for Ph[1]/*bcr-abl* positive ALL might be the use of biological response modifiers as maintenance therapy. There are single observations indicating that α-interferon applied in CR patients can maintain remission and also the combination of IL-2 and α-interferon may be beneficial.[20]

What do chemotherapy results imply for BMT in adult ALL? It seems that with recent intensive chemotherapeutic regimens similar overall results (when corrected for age), and similar tendencies for an improvement in ALL subgroups are obtained in adult ALL. The decision when to have a BMT differs widely.[21] Some indications for BMT could change since prognosis after chemotherapy has improved substantially, e.g., in T-ALL, pre-pre-B-ALL and B-ALL. In contrast, there are subgroups with a uniformly poor prognosis, such as Ph[1]/*bcr-abl* positive ALL, where the indication for BMT is unanimous.

In fact, there are at present two approaches to the use of BMT in adult ALL: one is to consider all adult ALL patients as high risk with poor outcome and to offer them a BMT in first CR. The other is to select only high risk patients for BMT in first CR and low risk patients for BMT in second CR, respectively.[22] Large cooperative national trials are underway to evaluate these approaches prospectively. Hopefully a clear indication of how chemotherapy and BMT can best be scheduled for an individual patient will soon be available.

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UPDATE OF A RANDOMIZED CONTROLLED TRIAL COMPARING AUTOLOGOUS BONE MARROW TRANSPLANTATION AND CHEMOTHERAPY AS POSTREMISSION THERAPIES IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA (LALA 87)

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Although the use of intensive combination chemotherapy regimens has led to noticeable improvement in prognosis of adult acute lymphoblastic leukemia (ALL), post-induction chemotherapy produces long-term disease free survival (DFS) rates of only 20-40%.[1,2,3] In an effort to improve this outcome, autologous bone marrow transplantation (ABMT) has been used, allowing dose escalation unlimited by hemopoietic toxicity.[1-8] Several groups have reported long term-DFS rates of 38-65%.[4-7] But the actual benefit of this approach remains questionable due to possible patient selection bias. So after the pilot study, the French Group of Therapy for adult ALL, proposed a trial with the first objective being to evaluate randomly, after consolidation therapy, the benefits of autologous BMT over classical maintenance chemotherapy.[9]

This study was a part of a large multicenter trial conducted between November, 1986, and July, 1991, by 43 French and Belgian institutions. General results have been already published and we shall focus on the controlled comparison of ABMT and chemotherapy as strategies for post-induction therapy, with a median follow-up of 60 months.

PATIENTS AND METHODS

The autologous BMT trial reported is a part of the LALA 87 protocol which is summarized in figure 1. Overall results have been published elsewhere.[10]

Patients were eligible if there was: (1) a previous inclusion in LALA trial, (2) achievement of complete remission (CR) with the induction or the salvage therapy regimen, (3) age from 15-40 and absence

of an HLA-identical sibling donor, or age from 40-50, (4) still in CR after the first course of consolidation.

The randomization process took place at the beginning of the second course of consolidation treatment if a bone marrow examination showed the persistence of CR. Patients were allocated to the auto-BMT arm or chemotherapy arm.

Treatment Regimen

In the autologous BMT arm, patients received 3 monthly consolidation courses (C1, C2, C3). Marrow was harvested between C2 and C3. The protocol assumed MoAb depletion of the harvested marrow by the MoAbs D66/CD2, A50/CD5 and I21/CD7 (provided by A. Bernard) for T-lineage ALL and the MoAbs ALB2/CD10 (Immunotech, Marseille) and SB4/CD19 (Sanofi, Montpellier, France) for B lineage ALL. Asta-Z (Asta Werke, Frankfurt, Germany) depletion was proposed for patients with less than two of these markers on their leukemic cells.

The conditioning regimen consisted of cyclophosphamide 60 mg/kg on days 1 and 2 and total body irradiation, either 10 Gy as a single dose, or 12 Gy in six fractions. No treatment was administered thereafter.[11]

In the chemotherapy arm, patients received the same 3 monthly consolidation courses which consisted of the same anthracycline administered during induction therapy (daunorubicin 60 mg/M^2 or Zorubicin 120 mg/M^2, day 1, Cytarabine 60 mg/M^2 from day 3 to 7, and Asparaginase 1000 U/kg from day 8 to 12.

Central nervous system prophylaxis was immediately delivered after the consolidation phase (18Gy delivery above C2 for 2 weeks). Maintenance chemotherapy consisted of a modified L10 regimen for eight cycles of 64 days spaced by a week.[12]

Analysis

All analyses were performed on an intention to treat basis. Disease free survival was calculated from the date of the second randomization until relapse, death (regardless of cause) or date when last known alive. Survival duration was calculated from the day of the first randomization (induction) until death or date when last known alive. When calculating DFS and survival, deaths in CR were counted as adverse events. Univariate analysis was performed using the X^2 test and the generalized Wilcoxson test of Gehan. Survival curves were estimated by the method of Kaplan and Meier.[13-15]

RESULTS

Pretreatment Characteristics

The figure 2 shows the chart flow of 436 patients achieving CR in the protocol. Two hundred sixty-two patients were eligible for randomization in auto-BMT versus chemotherapy, but between the achievement of CR and the time of randomization, 71 patients were excluded: 21 for early relapses, 10 for patient refusals, 8 for medical decisions, 10 for organizational reasons, 16 for severe infectious complications during induction or salvage therapy, and 6 over 40 for allogeneic bone marrow transplantation. Thus 191 patients were randomized, 96 in chemo arm and 95 in auto-BMT arm.

Patient characteristics were similar in the two groups with a median age of 28 and 25 in chemo and auto-BMT arms. Male sex in 55 and 61, T-ALL in 27 and 35, CNS involvement in 6 and 7 and white blood cells >30 G/l in 31 and 35 patients.

Outcome

The DFS is not statistically different between the two arms. The median DFS is 19 and 13 months for chemo and auto-BMT arms. There are more events in the first part of the evolution in auto-BMT arm and less in the second part so the 5-year DFS rate is respectively 25 and 35%. The total number of relapses is 68 in chemo and 54 in auto-BMT arm, relapses occurring late after 18 months, are 24 in chemo versus only 9 in auto-BMT arm. Four deaths in CR occurred in each arm, 2 fatal infections, one hemorrhage and one veno-occlusive disease in auto-BMT arm, 2 septic deaths, one myocardial infarction and one suicide in the chemo arm.

The survival rate at 5 years is 31% in chemo and 40% in auto-BMT arm and the median survival is 27 and 31 months. The difference is not yet significant. Figures 3A and 3B indicate DFS and survival of patients based on an intention to treat analysis.

Only 63 of 95 patients (66% of randomized patients in auto-BMT arm were actually transplanted). The reasons for non-compliance with the procedure were early relapse (n=19), medical complications (n=3), insufficient marrow harvesting (n=4), refusal of patient (n=4) or organizational reasons (n=2). B-cell depletion was performed in 25 patients, T-cell depletion in 19 patients and 8 cases were depleted with Asta Z. The median time between CR and autologous BMT reinfusion is 116 days. Outcome of this selected group of patients is much better with a

median DFS and 5-year DFS rate of 42 months and 45%, respectively. The median survival and 5-year survival rate are 62 months and 50%, respectively.

DISCUSSION

The main objective reported here of this large multicenter study was to test, in a controlled trial, the advantage of autologous BMT compared with classical chemotherapy in adult ALL. Impressive results have been reported from a single center trial obtained with autologous BMT performed in first CR with DFS rates of greater than 50%.[4-7] A retrospective analysis of 233 patients reported similar results.[8] However, the absence of a controlled trial rises the possibility of patient and treatment-related biases such as age, state of patient the duration between CR and transplantation.

The present study shows the natural selection of good risk patients for auto-BMT, excluding early relapses and poor clinical status. Outcome of patients actually transplanted in first CR is the same as other noncontrolled or retrospective published studies.

In conclusion, this trial emphasizes:

- the importance of selection bias when evaluating auto-BMT
- the necessity of randomized controlled trials with an intention to treat analysis
- similar outcomes for auto-BMT and chemotherapy as post remission therapy in adult ALL with a trend for less relapses in the autologous BMT arm.

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LALA 87 – Study design

Abréviations
R1 : First randomization
R2 : Second randomization
V.C.P. : Vincristine 1.5 mg/m2,
Cyclophosphamide 600 mg/m2,
Prednisone 60 mg/m2
C.V.P. : Cyclophosphamide 600 mg/m2,
Vincristine 1.5 mg/m2,
Prednisone 60 mg/m2
C : consolidation

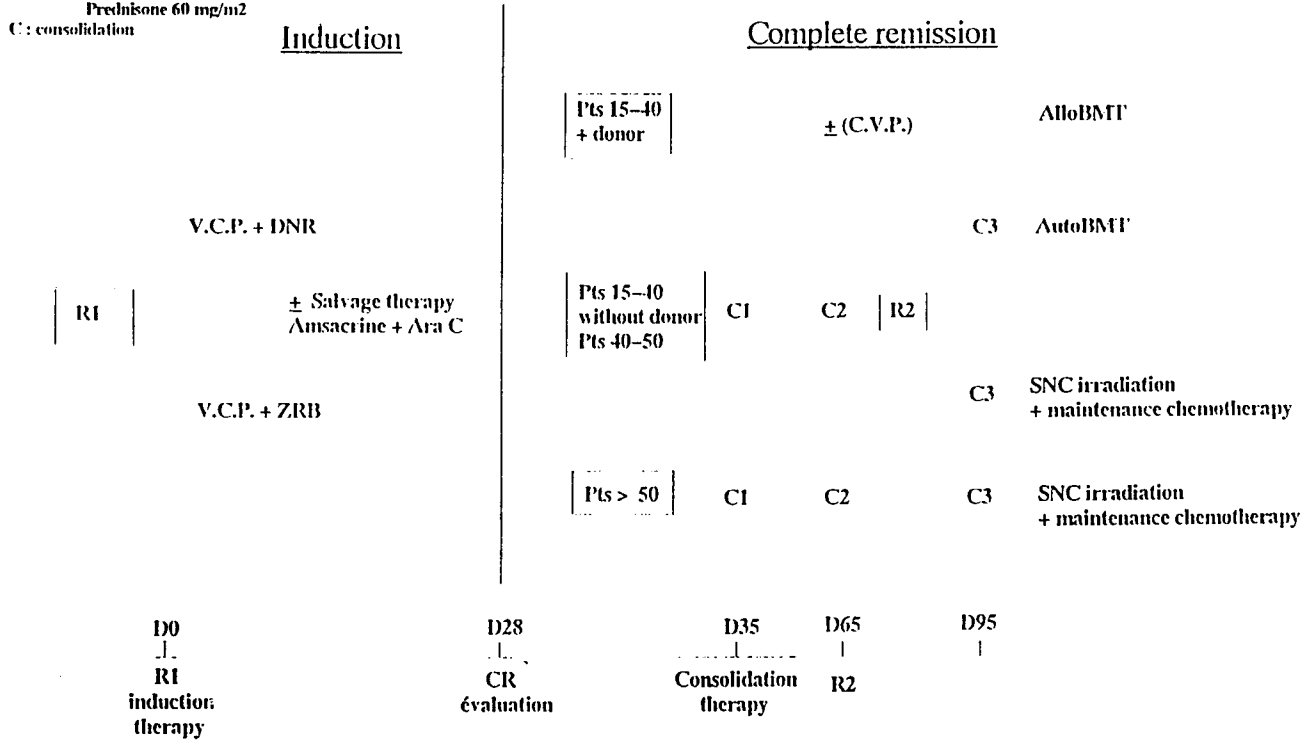
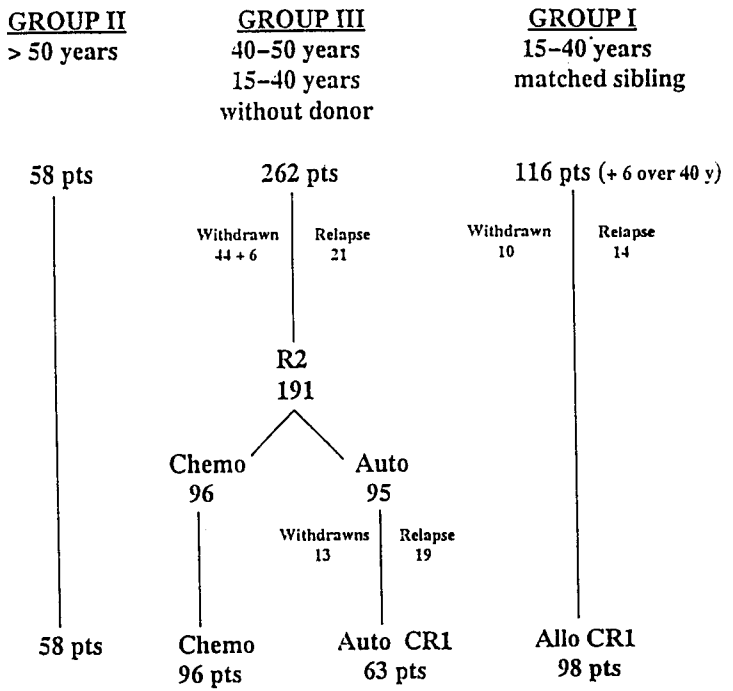


Figure 1.

LALA87 Protocol
Chartflow: 436 patients in CR



Chemo = Maintenance chemotherapy
Auto = Autologous Bone marrow Transplantation
Allo = Allogenic Bone Marrow Transplantation

Figure 2

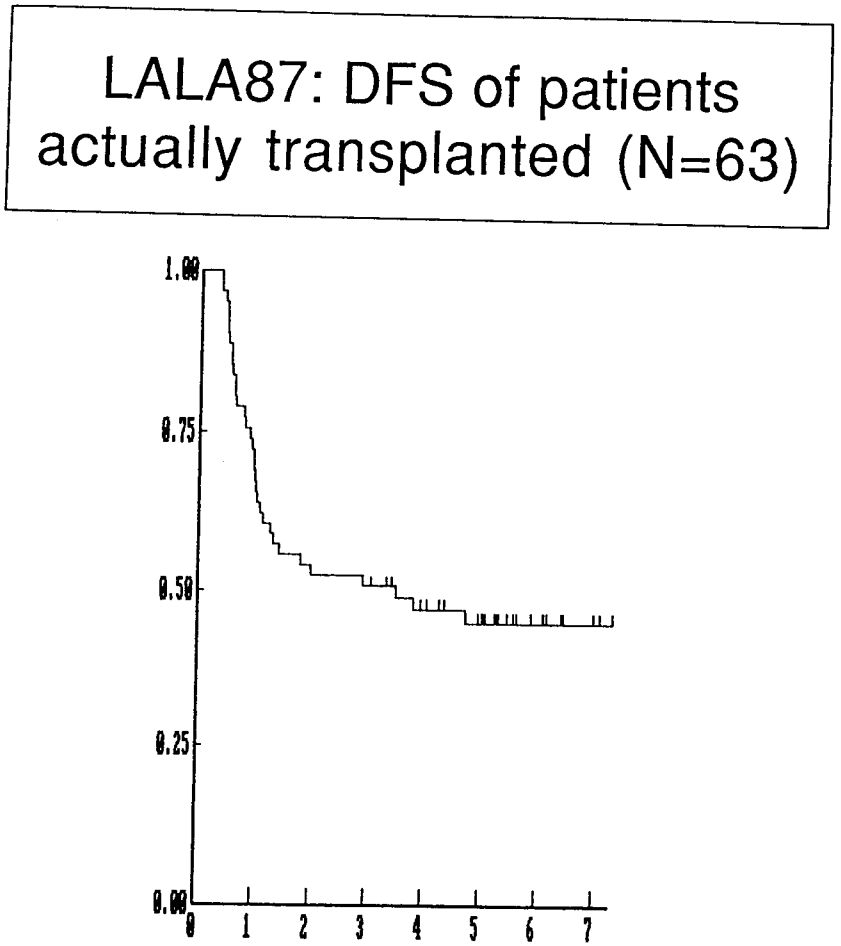

LALA87: DFS of patients actually transplanted (N=63)
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7

Figure 3A

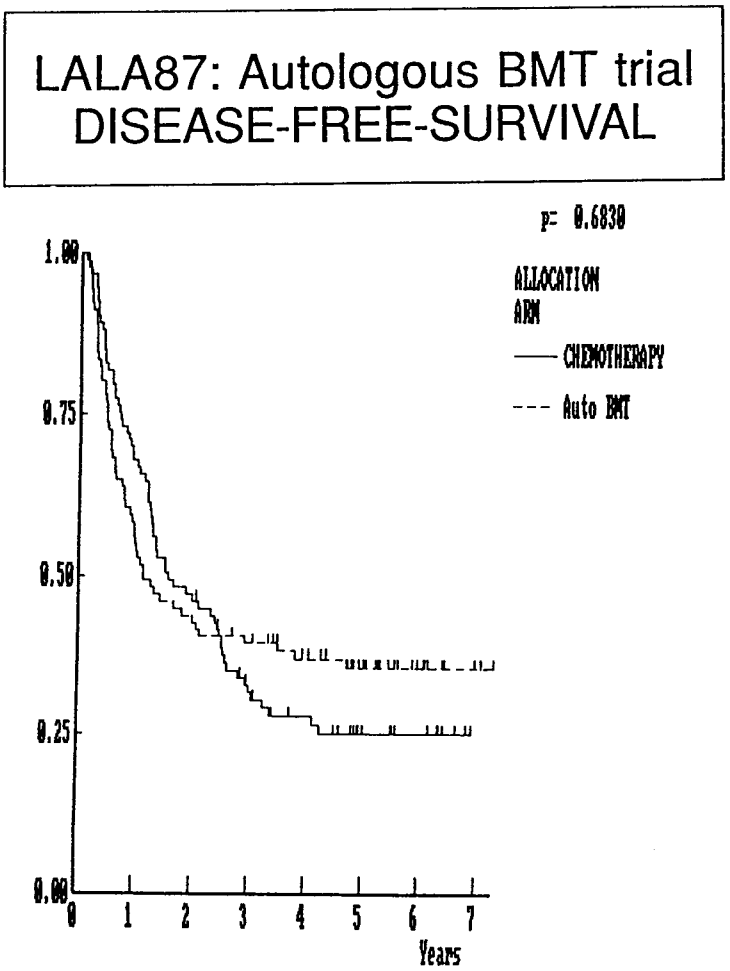

LALA87: Autologous BMT trial
DISEASE-FREE-SURVIVAL
p= 0.6830
ALLOCATION
ARM
CHEMOTHERAPY
Auto BMT
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Years

Figure 3B

SEQUENTIAL HIGH-DOSE THERAPY OF ADULT ACUTE LYMPHOBLASTIC LEUKEMIA: ROLE OF MAINTENANCE CHEMOTHERAPY AFTER PERIPHERAL BLOOD STEM CELL TRANSPLANTATION IN FIRST REMISSION

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ABSTRACT

A sequential therapeutic strategy employing a first complete remission (CR) peripheral blood stem cell autograft (PBSCT) followed by maintenance chemotherapy has been developed for adult acute lymphoblastic leukemia (ALL), where relapsing patients are salvaged by an autologous or allogeneic bone marrow transplantation (BMT) in second CR. Thirteen patients underwent PBSCT after high-dose melphalan (200 mg/M^2) in first CR. Eleven had high-risk disease (one of: age >35, leukocytes >30, CR after >4 weeks). Toxicity of PBSCT was minimal with 2 days (range 0-5) of fever and 18 days (range 17-23) in hospital. Neutrophils reached 0.5 x 10^9/L on day 15 (range 12-27), and platelets 50 x 10^9/L on day 16 (range 12-77. 6-MP was started in 12 patients on day +32 (range 15-132). The median daily dose of 6-MP tolerated, averaged over the entire post-PBSCT follow-up period, was 44.1 mg/M^2. Nine patients were started on MTX. Patients transplanted after December 1993 also received monthly vincristine and steroids. Ten patients (76.9%) are alive and well on chemotherapy in first CR at 12 months (range 2-20). Of 3 patients relapsing at 4-7 months, 2 are alive and well two and three months after allogeneic BMT from matched siblings in second CR. The third declined autologous BMT in second CR and is currently in third CR. Overall survival is 100%. We conclude that melphalan and post-PBSCt maintenance chemotherapy have significant antileukemic activity in adult ALL, patients relapsing after PBSCT can be salvaged by a second BMT, and this structured approach minimizes treatment-related toxicity by reserving allogeneic BMT for relapsing patients.

INTRODUCTION

Autologous or allogeneic bone marrow transplantation (BMT) in adult patients with acute lymphoblastic leukemia (ALL) in the first complete remission (CR) results in 20-70% long-term event-free survival.[1-9] Transplant-related mortality remains the most important cause of failure of allogeneic BMT,[3-7] while the predominant cause of failure of autologous BMT (ABMT) is relapse of the disease posttransplant.[7-9]

Maintenance chemotherapy with 6-mercaptopurine (6-MP) and methotrexate (MTX) is presumed to be useful in adult ALL because protocols omitting maintenance chemotherapy have been associated with a high relapse rate and relatively low disease-free survival.[10,11] We have previously shown that administration of maintenance chemotherapy after ABMT in ALL results in decreased relapse rates.[12]

To minimize transplant-related toxicity, which in our ABMT patients was mostly attributable to total body irradiation (TBI),[12] we have now devised a sequential treatment strategy utilizing peripheral blood stem cell transplantation (PBSCT) as the first step after a conditioning regimen that does not include TBI.[13]

PATIENTS AND METHODS

In the sequential therapy program, patients aged 15 years and over who are in first CR after a standard 4-drug induction therapy schedule (MRC UKALL Xa Regimen D), undergo PBSCT after high-dose melphalan (200 mg/M^2) irrespective of the availability of HLA-identical siblings. Blood cells are harvested after a second treatment cycle (intensification) in patients attaining CR at the end of 4 weeks of induction therapy, and after a third cycle (consolidation) in those attaining CR after intensification. Patients not in CR at the end of the intensification phases are ineligible, as are patients with Ph+ ALL, B-ALL, and CNS disease. CNS prophylaxis consists of 2400 cGy cranial irradiation in 15 fractions before PBSCT and 6 intrathecal injections of MTX.

Maintenance chemotherapy with daily 6-MP is begun when the leukocytes reach 3 x 10^9/L and the platelets 100 x 10^9/L. Target doses of 6-MP and MTX are 80 mg/M^2 daily and 20 mg/M^2 weekly respectively. Therapy is started with 25 mg 6-MP and this is increased weekly or fortnightly in 25-50 mg steps. MTX at the dose of 5 mg (increasing in 2.5-5 mg steps) is added when the dose of 6-MP reached 75 mg. The dose is adjusted to maintain the absolute neutrophil count over 1 x 10^9/L.

Maintenance chemotherapy is scheduled to be continued for a period of two years from the date of its commencement posttransplant. Patients receive folic acid and prophylaxis for *Pneumocystis carinii* pneumonia with oral trimethoprim-sulfamethoxazole or inhaled pentamidine while on maintenance chemotherapy. Patients transplanted since December 1993 have also been receiving monthly vincristine and prednisolone.

In relapsing patients, repeat induction of remission is attempted using standard or salvage regimens. Patients are offered allogeneic BMT from matched siblings or unrelated donors, or ABMT if no suitable donors are available using unpurged marrow harvested in second CR. The conditioning regimen for the second transplant is high-dose VP-16 (60 mg/kg) and TBI (1050 cGy as a single fraction).

Blood stem cells were initially mobilized with G-CSF (Neupogen, Amgen) at the dose of 125 $\mu g/M^2$ sc. q.12h on days 1 to 7 (7 consecutive days), and leukapheresis was performed on days 5 to 8 (4 consecutive days). This has now been modified to 12-16 μg/kg G-CSF sc. q.24h on days 1-4, and leukapheresis on days 4 and 5. The G-CSF dose is rounded off to the nearest vial size so that the actual dose administered is between 12 and 15 μg/kg. Leukapheresis is performed on a Cobe Spectra (Cobe Industries, Lakewood, CO) continuous-flow cell separator with 150-200% of the patient's calculated blood volume being processed at each session. Patients receive a single dose of 200 mg/M^2 melphalan with hydration on day -1. The entire cryopreserved PBSC collection is thawed rapidly at 37°C and infused on day 0; 24 hours after the administration of melphalan.

Patients are treated in protective isolation in rooms with positive-pressure ventilation. CMV-negative blood products are used for seronegative patients in view of the possibility of a future allograft. Broad-spectrum antibiotic therapy is started for fever in the neutropenic phase. Irradiated random platelets are transfused to maintain the platelet count at 20 x 10^9/L, and packed cells to maintain the hemoglobin at 100 g/L. Vascular access for PBSCT is usually through a central line and Hickman catheters are not routinely inserted.

Thirteen consecutive adult patients with ALL in first CR who satisfied the inclusion criteria have been enrolled on the program between January 1993 and June 1994. They were stratified into standard- or high-risk groups on the basis of previously published criteria.[14] Patients with one or more of the following features were classified as having high-risk disease: age >35 years, leukocyte count at presentation >30 x 10^9/L, and time taken to achieve CR >4 weeks.

The average dose of 6-MP or MTX administered was determined by calculating the actual total amount of drug administered after PBSCT. The last date for determining an individual patient's total chemotherapy was the date of relapse or the date of analysis (31 July 1994); whichever was the earlier. This amount was divided by the number of days (6-MP) or weeks (MTX) from the date of the transplant to the last date of the chemotherapy. The amount of chemotherapy delivered to these patients was compared to a group of historic controls who received ABMT after melphalan-TBI.

RESULTS

Table 1 shows patient characteristics at initial presentation. The diagnosis-PBSCT interval was 4-13 months (median 5), and the CR-PBSCT interval was 2-11 months (median 3). The first 7 PBSCT recipients were harvested on 4 days and consequently received 8.1-15.1 x 10^8 nucleated cells/kg (median 13.1). With the number of leukaphereses reduced to 2 from 4, the next 6 patients received 3.9-9.2 x 10^8 nucleated cells/kg (median 4.3).

Engraftment was rapid with neutrophils reaching 0.5 x 10^9/L on day 15.5 (range 12-27), and platelets reaching 50 x 10^9/L on day 17 (range 12-77). No transplant-related deaths were seen in the PBSCT group (P=NS). Morbidity of the procedure was acceptable with 2 days (range 0-5) of fever over 38°C and 18 days (range 17-23) in hospital.

Table 1. Patient Characteristics

Number of patients	13
Males	6
Females	7
Median age (range)	32 years (19-58)
Immunophenotype	
Common	10
T	2
Null	1
Karyotype	
Ph variant	1 (detected at relapse)
t(4;11)	1
t(11;14)	1
t(5;12)	1
Trisomy 8	1
Trisomy 7	1
Del 6q	1

Normal	4
Not available	2
Features at presentation	
Leukocyte count	0.7-260 (5.8)
Platelet count	13-260 (111)

Table 2 shows the time to starting maintenance chemotherapy, the proportion of patients starting it, and the doses of chemotherapy administered. Data on 38 adult ABMT patients are shown for comparison. The actuarial probability of starting maintenance chemotherapy was 65% at 3 months for ABMT recipients compared to 85% for PBSCT patients ($P<0.05$). Temporary attenuation of the dose of the 6-MP and/or the MTX was required in approximately half the patients due to myelosuppression. No primary or secondary failure of engraftment was seen.

Three patients relapsed 4-7 months posttransplant. All three attained a second CR with combination chemotherapy which was tolerated well. One refused ABMT in second CR and is currently in third CR 15 months after PBSCT. The other two are two and three months after allogeneic BMT from matched sibling donors with high-dose etoposide-TBI conditioning seven and eleven months after the first graft respectively. Allograft-related toxicity was acceptable and reversible in both patients. As on July 31, 1994, all 13 patients are alive and in CR, and 10 are alive on maintenance chemotherapy in continuous CR twelve months (range 2-20) posttransplant.

Table 2. Post-Autograft Maintenance Chemotherapy: Comparison of PBSCT with ABMT

Parameter	ABMT	PBSCT	P
Number of patients	38	13	
Day posttransplant to start maintenance chemotherapy Median (range)	58.5 (26-152)	32 (15-132)	0.002
Probability of starting chemotherapy at 3 months	65%	85%	0.05
Patients receiving 6-MP	30 (78.9%)	12 (92.3%)	
Patients receiving MTX	20 (52.6%)	9 (69.2%)	
Daily dose of 6-MP (mg/M^2) Median (range)	33.5 (7.9-91.3)	44.1 (14.8-59.9)	NS
Weekly dose of MTX (mg/M^2) Median (range)	4.7 (0.7-18.3)	7.6 (1.5-20.7)	NS

DISCUSSION

Relapse remains the most important cause of treatment failure after ABMT for ALL, occurring in 40-70% of patients.[2,7-9] Post-autograft strategies which have been employed in an attempt to decrease relapse rates in ALL have included cytokine-mediated immunotherapy with interleukin-2 infusions,[15] cell-mediated immunotherapy with haploidentical T-cells,[16] and intensive chemotherapy (repeat remission induction and intensification).[2,17] We have shown that the novel strategy of continuous maintenance chemotherapy with 6-MP and MTX after ABMT for a period of 2 years decreases relapse rates in ALL.[12]

Two important changes were made when we embarked upon the sequential therapy program: Firstly, TBI was omitted from the conditioning regimen to decrease peri-transplant morbidity and mortality,[12,18] and to avoid long-term consequences such as cataracts, sterility, organ damage and secondary neoplasms.[19] Secondly, allogeneic BMT was no longer offered in first CR to adult patients with ALL except Ph+ ALL and patients not in CR after 8 weeks of initial therapy. The rationale was to avoid the morbidity and mortality of allogeneic BMT in a situation where graft-versus-leukemia reactions in first CR are relatively less marked compared to other types of leukemia and advanced ALL.[20,21] It was decided to reserve TBI for use with a subsequent transplant (from a matched sibling or an unrelated donor, or autologous) in case of a relapse.

In the context of these exclusion criteria, it must be mentioned that of the two patients allografted in second CR on this program, one was found to have a variant Ph chromosome at relapse and the other was retrospectively found not to have attained CR after the intensification phase of initial therapy. These two patients therefore should not have been enrolled on the sequential therapy program, but should have been allografted in first CR. In future, PCR will be done for *bcr-abl* to identify Ph+ ALL.

In ABMT patients who relapsed after melphalan-TBI conditioning, second remissions were difficult to attain, and most patients died of progressive disease or toxicity of chemotherapy. The three patients relapsing after PBSCT attained second remissions very easily using standard combination chemotherapy. This is likely to be largely attributable to the attenuation of the intensity of the conditioning regimen.

It is noteworthy that the intensity of the UKALL Xa chemotherapy administered to these patients is considerably less than other protocols which have been associated with relatively lower relapse rates.[14,22,23]

Intensive consolidation has been shown to improve continuing CR rates significantly in the successive German multicenter adult ALL trials.[24] It is possible that the high-dose melphalan consolidation in this group of patients overcame some of the lack of efficacy of the induction-intensification therapy, and that the results obtained here may be improved further if the initial therapy is intensified.

Our data show that G-CSF-mobilized blood stem cells used to support high-dose consolidation chemotherapy are as durable as marrow-derived cells in tolerating maintenance chemotherapy posttransplant. As Table 2 shows, maintenance chemotherapy could begin earlier after PBSCT compared to ABMT due to faster hematopoietic reconstitution. Because MTX used within the first 2 weeks posttransplant for the prophylaxis of graft-versus-host disease after allogeneic BMT for ALL has been shown to reduce the risk of relapse, presumably by its direct antileukemic action,[25] early commencement of maintenance chemotherapy after autografting may be maximally beneficial. Also, a higher proportion of PBSCT recipients could receive maintenance chemotherapy compared to ABMT.

The average doses of chemotherapy that could be administered after PBSCT and ABMT were not significantly different. The projected cumulative doses of 6-MP delivered after PBSCT are high and are comparable to other studies.[14,22,23] Though it is difficult to assess the exact benefit of different cumulative levels of maintenance chemotherapeutic agents (6-MP),[26] studies with higher projected total dose-delivery of 6-MP[14,22,23] have been associated with better long-term outcome than those with lower dose.[10,11]

PBSCT is increasingly replacing ABMT due to rapid engraftment and significantly shorter periods of pancytopenia. However, as the number of nucleated and mononuclear cells reinfused at the time of PBSCT is much higher than that reinfused at the time of ABMT,[27] the issue of contamination of the infused product with malignant cells is likely to assume a great deal of significance. It is likely that PBSCT, as currently employed in the acute leukemias, may result in higher relapse rates than ABMT due to infusion of a greater number of malignant cells. The demonstration of durability of PBSCT grafts to maintenance chemotherapy in our study is crucial because posttransplant maintenance treatment of some sort (chemotherapy or immunomodulation) is likely to prove essential after PBSCT for the acute leukemias.

Much longer follow-up and a large randomized study will be required to determine if the overall survival with our strategy of

posttransplant maintenance chemotherapy is better or not. Our data demonstrate that this strategy is feasible with PBSCT as well as with ABMT. Being a relatively low-risk procedure compared to a standard TBI-containing transplant, high-dose chemotherapy with PBSCT is likely to find much wider application as an intensive post-consolidation procedure if found to be beneficial. In addition to the minimal morbidity, the median cost of these transplants is 30% less than the median cost of autologous BMT for AML at our center using a comprehensive template of transplant-related activities (unpublished data).

We conclude that high-dose melphalan with PBSCT in first CR followed by maintenance chemotherapy is a safe and feasible treatment strategy in adult ALL. Patients relapsing after PBSCT can attain second remissions easily, and may be able to undergo second transplants after TBI-containing regimens with acceptable morbidity. This sequential therapy exposes only relapsing patients to the high treatment-related risk of TBI and allogeneic BMT, but all patients benefit from the antileukemic effect of high-dose melphalan and posttransplant maintenance chemotherapy. Because blood-derived stem cells harvested using G-CSF in the steady-state are as tolerant of maintenance chemotherapy after autografting as marrow-derived cells, future studies evaluating the utility of autografting in ALL should employ high-dose chemotherapy and PBSCT to decrease transplant-related toxicity and maintenance chemotherapy post-PBSCT to decrease relapse rates.

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MONOCLONAL ANTIBODY-PURGED AUTOLOGOUS BONE MARROW TRANSPLANTATION FOR ACUTE LYMPHOBLASTIC LEUKEMIA IN CHILDREN AND ADULTS AT HIGH RISK OF RELAPSE: THE DANA-FARBER CANCER INSTITUTE EXPERIENCE

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INTRODUCTION

Although the prognosis for children with newly diagnosed acute lymphoblastic leukemia (ALL) has improved in recent decades, most children with relapsed ALL have a poor prognosis. Chemotherapy alone is rarely curative, especially for children with a short first remission.[1] The prognosis for adults with either newly diagnosed or relapsed ALL is far worse than for their pediatric counterparts.[2] Disease-free survival after allogeneic bone marrow transplantation from matched sibling donors ranges from 40-70% in children with relapsed ALL and from 15-45% for adults.[3-11] Allogeneic BMT from matched siblings is limited by lack of donors in 70-80% of patients.[12,13] Attempts to expand the applicability of allogeneic BMT, including the use of marrow from mismatched related donors and matched unrelated donors have met with mixed results.[14-17] Even if mortality from graft-versus-host disease and graft rejection could be decreased, the donor pool remains limited.[17]

The use of autologous marrow offers a therapeutic alternative for patients with ALL who do not have matched sibling donors. Autologous bone marrow transplantation (ABMT) can expand the proportion of patients eligible for BMT and is generally associated with less morbidity and mortality than allogeneic BMT. Autologous BMT has the theoretical disadvantage of the possible contamination of the harvested marrow with occult leukemic cells. Both pharmacologic and immunologic techniques to purge the marrow of residual leukemia cells have been developed.[18-22]

We have developed an *ex vivo* autologous marrow purging program which employs two monoclonal antibodies (J2, J5) which,

respectively, recognize the CD9 and CD10 surface antigens expressed on the majority of pre-B ALL blasts.[23] In this report, we describe our current experience with 93 pediatric and 22 adult patients with B-lineage ALL at high risk for relapse who underwent autologous BMT with the infusion of J2/J5 purged marrow.

PATIENTS AND METHODS

Patient Eligibility

All patients who underwent ABMT had B-lineage with expression of CD9 and/or CD10 on the surface of the leukemia cells and had no matched sibling donor for allogeneic transplantation and were in complete remission at the time of bone marrow harvest and transplantation. Additional eligibility criteria for pediatric patients were second or subsequent remission and age less than 18 years. Of note, one pediatric patient underwent ABMT in first remission after initial induction failure. Additional eligibility criteria for adult patients were initial induction failure or second or subsequent remission and age at least 18 years. Clinical protocols were approved by the Institutional Review Boards of the Dana-Farber Cancer Institute, Children's Hospital, and/or Brigham and Women's Hospital. All patients or their parents gave written informed consent prior to undergoing treatment.

Intensification Chemotherapy. Pediatric patients received a cycle of intensification chemotherapy after reinduction and prior to bone marrow harvest. The standard regimen used for all patients prior to 1986 and for patients with a long first remission of at least 24 months included intravenous cytarabine 300 mg/M^2, intravenous teniposide 200 mg/M^2, and intrathecal cytarabine with hydrocortisone twice weekly for two weeks. From 1989 on, patients with a short first remission of less than 24 months received intravenous cytarabine 3 gm/M^2 every 12 hours for 6 doses followed by weekly intramuscular asparaginase 25,000 IU/M^2 weekly x 4.[24] There was no specific requirement for post-induction chemotherapy for adult patients.

Marrow Purging. Ten to fifteen cc/kg of bone marrow was harvested and treated as previously described with three cycles of J5 anti-CALLA (anti-CD10) and J2 anti-GP26 (anti-CD9) monoclonal antibodies and complement.[25-27]

Preparative Regimen. All patients received total body irradiation (TBI) and cyclophosphamide 60 mg/kg x 2 days. The majority of pediatric patients received cytarabine 3 gm/M^2 q.12 hours x 6 with two

concurrent doses of teniposide 200 mg/M^2, cyclophosphamide 1800 mg/M^2 x 2 days, and TBI of 1400 cGy at 10 cGy/minute, 175 cGy/fraction twice daily for eight doses. Prior to 1986, different cytarabine and TBI doses were used for pediatric patients.[25,26] From 1989-1992, pediatric patients with a short first remission received TBI of 1400 cGy as above, followed by a single dose of etoposide 60 mg/kg, and cyclophosphamide as above. Adult patients received cyclophosphamide 60 mg/kg x 2 days followed by fractionated TBI of 1200-1400 cGy.[27] The first nine patients also received continuous infusion cytarabine, the first two with two concurrent doses of teniposide.

Statistical Methods. Remission was defined as <5% blasts in the marrow and no evidence of extramedullary leukemia. Relapse was defined as >25% lymphoblasts in the bone marrow or evidence of extramedullary leukemia. Results were analyzed as of August 1994 for pediatric patients and January 1993 for adult patients. For analysis of event-free survival (EFS) and disease-free survival (DFS), events were defined as relapses, remission deaths or second tumors. Times were calculated from the day of bone marrow infusion. The Kaplan-Meier method was used to estimate EFS and DFS distributions.[28] Greenwood's formula was used to estimate standard errors.[29] The two-sided logrank test of survival analysis was used to compare the various subgroups with respect to EFS or DFS.

RESULTS

Patient Characteristics

The characteristics of the 93 pediatric patients transplanted from November 1980 to June 1992, and of the 22 adult patients transplanted from September 1983 to January 1991, are listed in Table 1. For pediatric patients, sixty-four were male; the median age was 8 years; the median white blood cell count at diagnosis was 10.6 x 10^3 µl; the median duration of first remission was 32 months; and the median interval from diagnosis to BMT was 3.4 years. One pediatric patient was transplanted in first remission; 58 in second remission, 30 in third remission, and 4 in greater than third remission. Twelve pediatric patients were transplanted after isolated extramedullary relapse(s). For adult patients, sixteen were male; the median age at BMT was 28 years; the median duration of first remission was 16 months; and the median interval from diagnosis to BMT was 28 months. One adult patient was transplanted in first remission, eleven in second remission, 7 in third remission, and three in fourth

remission. One adult patient was transplanted after isolated extramedullary relapse.

TABLE 1. Patient Characteristics

	Pediatric	Adult
N	93	22
Sex Male:Female	64:29	16:6
Age at BMT (yr) median (range)	8 (3-18)	28 (18-54)
WBC at diagnosis ($x10^3/\mu l$) median (range)	10.6 (1.6-202)	----
Duration of first remission (mo) median (range)	32 (0-84)	16 (2-148)
Interval from diagnosis to BMT (yr) median (range)	3.4 (.5-13.1)	28 (6-170)
Remission number at BMT		
1	1	1
2	58	11
3	30	7
≥4	4	3
Isolated extramedullary relapse	12	1

Abbreviations: WBC = white blood cell count; BMT = bone marrow transplantation

Hematologic Engraftment. The median number of marrow mononuclear cells infused was .8 x 10^8 cells/kg (range .03-40) for pediatric patients and .48 x 10^8 cells/kg (range .25-.98) for adult patients. For pediatric patients, the median time to an absolute neutrophil count of 500 x 10^6/l was 28 days (range 10-68) and the median time to a platelet count of 20 x 10^9/l unsupported was 40 days (range 12-120). For adult patients, the median time to an absolute neutrophil count of 500 x 10^6/l was 22 days (range 19-161) and the median time to a platelet count of 50 x 10^9/l unsupported was 29 days (range 15-161). There was no correlation between the number of cells reinfused and rapidity of engraftment.

Pediatric Patient Outcome. The EFS at four years of pediatric patients was 38% (±7), as shown in Figure 1. Thirty-nine patients relapsed, 15 died in remission, 1 developed a second tumor, and 38 remain in continuous complete remission at a median follow-up of 3.9 years (range .9-12.6). Most remission deaths occurred early at a median time of 33 days. Only two occurred greater than 100 days from BMT. The cause of death included infection (5), pneumonitis (3), hemorrhage (3), and other (4). Deaths in remission occurred more frequently in patients whose first

remission was less than twenty-four months (9/31, 29%) than they did in patients who had a long first remission of at least twenty-four months (6/61, 10%). The one second tumor, acute myelogenous leukemia with an 11q23 translocation, occurred eighty months after BMT. Thirty-six of the thirty-nine relapses involved the bone marrow. Relapses occurred at a median of 7 months (range 2-37), and only five occurred more than 2 years after BMT.

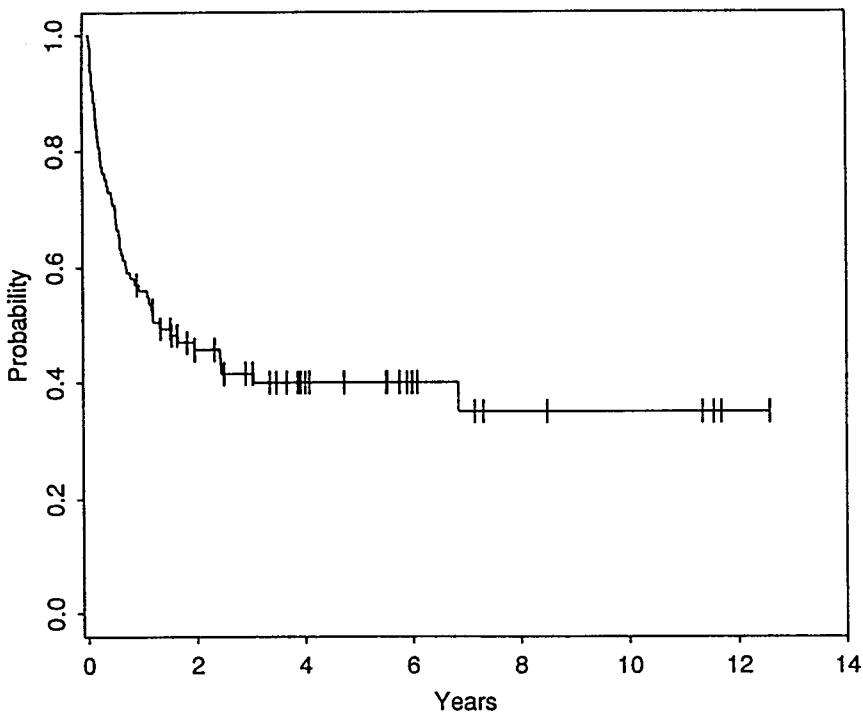

Figure 1. Kaplan-Meier plot of EFS for all pediatric patients from time of marrow infusion. Tick marks represent individual patients continuing at risk. Four-year EFS was 38% (±). Thirty-nine patients relapsed, 15 died in remission, one developed a second tumor, and 38 remained in CCR at a median of 3.9 years.

We examined several pre-transplant characteristics to determine if they exerted any influence on event-free survival. Decreased EFS was not related to white blood cell count at diagnosis, remission number at bone marrow transplantation, or number of mononuclear cells reinfused. As shown in Figure 2, however, there was a significant correlation between duration of first remission and outcome. EFS was 20% (±8) for the thirty-one patients with a short first remission of less than 24 months as compared to 44% (±8) for the sixty-two patients with a long first remission

of at least 24 months (p=.0004). Other than duration of first remission, there were no significant differences in patient characteristics between these two groups. Among patients with a long first remission, the 19 patients with a first remission longer than 48 months had an EFS of 66.6% (±13.8) as compared to an EFS of 36.2% (±10) for the 43 patients with a first remission of 24-48 months (p=.0008).

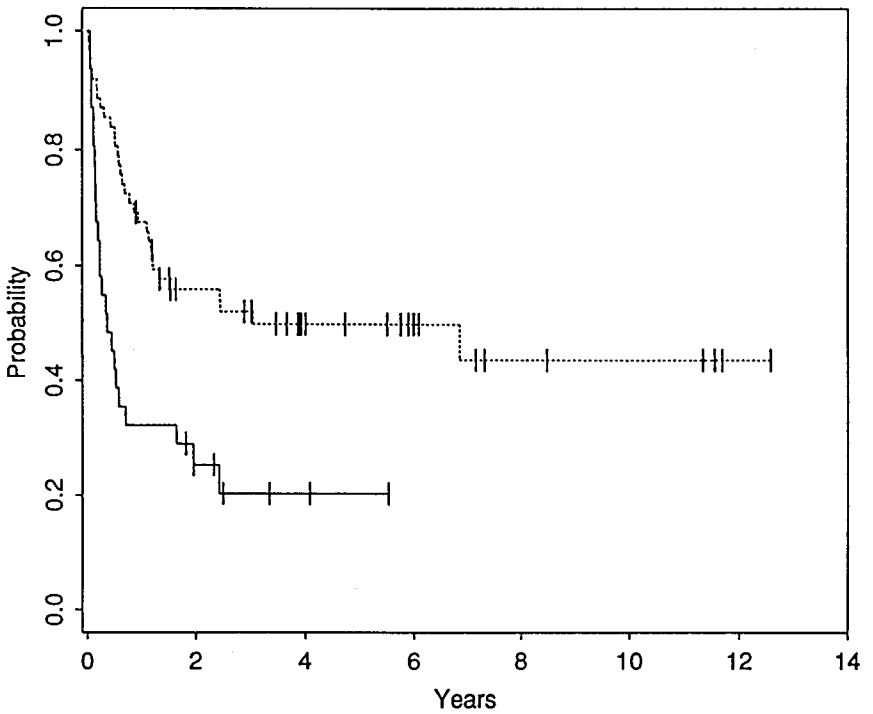

Figure 2. Kaplan-Meier plot of EFS for all pediatric patients by duration of first remission: ≥24 months (----) versus <24 months (____). Tick marks represent individual patients continuing at risk.

As shown in Figure 3, there was a very significant difference in EFS for the short first remission patients who received the standard treatment before 1989 as compared to the new treatment from 1989 to 1992. For the 20 patients treated prior to 1989, EFS was 5% (±5) as compared to 55% (±15) for the 11 patients treated subsequently (p=.008). There were no significant differences in patient characteristics between these two groups. The EFS of 55% for the patients with a short first remission who received the new treatment was not significantly different than the EFS of 44% for the 63 patients with a long first remission who received the standard treatment.

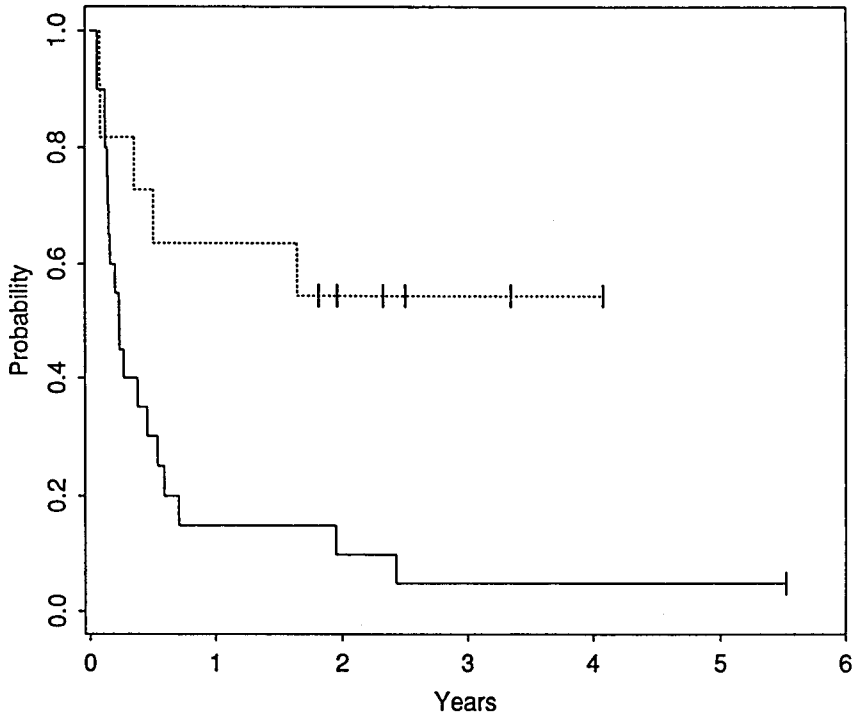

Figure 3. Kaplan-Meier plot of EFS for pediatric patients with a short first remission (<24 months) by treatment received: standard treatment from 1980-1989 (____) versus intensive treatment from 1989-1992 (----). Tick marks represent individual patients continuing at risk.

The EFS of the twelve patients transplanted after isolated extramedullary relapse(s) was 50% (±14). One patient died in remission, five relapsed, and five remain in continuous complete remission. Because of the small number of patients in this group, we did not examine the effect of pre-transplant characteristics on outcome.

Adult Patient Outcome. The DFS at four years was 20%. Thirteen patients relapsed, four patients died in remission, and five remain in continuous complete remission with a median follow-up of 6.5 years after BMT. The deaths during remission occurred early, three from infection at day 4-56, and one from myocardial necrosis on day 0. All four death in remission occurred among the 9 patients who received cytarabine during conditioning as compared to none among the 13 patients who did not receive cytarabine ($p<.02$). Twelve of the thirteen relapses occurred within one year of BMT, one occurred 42 months after BMT. Two of nine

patients who received cytarabine during conditioning relapsed, as compared to 11 of 13 who did not receive cytarabine ($p<.01$).

Decreased DFS was not correlated with remission number at BMT but was strongly correlated with age at BMT. As shown in Figure 4, DFS was 45% for the 10 patients less than 28 years of age as compared to 0% for the 12 older patients. Of note, there were no significant differences in other pre-transplant characteristics between these two groups of patients.

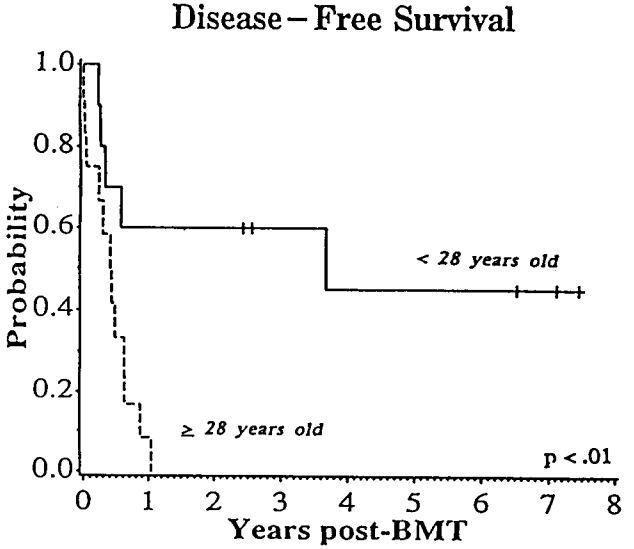

Figure 4. Kaplan-Meier plot of DFS for adult patients by age at transplantation: <28 years of age (____) versus ≥28 years of age (----). Tick marks represent individual patients continuing at risk

DISCUSSION

Our experience suggests that purged autologous bone marrow transplantation can be effective therapy for many pediatric and adult patients with ALL at high risk of relapse. Our ABMT results in children compare favorably to results from other centers.[30-33] For children with a long first remission, our EFS of 44% after autologous transplantation compares favorably with both chemotherapy and allogeneic bone marrow transplantation from matched sibling donors.[1-9,34,35] Compared to allogeneic transplantation from matched sibling donors at our own institution, autologous transplantation is available to more patients and has less treatment-related morbidity and mortality and has a similar overall outcome.[35] The major cause of failure for these patients remains relapse.

Although the outcome for children with a short first remission was dismal in the early years of our program, changes in our treatment program instituted in 1989 significantly improved outcome for the eleven patients we treated subsequently. The EFS of 55% for this group of eleven is not significantly different than the EFS of 44% for the sixty-two patients with a long first remission who received the standard intensification and conditioning regimens.

Management of pediatric patients with isolated extramedullary relapse is controversial. Patients with an early central nervous system relapse appear to have a worse outcome compared to those with a late relapse.[36] Our data with an EFS of 50% suggest that autologous bone marrow transplantation may be an effective therapy for patients with isolated extramedullary relapse.

It is difficult to compare our DFS of 45% for adults less than 28 years of age with the results of others since there were few reports of only adult patients undergoing ABMT for ALL in greater than first complete remission. The European Bone Marrow Transplant Group reported a four-year disease-free interval of 24% in 71 adult patients at 51 centers.[33] Overall results with autologous transplantation appear similar to allogeneic transplantation from matched sibling donors.[10,11,27] Unfortunately, our results with patients over age 28 years remain very poor.

In summary, we have demonstrated that antibody-purged autologous bone marrow transplantation is effective for many pediatric and adult patients. Future investigation will need to focus on a number of topics: 1) phase III trials in children with a long first remission comparing ABMT to chemotherapy, as is currently planned by the Pediatric Oncology Group, 2) phase II trials in children with a short first remission testing the efficacy of ABMT in a larger group of patients, as is currently planned by the Pediatric Oncology Group, and 3) strategies to reduce the risk of relapse in all patients such as more intensive pre-transplant chemotherapy, more intensive conditioning regimens, post-transplant induction of graft-versus-leukemia effect, or other post-transplant modifications.

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THERAPEUTIC CHOICES FOR ACUTE LYMPHOBLASTIC LEUKEMIA: AUTOLOGOUS VERSUS UNRELATED DONOR MARROW TRANSPLANTATION

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Modern therapy of acute lymphoblastic leukemia (ALL) is frequently successful in inducing prompt and durable complete remission. However, in patients with high-risk features or in patients who relapse, even contemporary intensive chemotherapy is unlikely to produce extended leukemia-free survival and these patients must seek other options for potentially curative treatment. In first complete remission, only those with recognizable high-risk features are suitable candidates for marrow transplantation. These may include those with extreme leukocytosis, Ph^1 positivity, or t(4;11) translocations, mature B-cell leukemia or older patients. Following relapse, most patients are considered candidates for marrow transplantion.

Matched sibling donor allogeneic bone marrow transplantation (BMT) may reportedly extend leukemia-free survival for between 30-50% of patients treated.[1-6] However, over 60% of patients have no available histocompatible related donor and must seek other options for transplantation.

Autologous marrow transplantation may be rapidly executed for patients in remission and is most often accompanied by modest peritransplant morbidity and mortality though its antileukemic efficacy is somewhat limited and leukemia-relapse posttransplantation is quite frequent. Only 20-40% patients achieve extended duration leukemia-free survival after autotransplantation.[7-15]

Alternatively, in recent years, closely matched unrelated donor (URD) bone marrow transplantation has been investigated. Donor identification through the National Marrow Donor Program in the United States and linkage to several international volunteer donor databases has improved in efficiency and success over the last several years. Currently, 30-50% of patients may have suitable unrelated donors identified. However, the time for donor searching, histocompatibility testing and scheduling is substantial and such testing is costly. In addition, allogeneic URD marrow transplantation is more often complicated by graft failure, graft-versus-host disease and infection leading to substantial increases in

peritransplant mortality. Notably, greater donor:recipient histo-incompatibility may enhance an immunologic graft-versus-leukemia effect and may thereby reduce the risks of posttransplantation leukemia recurrence.[16-18] The greater hazards accompanying URD transplantation may be offset to some degree by improved antileukemic efficacy of the therapy.

Barriers to Transplantation: Autologous Versus Unrelated Donor

Application of autologous transplantation requires a suitably cellular marrow in complete morphologic remission to be acceptable for aspiration and cryopreservation of sufficient hematopoietic progenitor cells. In some transplant centers, *ex vivo* purging is employed to deplete residual leukemia precursor cells before storage. After antileukemic chemotherapy and/or radiation, the autologous marrow progenitor cells are reinfused and multifaceted supportive care techniques are required to protect the patient until immunologic recovery is complete. For some patients, however, it may be difficult to delay the autologous marrow harvest until adequate marrow cellularity is present along with complete bone marrow remission. Recurrent ALL is often resistant to therapy and secondary or later complete remissions are often transient. These brief remissions or those accompanied by a hypocellular marrow often prevent the successful collection of an adequate marrow harvest and at times may be followed by early leukemia relapse, even before harvest and autotransplantation can be performed.

The search for a suitable unrelated marrow donor may involve different logistical obstacles. Though more than one million potential donors are accessible through international registries, the coordination of donor tissue-type searching, donor contact and recall for additional testing, donor medical evaluation and scheduling can all result in substantial delays in transplantation. These logistical steps may defer the performance of the allogeneic transplant and leave the potential recipient at greater risk for leukemia relapse over time.

Strategic Searching to Allow Consideration of Unrelated or Autologous Marrow Transplantation

In the University of Minnesota Bone Marrow Transplantation Program, we have pursued a dual option search strategy for all

transplantation candidates with ALL. The design of such strategy includes a plan to accelerate and expedite all administrative aspects of the patient and donor evaluation; to pursue both transplant options (autologous and unrelated donor) as rapidly as possible; and if possible to make a therapeutic choice within four months from the time of referral. After four months, if no unrelated donor is identified, then patients in remission proceed promptly to autologous transplantation. If an unrelated donor is identified, this transplant option is considered the primary choice.

Between 1991-1993, we evaluated over 100 consecutive referrals of potential transplantation candidates with ALL. Seventy-six had no related donor and were candidates for either autologous or URD marrow transplantation. Seventeen of these had no URD search initiated and eight proceeded to autotransplantation after a median of 12 weeks following referral (4-21 weeks). The other nine were either medically ineligible for transplantation, received alternative therapy at another institution, opted not to have an autotransplant or had no suitable third-party coverage for the treatment.

Formal unrelated donor searches were initiated for 59 patients and, for these, 22 unrelated donors were identified at a median of 10 weeks following referral (1-33 weeks). Fifteen proceeded to unrelated donor transplantation at a median of 17 weeks following referral (2-29 weeks) and four survive in continuing remission. One patient, who had a donor identified nonetheless chose to receive an eventually successful autotransplantation because of concern about additional delay, while six others with a donor identified were either medically ineligible or opted not to pursue URD transplantation.

During this same time, 37 patients who had formal searches initiated had no donor identified and only four of these proceeded to autotransplantation; two survived. Thirty-three others elected alternative therapy at another institution, were medically ineligible, uninterested in transplantation therapy, or had no financial coverage for this therapeutic option.

Of this consecutively-referred cohort of patients with ALL, 58 underwent marrow transplantation out of the 116 evaluated. The different transplant options, however, were exercised at different times following referral. Allogeneic related donor transplantation was performed after a median of 9 weeks (range 4-19 weeks) following referral while unrelated donor transplantation took place at a median of 17 weeks (2-29) following referral. The decision-making to allow time for unrelated donor searching

led to autologous transplantation being performed at a median of 12 weeks (4-21) following referral.

While this comparative search strategy has allowed evaluation of both unrelated donor allogeneic and autologous transplantation, its success is contingent on efficiency in several areas for it to be of practical value for patients with ALL. First, the referral and third-party payor authorization request for financial coverage must be initiated while the patient is in relapse and be underway while the patient is receiving remission induction therapy. Second, the unrelated donor search must be started promptly and sample collection for histocompatibility testing must take place before induction therapy renders the patient pancytopenic. Third, a histocompatible and promptly available unrelated volunteer donor must be identified for a sufficiently large fraction of the patients searched to justify the delays involved in the search process. Acceleration of the efficiency and success of donor searching is essential before greater numbers of patients with ALL can be evaluated after unrelated donor allogeneic transplantation.

Comparative Success of Autologous Versus Unrelated Donor Transplantation

Formal comparative analysis of autotransplantation or unrelated donor BMT must evaluate the conventional clinical endpoints of survival, leukemia-free survival, leukemia-relapse and treatment-associated mortality. Importantly, the result of time-to-treatment bias on patients selected for either transplant option must be acknowledged. Patients may be lost by virtue of relapse or death during the delays involved in an unrelated donor search process or during subsequent attempts to achieve durable clinical remission. These delays may result in transplantation therapy being applied in a later remission than would have taken place had an autotransplant been performed promptly. Beyond these selection bias features, comparative clinical analysis must also assess differences in patient demographic characteristics (e.g., age, gender, gender match with the donor, donor:recipient histocompatibility and CMV serologic history). Also, the phenotypic characterization of the leukemia which reflects its aggressiveness and hazard category must be addressed. This should include the complete remission number at the time of transplant, the surface immunophenotype of the leukemic cells, karyotype, diagnostic leukocyte count, duration of first complete remission, involvement of extramedullary sites with leukemia and duration of time from remission

until transplantation. Comparison of the two transplant options must formally evaluate these patients' leukemia characteristics as well as assess the time bias and differential loss of patients from each cohort while plans for transplantation therapy are underway. Conventional statistical comparison of survival or relapse time from the day of transplant may seriously underestimate the pretransplant selection bias inherent in any comparative analysis evaluating these two transplant techniques.

Essential Features of a Comparative Analysis

The formal statistical comparison of autologous marrow transplantation versus unrelated donor allogeneic transplantation must address the clinical heterogeneity and selection bias problems identified above. Matched case control analyses may permit such comparisons with reference to data from large multicenter registries such as the IBMTR, NAABMTR, EBMT and NMDP. Importantly, quality scientific reports should include and acknowledge the number of patients referred for possible transplantation as a means to reveal the depth of selection bias inherent in these different forms of transplantation. Analysis of the success of transplantation in context of the time from remission until transplantation may provide insight into the donor search interval. This interval is relevant as different delays in each cohort may distort the composition of either an autologous transplant or an unrelated donor allogeneic transplant group.

CONCLUSIONS

The differential application of autologous transplantation versus unrelated donor transplantation as therapy for patients with ALL is complex and the issues highlighted above identify some problems in a formal comparative analysis. This monograph has attempted to emphasize issues requiring careful thought and commentary regarding the interpretation of any given transplant report. When assessing these alternative transplant techniques with their inherent pretransplant delays and their differences in posttransplant morbidity and antileukemic effectiveness, it must be recognized that the patient's period of risk begins well before transplantation. Appropriate interpretation of posttransplant results must acknowledge the pretransplant hazards and the selection bias of any transplant series to properly improve decision-making about transplantation therapy for patients with ALL.

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HIGH DOSE THIOTEPA AND MELPHALAN WITH ABMIT RESCUE IN HEMATOLOGIC MALIGNANCIES

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Thiotepa is an alkylating agent known to have significant anticancer activity in a variety of solid tumors: HD, NHL, CLL and CML.[1] This drug is now being used as a single agent or in combination with busulfan as a conditioning regimen in pediatric solid tumors and breast cancer.[3] Furthermore, the Genova and Perugia BMT teams[4] have reported their experience with thiotepa associated with TBI and cyclophosphamide (CY) as conditioning regimen for allogeneic BMT in CML.

The aim of this study was to evaluate the toxicity and the efficacy of thiotepa (15 mg/Kg on day -4) combined with another alkylator (melphalan 140 mg/M^2 on day -3) as myeloablative regimen for ABMT.

From September 1992, the thiotepa/melphalan combination with autologous bone marrow rescue was used in 12 patients (4 males and 8 females), aged 18-48 years with a variety of hematologic malignancies: 7 NHL in CR II or PR; 3 ALL in CR II and 2 in CR I; 2 AML in CR I. No relevant toxicity was observed. All the patients engrafted with median time to ANC >0.5 x 10^9/L and PLT >50 x 10^9/L of 16 and 46 days, respectively. At a median follow-up of 365 days (range 671-90) no recurrence occurred.

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SESSION III: BREAST CANCER

M.D. ANDERSON CANCER CENTER ADJUVANT THERAPY TRIALS IN STAGE II OR III BREAST CANCER

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In four prospective studies, doxorubicin-containing adjuvant therapies were evaluated at our institute. Details of treatment, doses, and schedule of these protocols have been published previously.[1-5] The results of these studies are summarized in this paper.

PATIENTS AND METHODS

The first study was initiated in 1974; all patients (n=222) with either stage II or III disease, following completion of local therapy (total mastectomy) received a combination chemotherapy regimen of fluorouracil, doxorubicin, and cyclophosphamide (FAC) and nonspecific immunotherapy with BCG. After a total cumulative dose of doxorubicin of 300 mg/M^2, doxorubicin was discontinued and patients continued therapy for a total of two years. Most patients also had postoperative radiation therapy following mastectomy. The results of this group were compared to a group of historical control patients treated at our institution only with local therapies (surgery & irradiation) in earlier years.

In the second study (n=238), patients were randomized to receive FAC alone + BCG; and in the latter part, patients who were referred without prior postoperative irradiation were also randomized for post-operative irradiation. The dosage of drugs was the same as in the first protocol, but the interval between the treatment cycles was reduced from four weeks as in the first study, to three weeks in the second protocol.

In the third study (n=336), vincristine and prednisone were added to the FAC regimen. The interval between the cycles was kept at three weeks. Aftr completion of the FAC regimen all patients with positive or unknown estrogen receptor status were randomized to receive six months of alternate therapy, either with tamoxifen (T) alone, or tamoxifen and chemotherapy with methotrexate and vinblastine (TMV). Methotrexate and vinblastine were administered sequentially. All patients with estrogen

receptor-negative tumors received additional chemotherapy with methotrexate and vinblastine.

In the fourth trial, all patients (n=311) were treated with escalating doses of doxorubicin and cyclophosphamide. Doxorubicin was also administered as a continuous infusion over 72 hours through a central venous catheter. All treatment was administered in the outpatient setting by utilization of portable infusion pumps. Patients with positive or unknown estrogen receptor status also received concomitantly one year of tamoxifen therapy. After completion of chemotherapy, patients who were randomized to receive immunotherapy, were treated with partially purified leukocyte interferon for a total of one year, or no further therapy.

Disease-free and overall survival times were calculated by Kaplan-Meier's method, and the differences between the groups were measured by the generalized Wilcoxon test; calculations of significance were based on two-sided test. Deaths due to causes other than breast cancer were counted as deaths in completing survival curves. Estimated reduction in mortality was based on the ratio of relative death rates in relevant FAC and historical control groups.

RESULTS

At a median follow-up of 183 months for the first study, the estimated percent survival by stage, age, nodal status and percent reduction in mortality are shown in Table 1, compared to the historical control group. In each subgroup, FAC patients had higher survival than the historical control group. Estimated reduction in mortality in the FAC patients from that of control group varied from 28-53%. A more modest gain in survival in the ≥50 year age group treated with FAC could be partly explained by the observation that a sizable fraction of these die of competing causes other than cancer.[6] In the second study, the median follow-up of the patient population was 155 months, and there was no improvement in survival by addition of irradiation or BCG. The incidence of loco-regional recurrences was 2% for patients treated with postoperative irradiation, and 14% for those who did not receive postoperative irradiation. All patients in this study had total mastectomy. In the third study at a median follow-up of 123 months, the 10-year estimated disease-free survival by patient characteristics and type of alternate therapy are shown in Table 2. Overall estimated survival rates of patients treated with TMV were not significantly higher than those of patients treated with tamoxifen alone, but for patients who were known to have estrogen

receptor-positive tumors, 66% of the patients treated with tamoxifen alone and 72% for TMV were alive (p=.017).

Table 1. Estimated 15-year Survival and Percent Reduction in Mortality of the First FAC Study

Group	Treatment	% Alive	P Values	% Reduction in Mortality
Stage II	FAC	50	<0.01	44
	Control	27		
Age (yrs)				
<50	FAC	57		
≥50	Control	26	<0.01	53
Nodal involvement				
1-3	FAC	62		
	Control	31	<0.01	54
4-10	FAC	43		
	Control	31	0.05	34
>10	FAC	37		
	Control	13	0.62	28
Stage III	FAC	22		
	Conrol	17	0.05	26

Table 2. Estimated Percent Survival Rates at 10-year in the Third Study

Group	Tamoxifen	TMV	MV
Stage II	70	71	58
Age (yrs)			
<50	76	83	61
≥50	62	61	59
Nodal Status			
0	89	83	60
1-3	78	68	71
4-10	68	84	55
>10	NR	50	50
Estrogen receptor			
Positive	66	72	-
Unknown	68	48	-
Stage III	54	35	51
All Patients	67	64	5

The fourth study is at a median follow-up of 88 months; the addition of interferon had no impact on the disease-free or overall survival.

For comparably staged patients, estimated 7-year survival rates for the four protocols are shown in Table 3. Estimated survivals were not significantly different in four studies, but there was a trend that in the latter trials a higher fraction of patients were alive.

TABLE 3. Estimated Percent Survival Rates at 7-year of Four Adjuvant Trials

	Protocol #				
Trials	**1**	**2**	**3**	**4**	**P**
Stage II	67	65	74	72	0.17
Stage II only					
Nodal involvement					
1-3	77	70	80	80	0.64
4-10	65	70	76	68	0.43
>10	46	44	46	51	0.99
Stage III	48	46	55	58	0.41

Toxicities

Total cumulative dose of doxorubicin was increased in the third trial to 400 mg/M^2 for patients with more than ≥4+ nodes. Doxorubicin was administered by bolus method in the initial three studies. In the fourth trial, all patients received doxorubicin as a continuous infusion over 72 hours through a central venous catheter, and total cumulative dose of doxorubicin was increased to 430 mg/M^2. In our historial control group, five patients had congestive heart failure, whereas, in the FAC group eight patients had congestive heart failure. For patients who received doxorubicin by continuous infusion, the risk of cardiac failure was lower than for patients who received doxorubicin as a bolus. Two patients who received doxorubicin by continuous infusion had congestive heart failure, and both patients had preexisting symptomatic cardiovascular disease prior to initiation of therapy. Ten-year estimated incidence of second malignancies was 8.6% (SE ± .85) in the control group, and 6.5% (SE ± 1.1) in the FAC group. The risk of second malignancy was lower in the FAC population than in the historical control group. Ten-year estimated risk of developing leukemia was 0.8% (SE ± 0.6) in the FAC group, 1.2% (SE ± 0.8%) in the control group, and 2.1% (SE ± 0.8) in the FAC + irradiation group. Although the estimated risk of developing leukemia was

higher in the FAC + irradiation group than the control group and FAC alone group, the differences were not statistically significant (p=0.08).

DISCUSSION

Doxorubicin-containing adjuvant chemotherapy, as utilized at the M.D. Anderson Cancer Center, has resulted in significant reduction in the risk of recurrence and improvement in survival. Long-term data of these studies show that doxorubicin-containing regimens are effective in reducing the risk of deaths in stage II and III patients. A large number of patients with >10+ nodes were treated in these studies, and 37% of these patients were alive beyond 10 years.

Long-term follow-up of these studies also show that late sequelae of these treatments are acceptable, and doxorubicin administered by infusional method in the adjuvant setting has a reduced likelihood of cardiac dysfunction. A number of patients develop recurrent diseae following adjuvant therapy and need further therapy with additional potentially cardiotoxic drugs. Doxorubicin administered by this technique reduces the likelihood of cardiac dysfunction not only initially, but these patients are at reduced risk upon retreatment.

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USE OF NON-CROSS-RESISTANT CHEMOTHERAPY IN COMBINATION WITH IRRADIATION AND SURGERY FOR TREATMENT OF REGIONALLY ADVANCED PRIMARY BREAST CANCER

G.R. Blumenschein

The treatment of Stage II-B, III-A and III-B breast cancer with conventional-dose chemotherapy, surgery, and irradiation has resulted in improved relapse-free survival for such patients with approximately 45-55% of patients remaining relapse-free at five years.[1] The presumed reason 45-55% of patients fail conventional-dose chemotherapy in combination with irradiation and surgery is that conventional-dose chemotherapy may not be sufficient to eliminate all of the microscopic tumor burden containing drug-resistant clones of breast cancer. These clones later emerge as measurable metastatic disease.

Three strategies have evolved in an attempt to eliminate these microscopic clones of cancer resistant to first-line conventional-dose chemotherapy. These include the use of high-dose chemotherapy, often with autologous bone marrow or stem cell rescue, the use of non-cross-resistant combinations of chemotherapy, and the use of combinations of chemotherapy and P170 glycoprotein-blocking drugs to overcome drug resistance. A four-year 74% relapse-free survival has been reported for Stage II-B breast cancer patients treated with mastectomy, four induction courses of methotrexate/doxorubicin/5FU, one of high-dose chemotherapy with autologous bone marrow or stem cell rescue, and irradiation.[2]

We have evaluated the use of a non-cross-resistant combination of drugs following treatment of Stage II-B, III-A, and III-B breast cancer. After or before mastectomy, the patients received an initial doxorubicin combination times six followed by irradiation followed by a cytoxan/methotrexate/5FU plus cisplatinum combination (McCFUD). In patients who had inflammatory breast cancer, hyperthermia was administered in combination with radiotherapy. The non-cross-resistant program labeled "McCFUD" has been shown to achieve a 70% response in metastatic disease following response to doxorubicin combination.[3]

METHODS

Fifty-five breast cancer patients with diagnosed Stage II-B, III-A, or III-B breast cancer were entered on study between January of 1986 and

January of 1991. Generally, Stage II-B and III-A patients were seen after mastectomy and begun on chemotherapy. Stage III-B patients usually were started on chemotherapy and had mastectomy after receiving two or three courses of induction chemotherapy. The initial combination program consisted of cyclophosphamide, doxorubicin, and VP-16 (CAVe).[4] After six courses of CAVe, the patients received radiation therapy to the chest wall and peripheral lymphatics; then they were started on McCFUD chemotherapy. Three courses of McCFUD were administered with a 28-day treatment cycle. Patient characteristics and tumor prognostic factors are shown in Table 1. The chemotherapy doses and schedules are outlined in Table 2.

Table 1. Advanced Primary Breast Cancer Patient Characteristics

Age:	Range 28-72	Median 48
Histology:	Infiltrating Duct	35 (63%)
	Inflammatory	12 (22%)
	Other	8 (15%)
Stage:	Stage II (>9 + nodes)	17 (31%)
	Stage III-A	21 (38%)
	Stage III-B	17 (31%)
ER/PR:	+/+, 26 (47%)	+/-, -/+, -/-, 29 (53%)
Ploidy:	Aneuploid	14 (25%)
	Diploid	11 (11%)
	Unknown	30 (55%)
S Phase:	<5%	8 (15%)
	≥5%	16 (29%)
	Unknown	30 (55%)

Table 2. Advanced Primary Breast Cancer
CAVe Treatment Schema

	Day 1	Day 2	Day 3
Cytoxan 500 mg/M^2 IV	X		
VP-16 80 mg/M^2 IV bolus	X	X	X
Adriamycin 50 mg/M^2 IV continuous infusion	X	X	X

Repeat cycle every 21 days x 6

McCFUD Treatment Schema

	Day 1	Day 2	Day 3	Day 4	Day 5
Methotrexate 120 mg/M^2 IV over 1 hour	X				
Decadron 10 mg IV q.6h (8 doses)	X	X			
5-FU 1000 mg/M^2 IV over 1 hr, 6 hrs after methotrexate	X				
Cisplatinum 60 mg/M^2 IV		X			
Leucovorin 15 mg p.o. q.6h (8 doses)		X	X		
Cytoxan 300 mg/M^2 C.I. x 3 days			X	X	X

Cycle repeated every 28 days x 3

RESULTS

After a median follow-up of 42 months, Kaplan/Meier projection of relapse-free survival is 78% at 4 years and 74% at 5 years for the entire group of patients. There was a small difference between Stage III-A and III-B patients, although the subsets were small with results in the III-B group being superior to the results in the III-A and II-B subsets. The Stage II-B patients who were ER-positive appeared to continue to relapse later than the ER-negative II-B and III-B patients. When patients were ER/PR-positive, they received concomitant hormone therapy with chemotherapy

and irradiation. This usually consisted of tamoxifen; occasionally Lupron or oophorectomy was used in combination with tamoxifen in the premenopausal patients. The combined relapse-free survival curve for the three stages are shown in Figure 1. Figure 2 shows the projected overall combined survival for the three subsets. Table 3 describes sites of relapse. It is interesting to note that 29% of the initial sites of relapse occurred in the central nervous system. Other sites of relapse included liver, bone, lung, and chest wall. Regional relapses were minimal and occurred in only two patients. Toxicity was manageable. No patients died of chemotherapy-related toxicity.

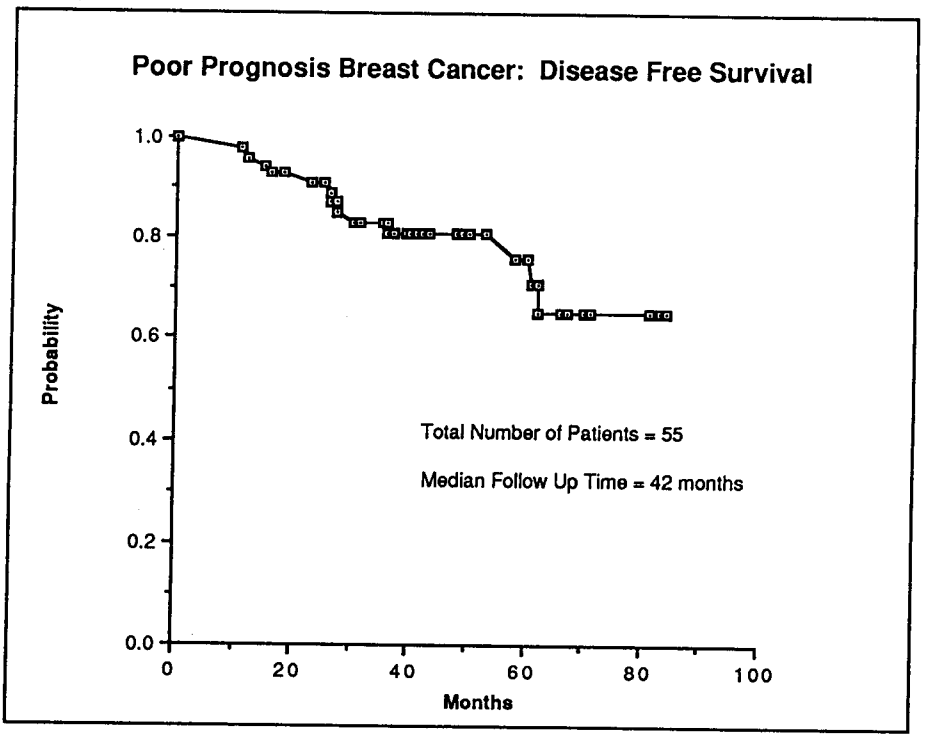

Figure 1.

Table 3. Relapse

Number of patients:	14
Sites and Frequency of Recurrence:	
Brain	4 (29%)
Bone	6 (43%)
Liver	2 (14%)
Lung	1 (7%)
Chest Wall	1 (7%)

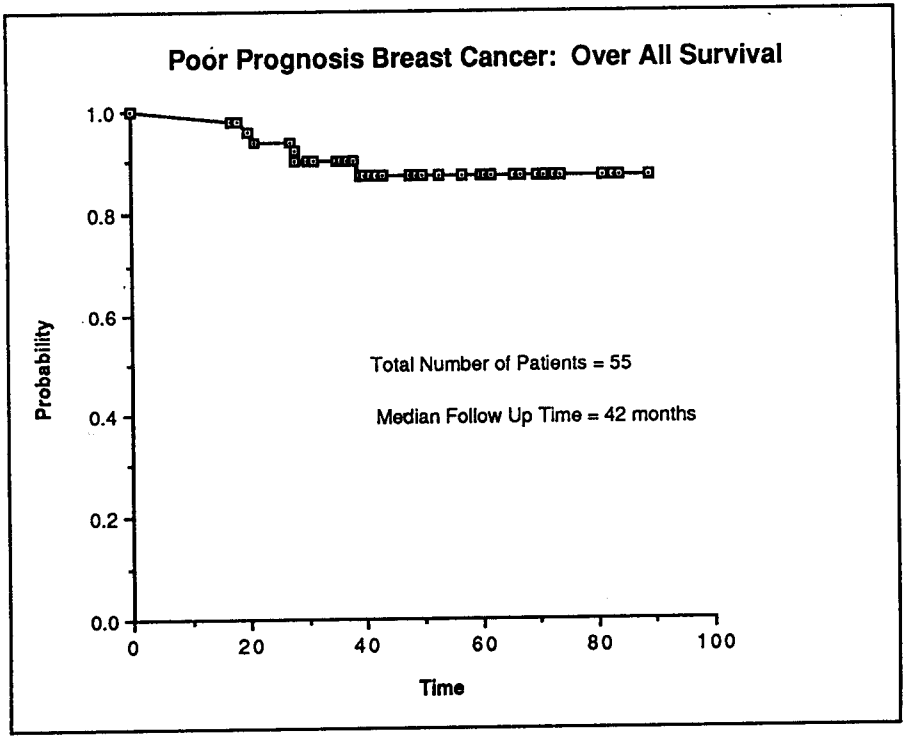

Figure 2.

DISCUSSION

Employing non-cross-resistant combination chemotherapy following doxorubicin-induction chemotherapy and irradiation after mastectomy appears to be as effective a strategy for prolonged relapse-free survival in regionally-advanced primary breast cancer as does using treatment strategy of dose escalation to eliminate microscopic tumor burden.

The program described appears to have very reasonable efficacy. It can be administered in the outpatient setting. Now, with the advent of Neupogen, it can be done with very little risk of infection. Because there is evidence for late relapse, we believe this strategy needs to be combined with the other popular strategy of dose escalation in an effort to further improve relapse-free survival beyond five years. The fact that 29% of the initial sites of relapse occur in the central nervous system indicates that while the program has significant effect against microscopic metastatic breast cancer, it does not cross the blood-brain barrier. This high recurrence rate in the central nervous system increases the requirement for follow-up of patients with MRI of the brain as a routine.

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HIGH-DOSE CHEMOTHERAPY WITH AUTOLOGOUS STEM CELL SUPPORT FOR STAGE IIIB AND IV BREAST CANCER

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INTRODUCTION

The overall median survival of women with advanced breast cancer (about 19 months) has not improved with conventional chemotherapy. Regimens employing high-dose chemotherapy with autologous stem cell (marrow and/or peripheral blood progenitor cell) support have been developed with the hope of optimizing tumor response and increasing survival.

Early phase I studies in patients with advanced refractory disease demonstrated the feasibility of administering agents in doses 5-30X higher than conventionally used. These studies achieved high response rates of short duration. Second generation studies combined an induction phase followed by one high-dose intensification at time of maximum tumor response. To date, between 10-20% of women with metastatic breast cancer are remaining progression-free with this dose-intensive approach, with median duration of follow-up approaching 50 months in larger series. With the advent of hematologic support such as peripheral blood stem cells (PBPC) and colony stimulating factors, the morbidity, mortality, and costs associated with this treatment have been substantially reduced. These supports now allow for two or more cycles of high-dose intensification to be employed, to exploit the potential of dose-intensity to optimize response.

DANA-FARBER CANCER INSTITUTE/BETH ISRAEL HOSPITAL EXPERIENCE

Advanced Breast Cancer

Single Intensification (CTCb). Initial phase II studies performed by the Dana-Farber Cancer Institute and the Beth Israel Hospitals were designed to treat women with unresectable or metastatic breast cancer responding to conventional dose chemotherapy, with one high-dose intensification and marrow or PBPC support.[1-3] Eligibility criteria included at least a minimal response to induction chemotherapy and absence of

marrow or central nervous system (CNS) involvement. The recommended induction regimen included doxorubicin (25 mg/M^2/day x 3), 5-FU (750 mg/M^2/day x 5) and methotrexate (250 mg/M^{2}on day 15) with leucovorin rescue (AFM). At the time of maximum response, one high-dose intensification of cyclophosphamide (6000 mg/M^2), thiotepa (500 mg/M^2) and carboplatin (800 mg/M^2) (CTCb) by 96-hour continuous infusion was administered. Autologous stem cell support (either marrow alone or GM-CSF- and chemotherapy-mobilized PBPCs) was reinfused on day 0.

Between May 1988 and March 1992, 62 women were enrolled on study. Four patients (3%) died of toxicity (CNS bleed, cardiomyopathy) or progressive disease while still platelet-transfusion dependent. For 27 patients receiving marrow alone, the median times to recovery of an absolute neutrophil count (ANC) ≥500/μl and to a platelet count ≥20,000/μl were 21 (range, 10-51) and 23 (range, 22-113) days, respectively. The median units of packed red blood cells (PRBC) and platelets transfused were 13 (range, 4-101) and 105 (range, 11-1350), respectively. For the remaining patients who received PBPCs alone, the median times to recovery of an ANC ≥500/μl and to a platelet count ≥20,000/μl were 14 (range, 10-57) and 12 (range, 8-134+) days, respectively. The median units of PRBCs and platelets transfused were 8 (range, 4-15) and 22 (range, 10-237), respectively. The use of PBPCs resulted in significantly improved time to engraftment, total antibiotic days, transfusion requirements and length of hospital stay.

With a median follow-up of 50 months, 20% of women remain progression-free (range, 24-63). Those remaining progression-free had a significantly higher incidence of complete response to induction chemotherapy, an increased interval from initial diagnosis of primary disease to first diagnosis of metastases, single site of metastasis, had estrogen receptor-negative tumors, and were aged greater than 40 years.

Double dose-intensive chemotherapy with marrow and chemotherapy and G-CSF mobilized PBPC support. In our previous studies, 20% of women with responding metastatic breast cancer remain disease-free a median 50 months following single high-dose CTCb. This trial studied the feasibility of administering two high-dose intensifications in an attempt to optimize disease response and duration.[4,5]

Women with at least a partial response to induction therapy (as determined after three or four cycles) underwent marrow harvest and then received two cycles of doxorubicin (25 mg/M^2/day x 3) and 5-FU (500 mg/M^2/day x 3), followed by G-CSF (5 μg/kg/day sc. on days 5-16). Three PBPC collections were obtained after each cycle of doxorubicin/5-

FU (AF) by three 2-hour leukaphereses when the total WBC was 1000/μl and rising (generally on days 17-19 and for a total of six leukapheresis products).

If patients remained in response, they then received high-dose melphalan (140-180 mg/M^2), followed 24 hours later by reinfusion of chemotherapy and G-CSF-mobilized PBPCs (four leukapheresis products) and additional G-CSF (5 μg/kg/day) post-reinfusion until granulocyte recovery. They were monitored daily as outpatients. After recovery, patients were hospitalized for a second intensification of CTCb with marrow, PBPCs (two leukapheresis products), and G-CSF post-reinfusion until granulocyte recovery. Local therapy was then given to accessible sites of bulk disease.

Forty-two women (median age 42 years) are entered and evaluable. Seventy percent required hospitalization for fever/neutropenia with or without mucositis after high-dose melphalan (median hospital stay: seven days). The median days of ANC <500/μl and a platelet count <20,000/μl were 6 (range, 4-17) and 5 (0-41), respectively. Twenty-one percent developed severe mucositis and 7% had overt infections. The median units of PRBCs and platelets transfused were 3 (range, 0-13) and 15 (range, 0-99), respectively.

Patients received CTCb a median of 25 days after starting melphalan, and the total CTCb hospital stay averaged 24 days. After CTCb, the median days of ANC <500/μl and a platelet count <20,000/μl were 11 (range, 7-31) and 22 (range, 5-300+), respectively. The median units of PRBCs and platelets transfused were 8 (range, 3-19+) and 63 (range, 13-232+), respectively. Grade 3 toxicities included: stomatitis (38%), hepatomegaly (5%), and mild reversible VOD (7%). There was a 26% incidence of overt infections (20%) and one death from venoocclusive disease (VOD). That death resulted in our fixing the interval between the start of melphalan to the start of CTCb to five weeks. With that increased interval, there have been no further incidences of hepatomegaly or VOD. To date, 15 patients (37%) remain progression-free a median of 19 months (range, 9-26) post CTCb. While this double dose-intensive approach for metastatic breast cancer is feasible with acceptable toxicity, longer follow-up is needed to determine whether responses are most durable and survival is enhanced.

Stage IIIB/Inflammatory Breast Cancer

Recent single institution studies using ABMT for high-risk stage II and III breast cancer have reported preliminary findings suggesting a

prolonged disease-free survival.[6,7] The cooperative groups have now begun prospective randomized studies in high-risk stage II and III women with ≥10 positive axillary lymph nodes, and will soon study the efficacy of ABMT in women with inflammatory or locally unresectable breast cancer (stage IIIB).[8]

Women with locally unresectable or inflammatory (stage IIIB) breast cancer have approximately a 30% 5-year disease-free survival with conventional multi-modality therapy. We designed a pilot study to test the feasibility of administering dose-intensive doxorubicin followed by high-dose CTCb with marrow and PBPC support to women with newly diagnosed disease.

Patients received three cyles of doxorubicin (30 mg/M^2/day IVB on days 1-3) every 14 days, followed by G-CSF (5 μg/kg/day sc. on days 4-12). It there was no evidence of disease progression, they then underwent marrow harvest, followed by a fourth and final cycle of doxorubicin/G-CSF. After the fourth cycle, two PBPC collections were obtained by two 2-hour leukaphereses when the total WBC was 1000/l and rising (generally on days 10-14). After completion of induction chemotherapy, the patients were hospitalized for a single intensification of high-dose CTCb with marrow and PBPC support. G-CSF was given post-reinfusion until granulocyte recovery. Mastectomy usually occurred after full recovery from BMT (generally two weeks after discharge from the hospital). Full-course radiotherapy was begun after recovery from mastectomy. Anti-hormonal therapy (for 5 years) was initiated for receptor-positive/borderline tumors.

To date, 28 women are evaluable. The median age was 38 years (range, 27-49) and 75% had palpable axillary adenopathy. Seventy-five percent received full-dose doxorubicin; the others required dose reduction or delay for mucositis or sepsis. All women have completed CTCb with acceptable toxicity. Mastectomy performed in seven patients (all had pathologic inflammatory breast cancer) prior to any chemotherapy revealed diffuse disease; surgery performed in two after doxorubicin induction and before CTCb showed persistent gross disease. Mastectomy done after CTCb in 19 patients revealed pathologic complete response in four (21%), intraductal carcinoma in three, microscopic foci only in four (21%), or gross disease in eight (42%). All were rendered disease-free with surgery. With a median follow-up of 21 months (range 4-33) since start of therapy, there has been one relapse. This dose-intensive multimodality approach for locally unresectable/inflammatory breast

cancer is feasible with acceptable toxicity, and will be one arm of a proposed randomized trial through the CALGB cooperative group.

FUTURE DIRECTIONS

Ongoing research is being directed toward development of more effective intensification regimens, both in terms of new chemotherapeutic agents and optimal delivery schedule. While alkylating agents remain the backbone of high-dose regimens, new agents worthy of dose escalation are being actively tested or contemplated for future trials. Agents such as amonafide, taxol or taxotere, and liposomal doxorubicin may be dose-escalated for use in either induction or intensification. Modulation of chemotherapy by drugs which can inhibit glutathione, decrease hypoxia, or inhibit topoisomerase II activity may lead to an additional log cell kill and is an area of active investigation. To make dose-intensive treatment safer, combinations of hematopoietins and peripheral blood progenitor cells are being investigated to decrease even further the period of absolute aplasia. With advances in PBPC technology, the techniques of gene transfer are proceeding to clinical trial in the near future.

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AUTOTRANSPLANTS FOR BREAST CANCER IN NORTH AMERICA

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The NAABMTR has collected data on over 3,000 patients receiving autotransplants for breast cancer since 1989, in over 100 centers in the United States and Canada. Over this period there has been a marked increase in the number of transplants performed for this indication (see below). Additionally, there has been increased use of autotransplant as adjuvant/neoadjuvant therapy and selection of patients with chemotherapy sensitive metastatic disease.

Autotransplants for breast cancer patients reported to the NAABMTR 1989-1993.

	1989	1990	1991	1992	1993*
No. of pts.	261	342	688	974	812
Disease State					
Stage II	1%	5%	8%	13%	13%
Stage III	2%	4%	7%	14%	14%
Metastatic					
Sensitive	64%	54%	49%	48%	45%
No Response	21%	20%	15%	13%	8%
Other	12%	17%	21%	12%	20%
Median age, yrs (range)	41 (23-72)	42 (24-66)	44 (22-69)	44 (19-65)	44 (22-70)
100-day mortality	22%	16%	11%	6%	6%

*Data incomplete

Over this period 100-day mortality has reduced significantly. The two year probability of survival (95% confidence interval) for autotransplant used as adjuvant/neoadjuvant therapy is 77 $\pm$ 6% and for metastatic disease 38 $\pm$ 3%.

HIGH-DOSE CHEMOTHERAPY AND AUTOLOGOUS STEM CELL SUPPORT IN METASTATIC BREAST CANCER: THE UNIVERSITY OF CHICAGO EXPERIENCE

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ABSTRACT

One hundred and two women with advanced breast cancer underwent high-dose intensification after obtaining a response to initial induction therapy. Median patient age was 43 years and all patients were required to have had a response or stable disease to induction therapy. Median follow-up was 27 months. The overall event free survival (EFS) was eight months with an estimated three year EFS of 13% $\pm$ 4%. Response to induction therapy seemed to be a predictor of outcome with patients in complete response versus partial response to induction with a median EFS of 12 months and six months, respectively. Three years EFS was 32% $\pm$ 10% and, 6% $\pm$ 5%, respectively. The presence or absence of marrow involvement or source of stem cell rescue did not affect outcome. Thus, approximately 10-15% of women achieved a durable response. Response to induction therapy appears to be an important predictor of outcome.

INTRODUCTION

Breast cancer remains a major health problem in the United States. About 180,000 cases will be diagnosed this year with about 46,000 deaths due to breast cancer. For metastatic breast cancer median survival is about 2 years.[1] Responses with currently available chemotherapy programs are of short duration. Recent clinical experience has been developed to intensify chemotherapy doses. Several studies in metastatic breast cancer have shown a correlation of dose intensity to response, which correlated with an improvement in survival.[2] We have attempted to exploit the steep dose response curve of alkylating agents in the setting of metastatic breast cancer. The following is a brief report of our results.

PATIENTS AND METHODS

Patient Characteristics

One hundred and two women with metastatic breast cancer were enrolled on 1 of 3 sequential high-dose chemotherapy (HDC) protocols between 1986-1993. All studies required response or stable disease following a standard dose induction chemotherapy regimen to proceed to high-dose chemotherapy. Written informed consent approved by the institutional review board was obtained in all cases. Patient characteristics are listed in Table 1.

Table 1. Patient Characteristics

Total Number of Patients	102
Age (years)	
Median	43
Range	20-63
Prior Adjuvant Chemotherapy (%)	
Yes	72
No	28
Bone Marrow Involvement (%)	
Yes	36
No	64
Number of metastatic sites (%)	
1	36
2	37
3 or greater	27
Source of stem cell rescue (%)	
Bone marrow	44
Peripheral blood progenitors	36
Both	21

Chemotherapy and Supportive Care

All 102 patients were required to obtain a response or have stable disease after a course of induction therapy.[3,4,5] After induction therapy patients underwent procurement of stem cells. Sixty-six patients had no histologic evidence of marrow involvement and underwent bone marrow harvest as well, 21 of these patients underwent peripheral blood procurement after G-CSF mobilization in addition to marrow. The remaining patients had histologic evidence of marrow involvement, thus they underwent only peripheral blood progenitor procurement. Patients

subsequently underwent high-dose intensification with either cyclophosphamide (7.5 gm/m^2) and thiotepa (675 mg/m^2) (79 patients) or the same cyclophosphamide, thiotepa, with carmustine (450 mg/m^2) (23 patients). All supportive care was as previously described.[4]

Statistical Methods

Time-to-event measures were estimated by the Kaplan-Meier method.[6] Overall survival was measured from date of reinfusion; an event was death due to any cause. Event-free survival was measured from date of reinfusion; progression of disease or death due to any cause were considered events.

RESULTS

The median follow-up time was 27 months from the day of reinfusion. The overall complete response (CR) rate for the entire induction plus high-dose intensification was 37%. The treatment-related mortality over the 7-year period was 12%. The median overall survival for the entire group was 16 months with an estimated 3 year survival of 26%$\pm$ 6%. The event free survival (EFS) was 8 months with an estimated 3 year EFS of 13% $\pm$ 4%. These results are summarized in Table 2.

Table 2. Summary of Treatment Outcomes

Group	Number of Patients	Median Event Free Survival (Months)	Estimated 3-year Event Free Survival
ALL Patients	102	8	13%
Induction CR	29	12	32%
Induction PR	42	6	6%
CR After High-Dose Therapy	38	12	29%
Total			
Induction CR	21	21	43%
Induction PR	15	10	0%

CR = Complete Response
PR = Partial Response

If patients are analyzed based upon their response to induction therapy, the median EFS is 12 months for patients in CR versus 6 months for patients obtaining a partial response (PR) to induction therapy. The corresponding estimated 3 year EFS(s) were 32%$\pm$ 10% versus 6% $\pm$ 5%.

Among patients obtaining a CR after high-dose intensification, response to induction therapy seemed to remain a predictor of outcome (Fig 1). The median EFS(s) among patients with a CR after high-dose intensification were 21 months and 10 months for those with a CR versus PR to induction therapy respectively. The corresponding estimated 3 year EFS(s) were 43% $\pm$ 13% versus 0%.

The presence or absence of marrow involvement did not appear to impact upon outcome. Likewise, the source of stem cell product did not appear to impact upon long term EFS. For women obtaining stem cells from marrow versus peripheral blood, the estimated 3 year EFS for both groups was 13% $\pm$ 4%.

DISCUSSION

To improve upon remission rates and, ultimately, survival in metastatic breast cancer, we explored the approach of initial cytoreductive induction therapy followed by high-dose intensification with autologous stem cell support. This approach theoretically would allow reduction of tumor bulk and treatment in a "minimal disease state" by selecting patients with chemotherapy sensitive disease. This approach yields high response rates but overall survival may not be better than conventional chemotherapy. In our experience, about 10-15% of women treated with this approach achieved a durable response. Others have reported similar results.[7,8] Response to initial induction therapy appears to be an important predictor of outcome. In those patients in complete remission prior to high-dose intensification, the EFS appears better than others even in partial remission.

In addition, neither the presence of marrow involvement nor the source of stem cell product appeared to impact upon outcome. This probably reflects the fact that most patients relapse in sites of prior disease.

Further studies are needed to improve upon anti-tumor efficacy. Newer chemotherapeutic agents can be incorporated into the design of these studies to improve upon induction CR rates prior to high-dose intensification. These studies need to be carefully designed so that we can determine the optimal criteria for patient selection in the future.

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REPETITIVE HIGH-DOSE THERAPY: TOLERANCE AND OUTCOME IN STAGE IV BREAST CANCER

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INTRODUCTION

In this manuscript we update the outcome of the tandem transplant approach[1-4] to high-dose intensification for Stage IV breast cancer, report the reduced mortality with this approach due to new and improved supportive care measures and clinical experience, and describe future approaches of high-dose therapy (HDT) for Stage IV disease.

GENERATION I: High-dose cyclophosphamide/etoposide/cisplatin (CVP) therapy for metastatic breast cancer

This report describes the outcome of 141 patients with Stage IV breast cancer known to have estrogen receptor (ER) negative tumors or to be hormonally-refractory (defined as progressive disease occurring while on hormone therapy in patients with either ER positive or ER status unknown tumors). All patients were in first relapse. A more extensive description of the clinical characteristics of these patients will be described in a future publication along with a multivariant analysis of clinical features predictive of outcome.

Disease staging was performed before the induction (conventional dose chemotherapy) phase, before the first (cycle 1 of HDT) intensive phase, and one month after completion of the second course of the intensive phase. Subsequent to the tandem transplant and after full recovery from all extramedullary toxicity and normalization of blood counts, follow-up disease evaluations were performed at 3-month intervals. Progression-free survival and overall survival were measured from commencement of the induction phase. Kaplan-Meier estimates of progression-free survival and overall survival were calculated.

ER negative patients began conventional dose chemotherapy (induction phase) at first evidence of recurrent or metastatic disease. ER positive patients were first challenged with hormonal therapy (except those patients who exhibited life-threatening visceral disease), and after evidence of disease progression entered into the induction phase. Attempts were made to initiate the intensive phase at the point of maximal tumor response to the induction phase. Intensification doses were administered at four successive dose levels in a phase I-II dose-escalating fashion (Table 1). Details of methods used to deliver dose-intensive chemotherapy, hydration, and supportive care have been reported in a previous publication.[2]

Table 1. Dose* Levels Studied in Generation I: CVP Protocols

Dose Level	Cyclophosphamide	Etoposide	Cisplatin
1	4500	750	120
2	4500	900	150
3	5250	1200	165
4	6000	1500	180

* mg/M^2

The response (complete and partial remission) rate to the induction phase and the "overall response" rate in patients who completed both the induction and intensive phases are summarized in Tables 2 & 3. One hundred forty-one patients are reported. The response to induction chemotherapy was 113/141 (80%). Of the 141 patients, 43 (30%) entered complete remission and 70 (50%) partial remission after induction chemotherapy. Twenty-one patients (15%) had stable disease and 7 patients (5%) had progressive disease after the induction phase and prior to HDT. The "overall response," defined as response of measurable disease following completion of both the induction and the intensive phases of this treatment program, was 123/141 (87%). Further analysis of these 123 patients revealed achievement of complete response (CR) in 86 (61% of total) and partial response (PR) in 37 (26%). Patients dying from the treatment-related causes were considered non-responders (Table 3).

Table 2. Response to the Induction Phase in First Generation CVP Studies

Response	Number (%)
CR	43 (30)
PR	70 (50)
Stable	21 (15)
Progression	7 (5)
Total	**141**

Table 3. Response to the Intensive Phase in First Generation CVP Studies

Response	Number (%)
CR	86 (61)
PR	37 (26)
Stable	8 (6)
Progression	2 (1)
Early Death	7 (5)
Clinical CR	1 (.1)

For these patients with metastatic disease, the median progression-free survival measured from the beginning of induction and of intensification therapy is approximately one year and 43 weeks, respectively. With follow-up that extends to 8 years, approximately 15% of the patients may be long-term disease-free survivors (Figure 1). Examining outcome after the intensive phase relative to response to the induction phase of therapy revealed an impressive plateau of disease-free survival at approximately 40% for patients entering intensification in CR. Patients in PR or with stable disease at time of transplant show a tendency to continuing late relapses but there is approximately 10% long-term disease-free survivors. Patients transplanted with progressive disease after induction therapy show a rapid and eminent relapse rate (Figure 2). Liver involvement predicted for a poor outcome in this expanded population of patients treated with CVP. An indepth univariate and multivariate analysis of prognostic factors for long-term disease-free survival has been published on a smaller series of patients.[2]

GENERATION II: CVP studies

This generation of studies has utilized dose level 3 of the CVP protocol in a tandem transplant approach and has incorporated major changes in supportive care measures. The basic change in supportive care has been the use of peripheral blood stem cells (PBSC) procured following growth factors in all patients. All patients received G-CSF (10μg/kg/day) and GM-CSF (5 μg/kg/day) subcutaneously (sc.) beginning 6 days before the start of apheresis and continued throughout apheresis. Apheresis was performed for a median of 4 consecutive days until at least 1 x 10^6 CD34+/kg were collected. PBSC were collected from patients through a double lumen subclavian catheter (Quinton Instrument Company, Seattle, WA) using continuous flow Fenwal CS3000 (Baxter Heatlh Care Products, Deerfield, IL) blood cell separator. Selected patients in which a single cycle of HDT was planned were apheresed for only 2 days.[5,6] The product of two alternate days of PBSC apheresis was infused after each cycle of high-dose therapy, alternating days 1 and 3 with days 2 and 4 between patients to insure a qualitatively similar PBSC product throughout the cohort. Patients also received autologous bone marrow transplant if there was no evidence of bone marrow or bone involvement by tumor. Additional modifications of the supportive care program included routine use of prophylactic antibiotics (vancomycin and ciprofloxacin) and rapid, early intervention in the setting of subclinical hypoxia, hypotension and renal impairment by algorithm management.

After administration of HDT, growth factor mobilized PBSC were given either alone (N=21) or with autologous bone marrow transplant (N=55). Patient (N=76) features included median age 42 years (range, 25-68), disease Stage II in 2, Stage IIIA in 4, Stage IIIB in 9, and Stage IV in 61. Of those patients with Stage IV disease, 7 were in CR, 14 in PR, 20 had stable and 9 had progressive disease at the time of transplant. Twelve patients with Stage IV disease had multiple relapses. Eighty-three percent of patients received a second transplant. Mortality within 30 days of the first cycle of HDT occurred in 2 (3%) patients, and with the second HDT cycle in 1 (1%) patient. Reversible Grade 3 nonhematologic toxicity included diarrhea in 20-30%, and nausea/vomiting in 50%. Fourteen percent of patients undergoing cycle 1 of HDT and 3% undergoing cycle 2 of HDT developed documented positive blood cultures (Table 4).

Table 4. Toxicities Observed with Generation II CVP

Mortality:	
Cycle #1 HDT	2/76 (3%)
Cycle #2 HDT	1/63 (1%)
Diarrhea*	25%
Nausea & Vomiting*	50%
Documented Infection:	
Cycle #1 HDT	14%
Cycle #2 HDT	3%

**Reversible Grade 3*

Excluding those with bone disease, 14 of 28 (50%) patients with either stable disease or in PR at the time of transplant entered CR after HDT. Median overall survival and progression-free survival (PFS) after PBSC and autologous bone marrow transplantation or PBSC alone for Stage IV patients was 143 and 49 weeks, respectively. From time of induction therapy, overall survival and PFS were 167 and 69 weeks, respectively (Figures 3 and 4). Examining overall survival only in the 41 patients who received high-dose CVP after responding to or remaining stable on induction therapy given for first relapse is encouraging and exceeds 3 years. Despite a lower frequency of patients in CR and a higher proportion of patients with stable disease after induction therapy (Table 5), the percentage surviving disease-free after HDT remains equivalent to older studies. The observed lower frequency of response to induction therapy is reflective of two changing patterns of patient referral for HDT: a higher frequency of patients aggressively treated during adjuvant therapy and a higher frequency of bone disease, in which it is difficult to evaluate response.

Table 5. Response to Induction Therapy Before CVP

Response	Number (%)
CR	7 (17)
PR	14 (34)
Stable	20 (49)

GENERATION III: CVPCT protocol

We have modified the CVP program to include carboplatin and taxol (CVPCT). The expectations are that severe nausea and vomiting will be lessened by dividing the dose between cisplatin and carboplatin, and

inclusion of taxol may increase the antitumor efficacy of this protocol. All patients receive cyclophosphamide (1.75 gm/M^2/day, day 1-3), VP-16 (400 mg/M^2/day, day 1-3), cisplatin (33 mg/M^2/day, day 1-3), and carboplatin (400 mg/M^2/day, day 1). Taxol is given by continuous infusion over 48 hours in escalating doses as follows: 6 patients received 100 mg/M^2, 3 patients received 150 mg/M^2, 3 patients received 200 mg/M^2 and 25 patients received 250 mg/M^2 (Table 6). No patient expired from procedure-related mortality. Response and follow-up are too short to determine if this approach improves outcome further.

Table 6. High-Dose CVPCT Program

Drug	Dose (mg/M^2)	Number of Patients
Cyclophosphamide	1750 x 3	
Etoposide (VP-16)	400 x 3	
Cisplatin	33 x 3	
Carboplatin	400 x 1	
Taxol (CI 48 hrs)	100	6
	150	3
	200	3
	250	25
Total		**37**

DISCUSSION

Much debate has centered on the usefulness of HDT with stem cell support for Stage IV breast cancer. We have also shared these doubts. However, the recent re-analysis and longer follow-up of our first generation of CVP studies, the ever decreasing and almost non-existent treatment-related mortality for ideal candidates and the possibility of further improving outcomes with the inclusion of newer active agents in the preparative protocols has changed our perspective for the use of this modality for Stage IV disease. As a consequence of the very mature follow-up of our initial CVP studies, it appears that indeed this therapy should be recommended for patients who enter CR with first-line chemotherapy for Stage IV disease. For those patients who enter PR or have stable disease after induction therapy, the small proportion of patients who remains disease-free after receiving HDT suggests that these patients

deserve the choice of HDT. The ever decreasing morbidity and mortality have removed a major criticism regarding the routine use of this approach and its recommendation as standard therapy. The remaining obstacle to an increase in the use of this modality is the cost of the procedure. Innovative approaches to this problem and use of HDT in earlier stages of disease may make this very economical.

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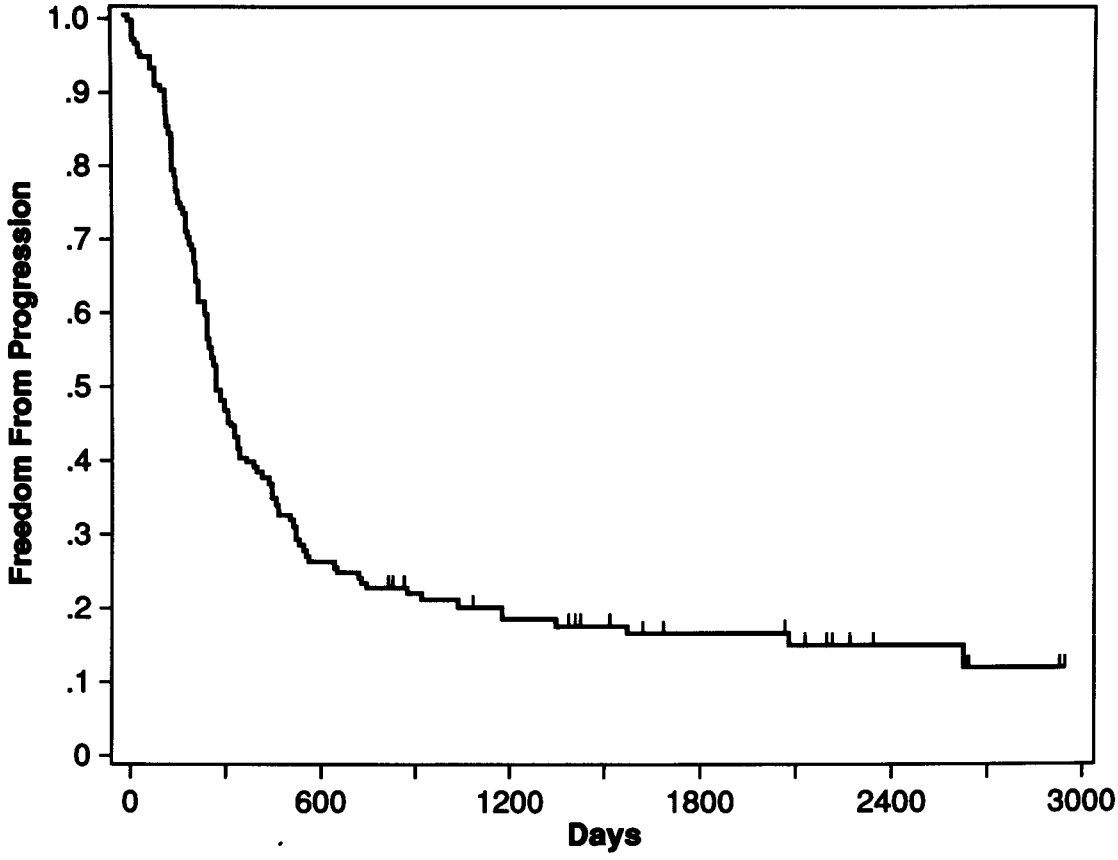
1.0
.9
.8
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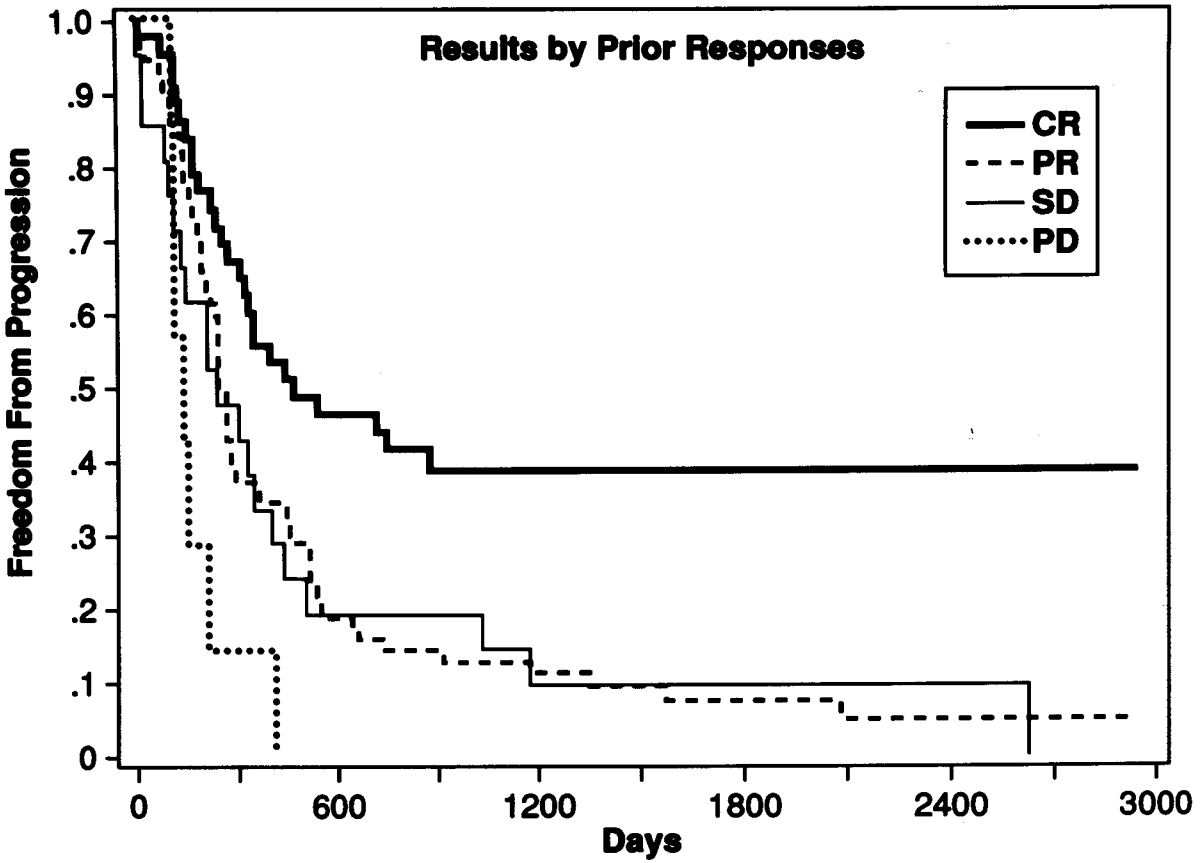
0
Freedom From Progression
0
600
1200
1800
2400
3000
Days

Figure 1.


Results by Prior Responses
CR
PR
SD
PD
Freedom From Progression
1.0
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600
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3000
Days

Figure 2.

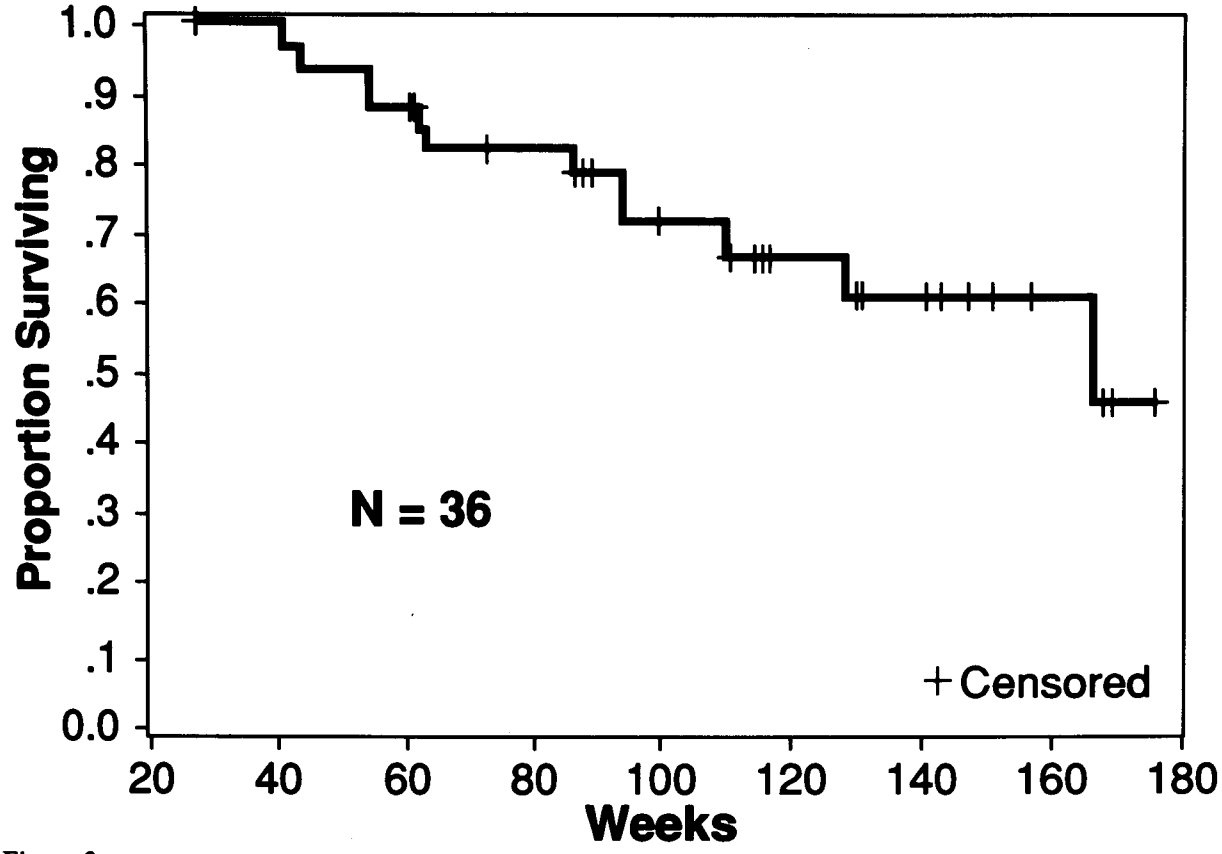
Proportion Surviving
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0.0
N = 36
+ Censored
20
40
60
80
100
120
140
160
180
Weeks

Figure 3.

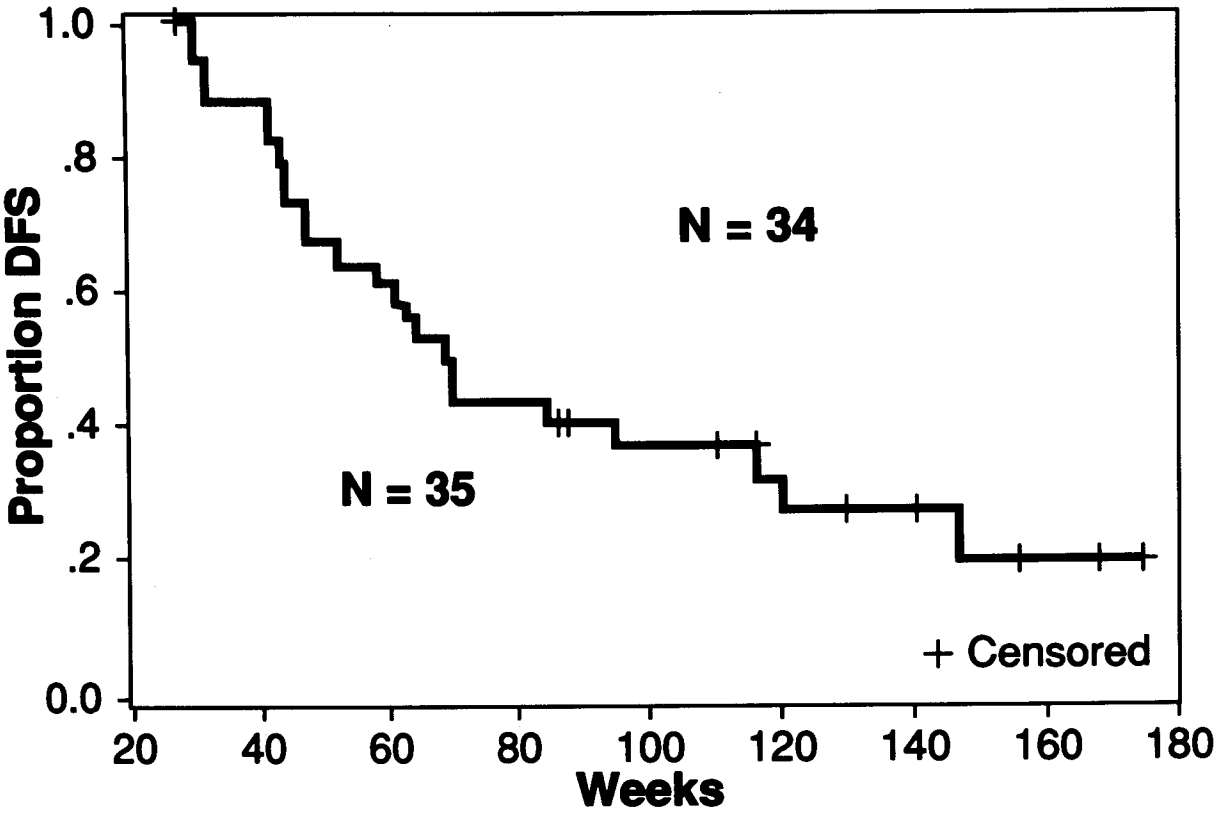
1.0
.8
.6
.4
.2
0.0
Proportion DFS
N = 34
N = 35
+ Censored
20
40
60
80
100
120
140
160
180
Weeks

Figure 4.

HIGH-DOSE CHEMOTHERAPY PROGRAMS FOR BREAST CANCER IN EUROPE

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High-dose chemotherapy programs have been extensively used in breast cancer patients around the world in the last few years.[1,2] In Europe the first experiences date back to the late seventies, usually involving multiple refractory patients with negative results. Nowadays, despite the fact that no randomized trials have been published on the exact role of high-dose combinations with ABMT/PBSCT support, every day in Europe a patient receives this sort of treatment for her breast cancer. In fact, during the year 1993, 360 patients underwent at least an ABMT/PBSCT procedure, which represents a marked increase if compared to the 252 patients of 1992 (+40%).[3]

In spring 1994, a questionnaire was sent to the chief European investigators involved in ABMT trials in Europe, even though they were not members of the European Bone Marrow Transplantation Group (EBMTG). Information requested included the main guidelines of their trials, along with inclusion criteria, type of conditioning regimens, previous chemotherapy and patients' status at graft. All the investigators responded. To date, we are aware of 35 ongoing or planned trials for 1994. Seventeen trials accrue patients for adjuvant therapy, while 14 deal with metastatic cases and 4 with inflammatory or locally advanced disease. Currently, seven trials are running or are planned for high-risk breast cancer patients. This figure represents a marked increase if compared to the three randomized studies known to run in Europe in 1992. Table 1 gives an outline of the seven adjuvant randomized trials (Milan, Scandinavian, Dutch, French Society of BMT, Italian GITADO, Germany, ICCG).

Table 1. The Ongoing Adjuvant European Randomized Trials for High-Risk Breast Cancer

Group	Minimum Positive Nodes	Chemotherapy (High-dose arm)	Mobilization	Standard Arm
ICCG	≥ 6	ECx4→CTCb	EC+G-CSF	EC x 8
Dutch	≥ 4	FECx4→CTCb	FEC+G-CSF	FEC x 5
Milan	≥ 4	HDS	CTX+G_F	Ex3+CMFx9
Scandinavian	≥ 6	FECx3→CTCb	FEC+G-CSF	FEC x 9
GITADO (It)	≥ 4	FECx4→CARBO-VP-L-PAM	FEC+G-CSF+EPO	FEC x 6
German	≥ 10	ECx4→MTZ-CTX-TT	EC+G-CSF	ECx4+CMFx3
French	≥ 8	FECx4→CMA	FEC+G-CSF	FEC x 4

ICCG = International Collaborative Cancer Group
EC = Epidoxorubicin and Cyclophosphamide
CTCb = Cyclophosphamide, Thiotepa, Carboplatin (STAMP V)
HDS = (High dose sequential) = Cyclophosphamide + PBPC harvesting, Vincristine and HD Methotrexate, Epidoxorubicin in sequence and then Thiotepa and Melphalan
G_F = Various combinations of growth factors
GITADO = Italian Group for high-doses in oncology
CMA = Cyclophosphamide, Mitoxantrone, Melphalan

From the data shown in Table 1, the definition of high-risk poor prognosis breast cancer suitable for ABMT/PBSCT programs is not homogeneous among the various investigators. In some instances (Milan and the Netherlands) four positive axillary nodes are a sufficient adverse prognostic factor, while other investigators (Germany) require at least ten positive nodes as entry criterium. Due to the fact that many trials now have fewer positive nodes for eligibility, the number of patients potentially accruable for high-dose programs in the next few years in Europe will dramatically improve. Except for the Dutch adjuvant trial, no other program is considering the cost-benefit analysis of the procedure. In Table 1, the standard arm regimens are also outlined. All of them are epidoxorubicin based schedules with a minimum of four to a maximum of 9 courses.

Age. For the majority of ABMT protocols (adjuvant and advanced) the upper age limit is 55 years, with some exceptions such as Moscow (65 years). In some instances (Milan), there is a less defined age for inclusion and depends on the general condition of the patient (55-60 years). It is noteworthy that an early analysis of the EBMT Solid Tumor

Working Party[4] did not show any correlation between age and outcome, even with a cut off at 40 years in that report.

Agents. The most widely used antineoplastic agents are alkylators, always employed in combinations. In our survey, cyclophosphamide is the most often chosen drug (by 74% of the groups), followed by carboplatin (58%), thiotepa (53%), L-PAM and etoposide (42%), mitoxantrone (26%) and ifosfamide (11%). Table 2 shows the different doses employed in some of the major trials, for these agents.

Table 2. Doses Employed by Different European Groups in High-Dose Chemotherapy Programs for Breast Cancer*

Group	VP-16**	L-PAM	TT	CTX	CARBO	MTZ
Dutch			480	6,000	1,600	
Milan		140	600	7,000		
Ravena			500	200/kg		40
Valencia	21/kg	140		100/kg		
Jerusalem	600	120	180		800	
ICCG			500	6,000	800	
French		140		120/kg		45
Moscow	1,600			6,000	1,600	
Germany			600	6,000		40

* *Doses in mg/sqm unless otherwise specified.*

** *VP16 = Etoposide, L-PAM = Melphalan, TT = Thiotepa, CTX = Cyclophosphamide, CARBO = Carboplatin, MTZ = Mitoxantrone*

Support. All groups now incorporate PBSCT in the place of or in combination with ABMT. No single trial is restricted to ABMT support alone (with or without growth factors), while before 1989 no trial was PBSCT based.

The means of mobilizing peripheral precursors varied among the centres. For example, in the Dutch adjuvant trials, PBSC are harvested after FEC + G-CSF, while in Milan the high-dose sequential program starts with cyclophosphamide at the maximum tolerable dose (7gm/M^2) supported with growth factors combinations. A new option is that used at the Ravenna Medical Oncology Department where Epidoxorubicin at high-doses (150 mg/M^2) is used as mobilizing agent,[5] with G-CSF starting 24 hours later.

From this survey of European high-dose programs some considerations may emerge: 1) there is a dramatic increase in the number of patients being offered high-dose chemotherapy and hematologic rescue

for breast cancer in Europe; 2) this trend seems to proceed not in a "wild" fashion due to the fact that good quality studies (i.e. randomized) are increasing year after year; 3) high-dose programs continue to be proposed to non refractory metastatic patients, even if generally in phase II studies; this policy is in line with a statement by the Jury of the First International Consensus Conference on Stem Cells Transplantation in Lyon.[6]

In the next few years valuable information regarding the role of high dose chemotherapy programs for breast cancer is likely to be provided in two ways: a) from good randomized data, and possibly b) by way of meta-analyses, when the final results of the randomized phase III trials will be published.

ACKNOWLEDGEMENTS

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DOSE-INTENSIVE CHEMOTHERAPY WITH ETOPOSIDE-CYCLOPHOSPHAMIDE WITHOUT STEM CELL SUPPORT FOR ADVANCED BREAST CANCER: PRELIMINARY RESULTS

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INTRODUCTION

Dose-intensive therapy with stem cell support has been used effectively in women with metastatic breast cancer.[1-5] Since marrow and/or extensive bone involvement and reimbursement issues limit the use of stem cell support, we developed a dose-intensive chemotherapy regimen for women with advanced breast cancer. From our experience in treating patients with hematologic malignancies, we selected a regimen of etoposide (VP) and cyclophosphamide (CY) which did not require the use of stem cell support.[6] The dose-limiting toxicity of this therapy is mucositis, although myelosuppression is quite severe but reversible without stem cell or hematopoietic growth factor support.

METHODS

Since 1989, 113 women with metastatic breast cancer received VP 2400-4200 mg/M^2 continuous IV infusion followed by CY 200 mg/kg (50 mg/kg/d x 4d). The protocol dose of etoposide was 4200 mg/M^2, but was reduced to 2400 mg/M^2 in 2 patients and 3600 mg/M^2 in 10 patients because of low serum albumen (<2.5 g/dl). Hematopoietic growth factors (including granulocyte-macrophage colony stimulating factor, granulocyte colony stimulating factor, interleukin-3, and/or erythropoietin) were usually, but not always, given to help accelerate marrow recovery. Patient characteristics: The median (range) age of women was 44 (24-60) yr. Only 55 (49%) of the women had one metastatic site; 39 (35%) had two sites, and 19 (17%) had 3 or more sites. The most frequent sites of metastases were: bone (52%), bone marrow (23%), skin and lymph node (37%), lung and pleura (22%), liver (22%), and CNS (5%). At the time the

dose-intensive regimen was started, 47 patients were still responding to standard therapy; 53 patients had progressive disease; 13 patients were treated without additional therapy (untested relapse). Toxicity was graded using the marrow transplant criteria. In general, grade 0 represented no toxicity, grade 1 was mild toxicity, grade 2 was moderate toxicity, grade 3 was severe, often life-threatening, but reversible toxicity, and grade 4 was fatal, treatment-related toxicity. Recovery of marrow function was determined by the recovery of granulocytes to >500 /μl and platelets to >20,000/μl untransfused. Responses were measured initially at 2 months after therapy and every 2-3 months thereafter. A complete response (CR) was defined as the complete disappearance of all measurable disease. A partial response (PR) represented at least a 50% reduction in measurable disease. All responses less than 50% reduction, stable disease, or progression were all considered as no response (NR). Patients with bone involvement who had normalization of laboratory parameters, clinical resolution of pain, but had stable (no new lesions) but persistent bone scan abnormalities, were considered responsive, but were designated with an asterix qualifier, i.e., CR* or PR*. Patients, without disease progression, who died from toxicity before marrow recovery were not evaluated for tumor response.

RESULTS

Toxicity

The regimen was well-tolerated, 66% of the women had mild to moderate organ dysfunction; 31% of the women had severe, but reversible toxicity, with the most common severe problem involving stomatitis (10%). There was 6% treatment-related mortality due to infection or hemorrhage (3.5%), venoocclusive disease of the liver (1.8%), and a single case of cyclophosphamide-induced myocarditis (0.8%). Recovery of marrow function was unaffected by the use of hematopoietic growth factors or by the presence of breast cancer in the marrow. For the 16 women who did not receive hematopoietic growth factors, the median (range) duration of granulocytopenia (PMN <500/μl) was 19 (10-38) days; and of thrombocytopenia (platelets <20,000/μl) was 14 (6-26) days. Women with marrow involvement had similar hematopoietic recovery, median (range) duration of granulocytopenia 18 (11-45) days and thrombocytopenia 19 (5-85) days. When growth factors were employed, the recovery in 75 women, the duration of the granulocytopenia was 17 (7-43) days and thrombocytopenia was 16 (2-85) days.

Response

Of the 113 women started on protocol, 103 were evaluable for response. Two patients are alive, but within two months of treatment and are not evaluable for response; one patient withdrew from study before completing all of the treatment; seven patients died from treatment. Sixty-three (61%) responded, with 22% CR (7% CR*), and 39% PR (23% PR*). The extent of disease, responsiveness to standard therapy, and dose of etoposide affected the response rate (sum of complete and partial responses). Patients with disease limited to bone, skin, and/or lymph node had a higher response rate (94%, 31/33) compared to patients with visceral metastases (46%, 32/70), $p<0.0005$. Women who were still responsive to standard-dose therapy, "responding relapse" had a higher response rate (86%, 38/44) than those patients who had failed conventional therapy, "refractory relapse," (40%, 19/48), $p<0.0005$. There was only a trend for a higher response rate with patients who received the higher doses of etoposide: 4200 mg/M^2 had a higher response rate (63%, 59/93) than patients getting <4200 mg/M^2 (40%, 4/10), $p<0.02$. The median (range) duration of CR was 18+ (2 - 60+) months and of PR was 6 (2 - 50+) months. None of the seven patients who had no evidence of disease when the high-dose therapy started continue to be free of disease between 8 and 40 months, with a median of 24 months. Of the patients who responded, nearly 40% have had responses that last for more than one year.

DISCUSSION AND SUMMARY

The treatment of metastatic breast cancer with dose-intensive therapy with marrow rescue has been moderately successful. A potential problem with this approach is the reliance on stem cell support for hematopoietic recovery. Many women have extensive bone and/or marrow involvement which either will preclude stem cell harvest or compromise the procedure. Furthermore, the results with dose-intensive regimens are better if the women are still responsive to chemotherapy. The present study represents a suitable alternative to regimens that require stem cell support. The regimen is well-tolerated with an acceptable treatment-related mortality (6%), which is similar to dose-intensive therapy with stem cell support. The results are comparable to a number of studies treating a similar group of women, with a 63% response rate and 22% complete response rate. The women who were still responsive to chemotherapy did quite well with an 86% response rate. The duration of

responses is also quite similar to other reports, with CR now having a median duration of 18+ months. Of equal importance is the fact that 40% of patients refractory to standard or conventional therapy had a response to this regimen, possibly permitting a second dose-intensive therapy. There were 16 women who were able to go on to another regimen with stem cell support.

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TANDEM HIGH DOSE CHEMOTHERAPY (INTENSIFICATION) SUPPORTED BY HEMATOPOIETIC PROGENITOR CELLS IN METASTATIC BREAST CANCER: A PHASE II STUDY

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INTRODUCTION

Breast cancer is the leading cause of cancer related deaths in American women; in 1994, 46,000 women within the United States will die from breast cancer. Once disseminated, the treatment of breast cancer is palliative and survival is determined by the dominant site of metatstatic involvement and the hormonal dependency of the breast cancer as reflected by the presence or absence of binding hormone receptors (estrogen [ER] and progesterone [PR]). Patients with ER/PR negative breast cancer and visceral or bone metastases who fail to attain a complete response when treated with conventional chemotherapy, have a particularly grim prognosis.[1]

Many groups, including our own, have reported on the use of high dose chemotherapy with autologous bone marrow or peripheral blood hematopoietic progenitor support (ABMT). The published results of phase II trials employing high dose chemotherapy and ABMT in stage IV breast cancer have yielded complete response rates of 50% and disease-free survivals of 15-20% at 3-5 years.[2-9]

We have previously published our results in high dose chemotherapy with ABMT in women with stage IV breast cancer.[4,6] In an attempt to increase the number of long-term survivors, we embarked on a double or tandem high-dose chemotherapy (intensification) with ABMT phase II trial in 1992. In planning this trial, we chose to continue to use cyclophosphamide and thiotepa as the first intensification as we have previously reported.[4,6] We chose melphalan as the drug to be used during the second intensification course. The objectives of this ongoing phase II study are to evaluate the feasibility of tandem high-dose chemotherapy and ABMT in stage IV breast cancer; to determine the feasibility of giving melphalan on an ambulatory basis; to determine the cumulative toxicities;

and to determine if tandem high-dose chemotherapy can increase long-term survival. This interim analysis examines on the first 22 patients enrolled on study.

PATIENTS AND METHODS

Patient Eligibility

Women aged 18 to 65 years with hematological documented metastatic or recurrent breast cancer, Stage IV, were eligible if they had attained a complete or partial response to chemotherapy used to treat stage IV breast cancer. For patients with bone metastases, a reduction of tumor markers (>75%) in conjunction with a stable radiographic appearance of lytic lesions or sclerosis of lytic lesions were required. Additionally, patients were required to have a good performance status. ECOG $\leq$ 2, normal renal function (creatinine clearance >60ml/min), normal liver function (bilirubin $\leq$1.5 mg/dL), normal cardiac function (gated heart scan ejection fraction >50%), normal pulmonary function (DLCO >60%), were to be negative for hepatitis B antigen, hepatitis A, B, and C antibody, and HIV. Bone marrow involvement did not preclude participation in this study. All patients were required to give written informed consent. The study they were enrolled in was LGH#1096 and was conducted with the approval of the IRB of the Lutheran General Health Care System.

Treatment schema. The initial chemotherapy used was left to the discretion of the treating physician. Following attainment of a CR and PR, generally 4-8 courses of therapy, patients were enrolled on study.

Bone marrow harvests, leukapheresis, and cryopreservation. Bone marrow was harvested from the pelvis and an attempt was made to obtain 5-6 x 10^8 mononuclear cells/kg from the marrow cavity. The marrow was then divided into 2 parts and cryopreserved. For patients with marrow involvement, cyclophosphamide was administered at a dose of 4 gm/M^2 intravenously with mesna 8 gms/M^2 divided into 3 doses was used as a uroprotectant prior to and at 4 and 8 hours following cyclophosphamide. GM-CSF (Leukine®) was administered at a dose of 5 mcg/kg sc. beginning 48 hours after cyclophosphamide and continued until the leucopheresis was completed. The leucopheresis was performed on a CS-3000 as we have previously described[6] and the intent was to collect a minimum of 4 x 10^6 CD34 positive mononuclear cells (PBPC)/kg. Once collected, the PBPCs were divided into 2 equivalent parts and cryopreserved. The last 10 patients enrolled on study, regardless of

whether the marrow was involved or not, received cyclophosphamide and GM-CSF (described above) to mobilize PBPCs.

Chemotherapy. Patients entered the hospital after marrow or PBPCs were harvested and received cyclophosphamide 2500 mg/M^2IV on day -6, -4, -2 and thiotepa 225 mg/M^2 days -6, -4, -2. All patients were given mesna at 5000 mg/M^2 in 3 divided doses that preceded and followed cyclophosphamide at 4 and 8 hours on days -6, -4, -2. On day 0, the cryopreserved autologous bone marrow or PBPCs were reinfused and GM-CSF 5 mcg/kg was started. Analgesic (I.V. morphine) was used to treat pain from mucositis. In the event of a temperature spike, patients were pancultured and ciprofloxacin was discontinued, ceftazidime, vancomycin and tobramycin were administered. If the fever continued despite the aforementioned antibiotics, fluconazole was discontinued and amphotericin-B was started.

Within 6 months (180 days) cyclophosphamide and thiotepa patients received high dose melphalan 140 mg/$M^2$and if, after a complete restaging, they remained in CR and PR. Melphalan was preceded and followed by 1L of normal saline and given at a dose of 140 mg/M^2IVPB over 1 hour on an ambulatory basis on day -2. Patients returned 48 hours later to receive their autologous marrow (4 patients) or PBPCs (10 patients). They continued IV hydration at home and returned on day - (Friday). They were instructed to start ciprofloxacin 500 mg p.o. q. 12h, fluconazole 100 mg p.o. daily, along with Leukine® 5 mcg/kg sc. daily on day +1 (Saturday). Patients were seen 3 times weekly on an ambulatory basis until hematologic recovery took place.

Response criteria. For patients with measurable disease (chest wall, lymph nodes, liver and lung) lesions were measured bidimensionally prior to and following initial chemotherapy, after cyclophosphamide PBPC mobilization, after intensification Nos. 1 and 2. A complete response (CR) required complete disappearance of all measurable disease for at least 30 days. Partial response was defined as a greater than 50% reduction of the sum products of all measurable lesions for at least 30 days.

For patients with primarily evaluable disease (patients with dominant sites of metastases to bone), a PR* defined those patients whose measurable disease completely disappeared, and whose tumor markers (CEA, CA15-3) fell to normal but had persistent abnormalities on bone scans or only partial recalcification of lytic bone lesions.

Statistical analysis. The time to treatment failure (TTF) and the estimate of TTF at 18 months was calculated from the date of the first reinfusion of marrow or progenitor cells (day 0, cyclophosphamide and

thiotepa) by means of the Kaplan-Meier product limit method.[15] Survival was calculated and the estimate of 24 months was calculated by means of the Kaplan-Meier product limit method.

RESULTS

Patient Characteristics

From April 1, 1992 to June 30, 1994, 22 women were enrolled on study. The patient characteristics are shown in Table 1. The median age was 45 years and the median performance status was 0. Sixteen of these 22 patients received adjuvant chemotherapy (13 CMF, 3 FAC) and the median time to relapse was 18 months. After initial chemotherapy, 3 patients were in CR (14%) and 19/22 in PR (86%).

Table 1. Patient Characteristics (n=22)

Median age	45 years (range 32-56 years)
Median PS	0 (range 0-1)
Prior adjuvant chemotherapy	16/22
Median time to relapse after adjuvant therapy	18 months (16-66 months)
Dominant metastatic sites:	
Lung	7 patients
Nodal and chest wall	6 patients
Bone and bone marrow	5 patients
Liver	4 patients
Initial chemotherapy:	
FAC	7 patients
Ifosfamide & doxorubicin	5 patients
AFM	4 patients
Cisplatin+5FU±Mito-C	2 patients
Taxol	1 patient

The median follow-up time from initial chemotherapy is 18 months (7-30 months), and the median time to treatment failure from day 0 of the first intensification is 12 months (1-26 months).

High dose cyclophosphamide and thiotepa. All twenty-two patients received high dose cyclophosphamide and thiotepa (C+T). Two women died following cyclophosphamide and thiotepa on days +46 and +50 from staphylococcus aureus and pseudomonas auerginosa sepsis with multiorgan system failure. Both patients died despite achieving granulocyte engraftment. The toxicities from C+T are shown in Table 2. C+T were well tolerated and the most frequent toxicities were mucositis

and diarrhea. Infections were documented in 6 patients and included 4 patients with staphylococcal epidermitis bacteremia, and one patient each with staphylococcal aureus and pseudomonas auerginosa.

Table 2. Toxicities Following Cyclophosphamide and Thiotepa

Organ System	Toxicity Grade				
	0	1	2	3	4
Gastrointestinal					
nausea and vomiting	0	21	1	0	0
mucositis	4	10	8	0	0
diarrhea	4	14	4	0	0
hepatic	21	1	0	0	0
Genitourinary					
bladder	19	3	0	8	8
Skin	19	3	0	0	0
Central Nervous System	21	0	1**	0	0
Cardiac	21	1	0	0	0
Pulmonary	21	0	0	1**	0

***Patient died of staph sepsis and multiorgan system failure.*

The median for the ANC to exceed 500 cells/μl was 12 days (range 10-29 days) and for the platelets to exceed 20,000 cells /μl was 23 days (range 11-92 days). Patients required a median of 4 units of PRBCs (range 2-8) and 84 units of platelets (range 24-280). The median hospital stay was 23 days (range 21-58 days). No patient was readmitted following discharge.

High dose melphalan. Of the eighteen patients who were eligible for high dose melphalan, only 14 (78%) actually received it. Four patients were ineligible because of tumor progression at day +125 - 1 patient; inadequate cardiac function - 1 patient; and persistent thrombocytopenia (platelets <20,000/μl) - 2 patients. The toxicities following high dose melphalan are shown in Table 3. The predominant toxicity was mucositis and the subsequent decreased fluid intake lead to inadequate oral hydration that resulted in hospital stay for 5/14 patients (36%). The median length of stay was 6 days (range 4-28). These patients developed grade 2 CNS toxicity. One patient developed bilateral paraparesis secondary to an aseptic meningitis (Guillain-Barre) that gradually improved over 2-3 weeks. Infectious complications were documented in one of 14 patients. This sole patient was admitted with volume depletion and lethargy. She was the sole patient with hepatic toxicity (Table 2). A staphylococcal

epidermitis bacteremia was documented and the patients hepatic toxicity cleared in response to antimicrobial therapy.

Table 3. Toxicities Following High Dose Melphalan

Organ System	Toxicity Grade				
	0	1	2	3	4
Gastrointestinal					
nausea and vomiting	4	9	1	0	0
mucositis	1	7	6	0	0
diarrhea	7	7	0	0	0
hepatic	13	0	1	0	0
CNS	11	0	3	0	0
Renal	12	1	1	0	0

The median time to an ANC >500 cells/μl was 13 days (range 11-61 days) and platelets >20,000 cells/μl was 21 (range 11-120 days). The median number of units of PRBCs used was 3 (range 2-25) and the median number of platelets transfused were 32 (range 20-200+ units).

The overall objective response was 11/22 CR (50%) and 3/22 PR* (14%).

The median TTF has not been reached at 18 months and 56% of all are estimated to be failure free. The median survival has not been reached at 24 months and the estimated survival is 65%.

DISCUSSION

Multiple studies have examined the feasibility of administering dose intensive chemotherapy with ABMT in women with stage IV breast cancer who have attained a complete or partial response to conventional chemotherapy. The results of such trials have shown that 18-30% of patients remain in continuous complete response at 3-5 years.[2-9]

Few studies have addressed the feasibility of delivering two courses of high dose chemotherapy in women with stage IV breast cancer.[5,16] Dunphy et al[5] reported on two courses of cyclophosphamide, etoposide, and platinol (CEP) in 58 women with disseminated breast cancer; however, their reported 3-year survival is not better than those studies using only one high dose chemotherapy treatment. Ayash et al[16] has reported on the use of high dose melphalan (140-160 mg/M^2) supported by PBPCs and G-CSF followed by cyclophosphamide, thiotepa, and carboplatin supported with marrow, PBPC, and G-CSF. This study is

similar to our approach with the exception of the high dose chemotherapy sequencing and the omission of carboplatin from our study. While direct comparisons between these two phase II trials is difficult, some comparisons are warranted. In both studies the overall CR rate is virtually identical, 11/20 in the series by Ayash; 11/22 in this series, the number of patients defined as PR* are similar in both trials. Notable differences between these two studies include the fact that we were able to deliver the second intensification to only 14 of 22 patients (63%) as opposed to Ayash. While Ayash reported a 30% incidence of V.O.D. and/or hepatic toxicity, we observed none. Our current TTF and survival at 18 months and 24 months are better than the results we previously reported.[4,6]

Double dose high dose chemotherapy appears to be a promising approach in women with advanced stage breast cancer and may be applicable to patients with less advanced stage disease.

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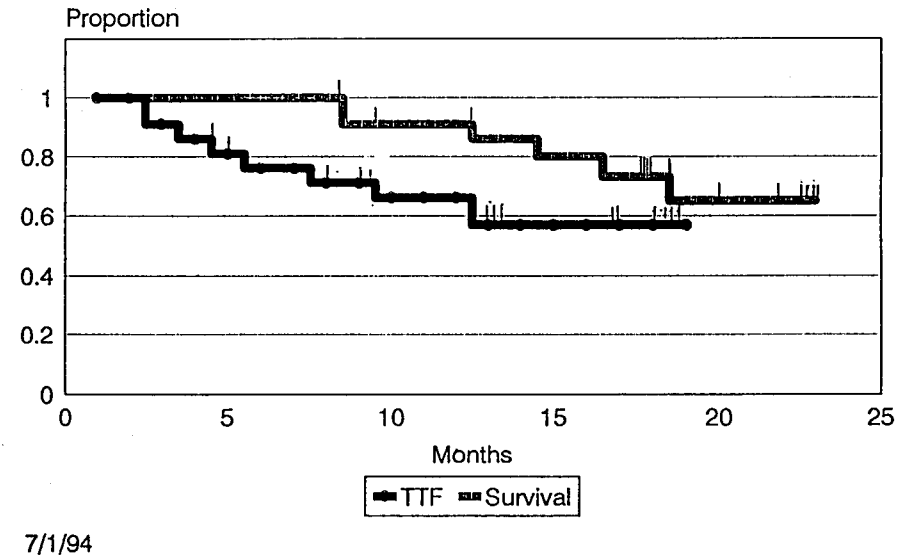

Figure 1. *Survival and time to treatment failure (TTF).*

A PHASE III STUDY OF HIGH DOSE THERAPY WITHOUT MARROW OR STEM CELLS SUPPORT FOR PATIENTS WITH HIGH RISK PRIMARY BREAST CARCINOMA

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ABSTRACT

Breast cancer patients with >9 node involvement or inflammatory carcinoma without distant metastasis were randomized to receive either standard dose 5FU, doxorubicin, and cyclophosphamide (FAC) every 3 weeks for 9 courses (control) or 6 courses of FAC followed by 2 courses of cyclophosphamide (5.25 gm/m^2), etoposide (1500 mg/m^2), and cisplatin (165 mg/m^2) (HDCVP). Patients with inflammatory carcinoma received radiotherapy and surgery between the third and fourth course of FAC. Twenty-four patients were entered into the protocol. Two patients were not analyzed: one had metastatic disease at diagnosis, three did not have inflammatory carcinoma or >9 nodes involvement. Twelve were randomized to the control arm and 10 to the HDCVP arm. The median age was 47 (range 36-64) and 45 (36-59) for the control and HDCVP arms respectively. There was no mortality from FAC or HDCVP. After a median follow-up of 16 months four patients on the control arm had relapsed, three with >9 nodes and one with inflammatory carcinoma at 366, 366, 576, and 518 days respectively. One patient with >9 nodes on the HDCVP arm had relapsed after 3 courses of FAC before HDCVP (140 days) and one with inflammatory carcinoma had relapsed after completion of therapy (518 days). Patient accrual continues for this study.

Patients with breast carcinoma and more than nine ipsilateral lymph node involvement have a five-year disease-free survival of less than 40%.[1,2] T3b tumors have a similar prognosis if local therapy alone was given.[2] Multinodal approaches for advanced stage III patients have prolonged overall survival in non-randomized trials as compared to historical controls.[3] Adjuvant doxorubicin-containing combinations improved the disease-free and overall survival. The five-year disease-free survival increased to 41% with doxorubicin-containing combinations.[4] Doxorubicin-containing combination chemotherapy is considered today

the best standard adjuvant chemotherapy for this group of patients. Obviously, a majority of patients still relapse within five years.

Dose intensified combination chemotherapy supported by progenitor cell support has produced improved response rates in patients with refractory malignancies.[5,6] For breast cancer patients with >9 node involvement, high dose chemotherapy with autologous marrow support as consolidation after standard dose chemotherapy appeared to decrease relapse compared to historical controls.[7] Non-myeloablative regimens with high-dose cyclophosphamide, etoposide, and cisplatin (HDCVP) had been developed at doses similar to those in transplantation regimens.[8,9] Marrow reinfusion had not been shown to alter the morbidity or mortality after HDCVP.[9] Repeated courses of HDCVP can provide a means to achieve a higher dose rate and intensity. This approach had achieved similar response rates and outcome as those achieved by a single course of myeloablative regimen with progenitor support in patients with metastatic breast cancer.[10,11] We are conducting a study to compare this intensification strategy with standard treatment in patients with high risk primary breast cancer.

PATIENTS AND METHODS

Patient Selection

Patients with histologically confirmed high risk primary breast carcinoma (more than nine ipsilateral lymph nodes involvement or inflammatory carcinoma) with no distant metastasis and no prior systemic therapy were accrued from two centers: the Ontario Cancer Foundation, Ottawa, Ontario, Canada and the Community Cancer Center, Coldwater, Michigan, USA. All patients were <65 years of age, performance status <2, adequate hematologic function (hemoglobin >100 gm/l, absolute neutrophil count $>1.5 \times 10^9$/l, platelet count $>100 \times 10^9$/l), serum creatinine level <140mmol/l, total bilirubin <26mmol/l, adequate pulmonary and cardiac functions.

Pretreatment Evaluation

All patients had complete history and physical examination, complete blood counts, biochemistry, urinalysis, EKG, chest radiograph, pulmonary function tests, multigated cardiac scan, and dental assessment. Physical examination, chest x-ray, bone scan, and abdominal ultrasound were performed to rule out metastasis.

Treatment Plan

As approved by each Institutional Review Board, written informed consent acknowledging the investigational nature of this study was obtained from all patients. Patients were randomly assigned to nine courses of 5FU, doxorubicin, cyclophosphamide (FAC) (control arm) or six courses of FAC followed by two courses of HDCVP (HDCVP arm). FAC consisted of 5-Fluorouracil 500 mg/m^2 on days 1 and 3, Doxorubicin 50 mg/m^2 over 3 days, and Cyclophosphamide 500 mg/m^2 on day 1. FAC was repeated every 21 says. HDCVP consisted of Cyclophosphamide 1,750 mg/m^2 daily for 3 days, Etoposide 250 mg/m^2 every 12 hours for 6 doses, and Cisplatin 55 mg/m^2 daily for 3 days. No MESNA or hematopoietic growth factors were administered. The second course of HDCVP was repeated 5-7 weeks after the first course. Patients with inflammatory breast cancer diagnosed by needle or incisional biopsy received local and regional radiation therapy after 3 courses of FAC. Megavoltage radiotherapy was started 3 weeks after FAC to a total of 46 Gy over 23 daily fractions, to the involved breast by opposed tangential fields and to the ipsilateral axillary and superclavicular areas by a direct anterior field with posterior axillary boost. No internal mammary irradiation was given. Modified radical mastectomy with axillary node dissection was performed three weeks after the completion or radiotherapy. Adjuvant FAC resumed three weeks after surgery. For patients with lumpectomy or mastectomy before randomization, radiotherapy was administered after completion of chemotherapy.

Supportive Care for HDCVP

All patients receiving HDCVP had central venous catheter and were admitted to hospital for chemotherapy. Patients were discharged two days after completion of chemotherapy provided that emesis was under control. Oral prophylactic regimen starting on Day 5 consisted of sucralfate, ciprofloxacin 500 mg twice a day, and fluconazole 100 mg daily. All blood products were irradiated with 25cGy. Patients received four units of random donor platelets for platelet counts $<10x10^9$/L, and two units of packed cells for hemoglobin <80 gm/L. Patients who developed fever (Temp >38.5°C) were admitted to hospital and treated with a combination of intravenous vancomycin and ceftazidine.

RESULTS

Twenty-four patients were entered in the study as of July, 1994. Two patients were not analyzed: one had metastatic disease at diagnosis, the other did not have inflammatory carcinoma or >9 nodes involvement. Twelve were randomized to the control arm and 10 to the HDCVP arm. The median age was 47 (range 36-64) and 45 (36-59) for the control and HDCVP arms respectively. In the control arm, there were seven with inflammatory carcinoma and five with >9 nodes involvement. In the HDCVP arm there were three with inflammatory carcinoma and seven with >9 nodes involvement.

In the control arm, one patient was hospitalized for mucositis, one for deep vein thrombosis, one for thrombosis of subclavian vein from central venous catheter, and one for non-neutropenic pulmonary infection during FAC chemotherapy. Two patients refused further FAC after 4 and 6 courses of FAC due to nausea and vomiting. There was no treatment-related mortality.

In the HDCVP arm, one patient was hospitalized for deep vein thrombosis and two for neutropenic fever during FAC chemotherapy. During HDCVP all patients require admission for neutropenic fever or platelet and blood transfusion. Two patients received only one course of HDCVP. One patient developed Grade 3 skin toxicity and one developed hearing loss after one course. Two patients refused HDCVP after 6 courses of FAC and both received 3 more courses of FAC. None developed hemorrhagic cystitis. There was no treatment-related mortality and none required oxygen supplement or intensive care support.

The outcome of patients are shown in Table 1. The number of patients is too small and the follow-up too short for analysis at this time.

Table 1. Patient characteristics and outcome

Diagnosis	Age	Relapse	Sites	DFS (days)	Survival	Status
HDCVP						
Inflammatory	36.2	Yes	liver,bone	518	567	Dead(PD)
Inflammatory	52.9	No		465+	465+	Alive
Inflammatory	55.3	No		147+	147+	Alive
14 nodes	40.0	Yes	breast,nodes	140	315	Dead(PD)
22 nodes	59.1	No		830+	830+	Alive
10 nodes	42.9	No		656+	656+	Alive

Table 1 (cont'd)

Diagnosis	Age	Relapse	Sites	DFS (days)	Survival	Status
11 nodes	48.8	No		613+	613+	Alive
10 nodes	56.9	No		333+	333+	Alive
11 nodes	42.3	No		314+	314+	Alive
26 nodes	37.6	No		266+	266+	Alive
CONTROL						
Inflammatory	51.3	Yes	bone,brain	518	628+	Alive
Inflammatory	47.4	No		889+	889+	Alive
Inflammatory	55.6	No		790+	790+	Alive
Inflammatory	40.5	No		789+	789+	Alive
Inflammatory	46.0	No		774+	774+	Alive
Inflammatory	44.2	No		291+	291+	Alive
Inflammatory	63.9	No		82+	82+	Alive
11 nodes	61.2	Yes	bone,brain	366	416	Dead(PD)
17 nodes	33.9	Yes	bone,nodes	366	421	Dead(PD)
14 nodes	34.1	Yes	bone,liver	576	668	Dead(PD)
15 nodes	59.8	No		516+	516+	Alive
14 nodes	42.9	No		432+	432+	Alive

DISCUSSION

The dose-response relationship was first demonstrated by Skipper and Schabel in preclinical models.[12,13] The importance of this relationship was confirmed in several prospective clinical studies.[14,15] The doses of some chemotherapeutic agents can be escalated several-fold above the standard doses and when used with hematopoietic progenitor cell support can produce improved response rates in patients with refractory metastatic breast cancer.[5,6]

HDCVP can be administered for two to three courses to achieve greater dose rate intensity and total dose intensity without hematopoietic progenitor cell support.[8,9] The efficacy of this regimen has been updated recently.[10] The long-term outcome of patients treated appeared to be similar to those treated with high-dose chemotherapy and autologous hematopoietic progenitor cell support. The morbidity and mortality of HDCVP is low compared to the three myeloablative regimens. In our pilot study, patients were followed in the out-patient setting after chemotherapy until signs of fever. No mortality developed in these patients and none developed septic shock.[16]

Bone marrow or stem cells from patients with malignancies had been shown to contain tumor cells even when they were histologically normal.[17,18] In patients with acute leukemia or non-Hodgkin's lymphoma, these occult tumor cells may predict for poor long-term survival after autologous bone marrow transplantation.[19,20] With gene-marking technique, it was shown that reinfused occult tumor cells contribute to the relapse of leukemia and neuroblastoma after autologous bone marrow transplantation.[21,22] Although the significance of reinfusing occult tumor cells after high dose chemotherapy in patients with breast cancer has not been studied, it is likely that they may also contribute to the recurrence of disease. Advantages of hematopoietic progenitor cell support may, therefore, be compromised by the reinfusion of occult tumor cells. The doubling time for human solid tumors is in the range of 4-14 weeks.[23] A single tumor cell can grow to a clinically detectable tumor ($1x10^9$ cells) in 20-90 months with these growth rates. Therefore longer follow-up will be needed to detect the relevance of reinfusion of occult tumor cells especially for patients with high-risk primary breast cancer treated with hematopoietic progenitor cell support.

We choose HDCVP as our intensification regimen to avoid reinfusion of occult tumor cells which may be present in the marrow of these high-risk primary breast cancer patients. The regimen is well tolerated with no mortality in this group of patients. The number of relapses in the control arm is similar to those treated with adjuvant standard dose chemotherapy. The small number of relapses after HDCVP is encouraging. The number of patients entered is too small for analysis at this time. Patient accrual continues for this randomized study.

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SESSION IV: NEW AVENUES

We dedicate the New Avenues Session of these Proceedings to

Dr. Joseph Sinkovics

It is a great honor to include Dr. Sinkovics' manuscript in these Proceedings (see page 305) as he was the first scientist to observe the graft-versus-leukemia effect in an experimental model.

HIGH DOSE CYCLOPHOSPHAMIDE, TOTAL BODY IRRADIATION AND AUTOLOGOUS BONE MARROW TRANSPLANTATION (BMT) FOR CHRONIC LYMPHOCYTIC LEUKEMIA

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is incurable with available treatment. Although fludarabine has been established as an effective agent especially in the previously untreated population with CLL, all patients ultimately relapse.[1,2] CLL is generally sensitive to alkylating agents and radiation. In this study, we analyzed the effect of high dose cyclophosphamide (CY) and total body irradiation (TBI) in patients with advanced CLL whose disease progressed after treatment with fludarabine-based therapy.

PATIENTS AND METHODS

Patients with CLL were eligible to undergo autologous marrow harvest at any time, provided that malignant cells in the bone marrow biopsy constituted less than 15% of the total cells present. Patients whose disease progressed after chemotherapy with fludarabine were eligible for BMT. They were required to have a good performance status with no severe concomitant medical or psychiatric illnesses.

The preparative regimen consisted of cyclophosphamide (60 mg/kg/d for 2 days) and fractionated TBI (10.2 Gy in six fractions over 3 days or 12.0 Gy in four fractions). Patients were treated in the laminar flow protected environment until the recovery of a granulocyte count greater than 0.5 x 10^9/L. The bone marrow cells of eight patients underwent depletion of B lymphocytes using an anti-CD19 monoclonal antibody and immunomagnetic separation (Dyna-beads M-450; Dynal, Lake Success, NY.[3]

RESULTS

Thirteen patients with B-lineage advanced CLL received autologous BMT. Their median age was 57 years (range, 37 to 66). The

median number of prior chemotherapeutic regimen was 3 (range, 2 to 6) and the median time from diagnosis to BMT was 48 months (range, 15 to 198). The disease status of patients at BMT is summarized in Table 1.

Table 1. Patient Status at BMT

Primary resistant to frontline therapy with Fludarabine	1
Resistant relapse	3
Untested relapse	5
Sensitive advanced relapse	3
Sensitive first relapse	1

In eight patients, the bone marrow was depleted of B-lineage cells to reduce residual disease, resulting in a 0.9 to 1.9 log reduction of leukemic cells. The majority of patients had detectable leukemia cells by flow cytometry post purging.

All patients achieved engraftment. The median interval from BMT to recovery of a granulocyte count greater than 0.5 x 10^9/L was 20 days (range, 10 to 28). The median time for a recovery of a platelet count greater than 25,000/μL was 31 days (range, 14 to 53).

One patient died of cytomegalovirus pneumonia 3 months post BMT. There was one incidence of transient pulmonary hemorrhage in another patient. The remaining patients recovered with minimal toxicity.

CLINICAL OUTCOME

All patients achieved major responses. Nine had complete remissions (CR), 3 had nodular CR (i.e., CR by all criteria except for persistence of lymphoid nodules in the bone marrow). Two patients died in CR at 2 and 3 months post BMT. All patients who had primary refractory disease to fludarabine, or had resistant or untreated relapse at BMT, had recurrent CLL (five patients) or transformed into Richter's (three patients). The recurrence usually occurred within one year of BMT.

Of the patients who received BMT in a stage of sensitive relapse, two out of 3 remain in remission at 15 and 17 months post BMT.

DISCUSSION

CLL is an indolent disease that affects predominantly older individuals and characterized by the involvement of bone marrow as part of its natural history.[4] Recent advances in chemotherapy with fludarabine-based regimen have produced CRs allowing to collect autologous marrow when it is minimally involved by the disease. Despite this response, conventional chemotherapy does not offer a cure for CLL and there is no long-term effective therapy for patients who relapse after a prior fludarabine response.[2] Therefore, high dose chemotherapy and autologous transplantation is a logical approach to improve the outcome.

Since the efficacy and safety of autologous transplant procedure in patients with CLL are not established yet, in this earlier experience, patients were eligible for transplantation only if they had CLL in relapse or refractory to conventional therapy. In this setting, 13 patients with up to 66 years of age were transplanted using CY/TBI as preparative regimen. Treatment-related mortality occurred in one patient. The remaining patients had acceptable toxicity.

The majority of patients achieved complete remission immediately posttransplant. However, the long-term outcome varied (Table 2). The response to BMT can be continuously improving over several months posttransplant. In particular, one patient who had detectable clonal cells by flow cytometry and molecular southern blot analysis at 1 and 2 months post BMT, had complete disappearance of his disease at 5 months.

Table 2. Long-Term Clinical Outcome

Status at BMT	Early Death	Relapse	Remission
Resistant or untreated relapse	1	8	0
Sensitive relapse	1	1	2

Now that the feasibility of this procedure is established, future studies need to focus on transplantation earlier in the course of this disease in patients who have CLL still sensitive to conventional chemotherapy, and also to focus on more effective strategies to eliminate malignant cells from the autologous graft.

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A MARROW HARVEST PROCEDURE UNDER LOCAL ANESTHESIA

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INTRODUCTION

Harvest of marrow occurred thus far under general anesthesia. Although the mortality of the procedure is low (less than 1 in 1,000, unpublished M.D. Anderson data), patients are being excluded from the procedure because of potential low tolerance of general anesthesia. Another disadvantage of general anesthesia is cost; general anesthesia requires operating room facilities which are cost-intensive. For these reasons, we developed a harvest protocol which does not require general anesthesia. The method is reported in this paper. Since the goal of marrow harvest is collection of sufficient numbers of stem cells for transplantation, we report in this manuscript the yield of hematopoietic progenitors as well as hematopoietic recovery *in vivo* after infusion of these cell suspensions. The colony-forming assays have been used in the past two decades as number of hematopoietic progenitors in marrow cell suspensions.[1] More recently, it was found that the CFU population belonged to a small subpopulation of bone marrow cells which can be identified by the reactivity with the CD34 monoclonal antibody using flow cytometry.[2,3,4] Recent data suggest that the CD34 progenitors correlate with hematopoietic reconstitution capability *in vivo*. This is the reason that we use this as a hallmark for progenitors.

METHODS

Marrow Harvest Procedure

The site of harvest is either the right or left posterior iliac crest. Per harvest, one or two skin punctures are used and from six to nine bone sites. Approximately 500 ml bone marrow is harvested which is 60ml per bone site. For local anesthesia 1% as well as 2% xylocaine is used, during the procedure IV lorazepam (Ativan) and hydromorphone hydrochloride (Dilaudid) are administered. In order to maintain an adequate blood pressure, 1500cc Ringer's lactate with 12.5 gm albumin is administered

before the harvest and another 1500cc during the procedure. The entire procedure lasts 1-1½ hours for the patient. The actual harvest time is 30-45 minutes (Table 1).

Table 1. Outpatient Marrow Harvest Procedure

1.	Location	:	Posterior iliac crest/sternum	
2.	Volume	:	500 ml	
			1-2 skin sites	
			6-9 bone sites	
			60 ml per bone site	
3.	Local anesthesia:		1% xylocaine:	soft tissue
			2% xylocaine:	periost/bone
4.	IV medications	:	RL + alb.:	2-3000 ml
			Ativan:	1 mg
			Dilaudid:	1 mg

CD34 Assay

Whole marrow or processed cells (1 x 10^6 total WBC) are incubated with 20 µl of an IgG1-PE/FITC isotype or, anti-CD34-PE (HPCA2) and anti-HLA-DR-FITC antibodies (Becton Dickinson, Mountainview, Ca.) at room temperature in the dark for 20 minutes. Red cells are lysed by adding 1 ml of Facslyse to each tube and vortexing briefly. Labeled cells are washed 2x with phosphate buffered saline (PBS) and resuspended in 1 ml of PBS for flow cytometric analysis. Cells are analyzed on an Epics Profile (Coulter Corp., Hialeah, Fl.) flow cytometer. Markers and quad stats are set for each specimen based on their isotypic control. Background staining is kept to <0.1%. At least 50 x 10^3 cells in each specimen are analyzed using a live gate. Results are reported as percentages of all cells stored for the patient (%CD34+, %HLA-DR+, %CD34+ HLA-DR-).

CFU-GM Assay

Triplicate plates are set up containing 1% methylcellulose, Iscove's medium plus 12.5% fetal calf serum and 12.5% horse serum, 10^{-5} M mercaptoethanol, 10 U/ml penicillin, 5 µg/ml streptomycin, 5 mg/ml amphotericin, 10% lymphocyte conditioned media, and 10^5 mononuclear cells/plate. Plates are incubated at 37°C in a humidified, 5% CO_2 atmosphere for 12-14 days, after which they are scored for colony (>50 cells) formation.

Preparation and Storage of Marrow Cell Suspensions

All procedures are carried out in a laminar flow hood. The transfer pack of marrow is gently mixed, equally divided into 100 ml centrifuge tubes and spun down at 1000 x g for 15 minutes. Serum samples (20 cc) are cryoprecipitated and buffy layers from each tube are pooled. Counts are performed on the whole marrow and pooled buffy using a Sysmex F800 (Baxter). Cells are frozen in 4.5 ml vials at a concentration of 200 x 10^6 cells/ml using controlled rate freezing (Cryomed). The final freezing solution contains 10% DMSO and 10% autologous serum. Vials are stored in liquid nitrogen vapor phase.[5]

MTVB Program

Patients treated with a combination of Mitoxantrone 15 mg/M^2, Thiotepa 150 mg/M^2, VP-16 600 mg/M^2 and BCNU 300 mg/M^2 are myelosuppressed, granulocytes <100/mm^3, platelets <20,000/mm^3 for at least 20 days and 30 days respectively. This regimen is administered over 4 days and has no other toxicity than marrow toxicity and is used for determining the restoration capability of infused marrow cells. Marrow cells are infused on day 5 after onset of chemotherapy.

RESULTS

At the time of presentation, 71 procedures were analyzed in Table 2. A median volume of 470 ml per procedure is collected. The number of cells per harvest is 4 x 10^9. The number of CD34 collected is 34.5 x 10^6 and that of CFU-GM is 200 x 10^6 (Table 2). Collection of 60 ml per bone site is different from the standard 15 ml. The reason for such large collections is to minimize the number of punctures per procedure.

Table 2. Outpatient Marrow Harvest Results*

Procedures	:	71	
Volume (ml)	:	470	(400-500)
Cells x 10^9	:	4	(1.9-43)
%CD 34+	:	0.8	(0.2-3.9)
CD34 x 10^6	:	34.5	(4.2-345)
CFU-GM x 10^4	:	200	(70-450)

*Results are presented as median number with range.

In Table 3, the yield of CD34 per ml harvest of 15 ml punctures and 60 ml punctures has been documented. As can be noted from Table 3,

the yield of CD34 cells per ml harvest in case of 60 ml harvests is 66% of the yield per ml when 15 ml is collected per bone site. The bone marrow cells collected by this harvest procedure were used for transplantation after the MTVB program.

Table 3. Effectiveness of the Outpatient Harvest Procedure Per ml Harvest

Volume	No. of Cells*	CD34+		Efficiency
		%	No.*	
15 ml	19.9 (7.9-66)	0.9 (0.2-4.2)	0.24 (0.03-0.88)	100%
60 ml	14.3 (6-43.5)	0.7 (0.4-4.1)	0.16 (0.02-0.41)	66.7%

*$x10^6$

The hematopoietic recovery data are listed in Table 4. These recovery data are not different from previously published results.[6]

Table 4. Hematopoietic Recovery after MTVB Regimen (8 patients)

No. of CD34+/kg	3.9×10^6	(1.1-12.6)
Days ANC <100	6	(4-8)
Recovery to ANC:		
≥100	11	(8-17 days)
≥500	13	(12-21 days)
≥1000	14	(12-19 days)
Platelets:		
≥50,000	33	(17-110 days)

MTVB = Mitoxantrone, Thiotepa, VP-16, BCNU

The minimum number of CD34 cells/kg body weight needed for transplantation has been estimated to be 1-2 x 10^6, which is a total of 70-140 x 10^6 in the case of a 70 kg person. This yield can be obtained with 3 harvests. Our goal is to limit the harvest procedures to 1-2 per patient and therefore the number of CD34 cells per harvest has to be increased. Twenty patients had steady state harvests followed by the administration of G-CSF at a dose of 5 μg/kg q 12 sc. x 3 or 4 (10 patients per dose level) prior to their second harvest. After either 3 or 4 doses of G-CSF, the yield increased from 29 x 10^6 to 85 x 10^6, nearly a factor 3.

Table 5. CD34+ Population in the Marrow Harvest Before and After G-CSF Stimulation *In Vivo*

	Volume Harvest (ml)	No. of Cells ($x10^6$)	CD34+ %	CD34+ No. x 10^6
Steady state	433	3737	0.77	29
Stimulated (3 shots)	480	13,518	0.62	85*
Stimulated (4 shots)	484	13,605	0.73	85*

* $p < 0.01$

In Table 6, the yield of the CD34DR- subpopulation has been listed since this cell population might be responsible for long-term engraftment. After 3 doses the CD34DR- population increased by a factor 4 which is in contrast with the results after 4 doses of G-CSF.

Table 6. CD34+ Population in the Marrow Harvest Before and After G-CSF Stimulation *In Vivo*

	Volume Harvest (ml)	CD34+ DR- $x10^6$	CD34+ Number $x10^6$
Steady State	433	9	29
Stimulated (3 shots)	480	35 p=0.014	85
Stimulated (4 shots)	484	16 p=0.19	85

The issue of patient friendliness of the procedure was addressed by the answering of a questionnaire by patients at the time of the harvest. The level of pain was evaluated during the various steps of the procedure (Figure 1). Pain levels ranged from 0-10, i.e. from no pain to intolerable pain. The injection of 1% xylocaine with a 25 gauge needle was experienced at a level 3 pain. The pain level at time of puncturing the

bone with the marrow harvest needle increased to level 5, which is similar during the initial 5-10cc marrow withdrawal per site.

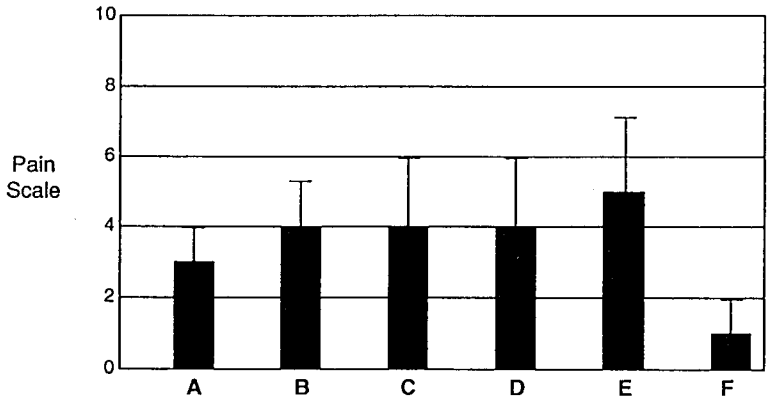

Figure 1. *Pain levels at different points of the procedure have been documented. Results were obtained from 10 patients.*

A = Anesthesia to soft tissue
B = Anesthesia to bone
C = Marrow needle into marrow cavity
D = Stylette out
E = First 10ml harvest
F = Second 15cc harvest

DISCUSSION

The harvest procedure as it stands now is short, effective and well tolerated. By avoiding general anesthesia, the procedure is cost-effective and is estimated to be 25% of the cost of the procedure under general anesthesia. The use of 60 ml per puncture site limits the number of bone sites to 8, which is important for the patient friendliness of the procedure. Also, when only 8 puncture sites are necessary, it is possible to use one skin puncture and have 500 cc marrow collected from multiple bone sites at one location. This limits the use of 1% xylocaine to 15-20 cc and of 2% xylocaine to 10-15 ml. By adding the IV medications, Ativan and Dilaudid, the procedure is well tolerated; very little discomfort after the procedure is experienced. Patients go back to work shortly after the procedure.

The effectiveness of 60 ml punctures in terms of CD34 yield per ml puncture is surprisingly high, 66%, when compared with the results of 15 ml bone punctures. Although the CD34 yield per ml is only 2/3 of the

number of CD34 per ml in 15 ml harvest, the total yield of CD34 in 60 ml is close to three times higher. Still the number of CD34 per 500 ml harvest is not sufficient for one transplant, which led us to look into methods to increase the CD34 population in the bone marrow. Stimulation with G-CSF starting 24-36 hours before the harvest procedure resulted in a 3-fold increase which reduces the total number of harvests per patient to two if infusion of 1-2 x 10^6 CD34/kg body weight is considered to be the minimal number of cells for engraftment. An increase in volume from 500 ml to 750 ml per patient is considered which may further reduce the number of harvests per patient. It is remarkable that the short (3 shots) G-CSF administration schedule results in an increased yield of immature CD34, CD34DR- whereas additional G-CSF hardly increases the yield of this population over baseline. It may well be that the spill-over or mobilization into the bloodstream is taking place at that moment since an increase in the CD34 population in the peripheral blood has been noticed at that time or shortly thereafter (data not presented).

The hematopoietic restoration capability of the CD34 population harvested by the method described does not differ from those collected under general anesthesia.[6] Studies are in progress to determine the hematopoietic restoration capability of marrow cells collected after G-CSF stimulation.

We conclude that this harvest method may be a valuable alternative for the conventional procedure especially in situations where there are contraindications for general anesthesia and when costs are a major determining factor.

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GENE TRANSFER INTO HEMATOPOIETIC STEM AND PROGENITOR CELLS

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INTRODUCTION

Over the past four years, there has been an explosion of interest in using genetic approaches to treat human disease.[1] The hematopoietic stem cell has been a primary target because of the potential to cure congenital hematologic, immune and metabolic disorders, as well as to offer new approaches to acquired diseases such as cancer and the acquired immunodeficiency syndrome.[2]

A number of requirements for successful gene transfer to hematopoietic progenitor and stem cells determine the clinical applicability of various vector systems to human disease. Chromosomal integration of the transferred gene and passage to all progeny cells is necessary in hematopoietic targets, which must continuously replenish daughter effector cells. The gene transfer procedure must not be toxic nor disrupt the self-renewal properties of the target stem cell pool. Gene transfer efficiency to true repopulating stem cells must be high enough to impact on the disease phenotype; the required efficiency varies from as low as a few percent corrected cells in some congenital enzyme deficiency disorders to much higher levels in other situations such as sickle cell anemia. Gene products must be stably expressed from the vector construct at adequate levels in the correct cell lineage, requiring strong constitutive or appropriately regulated transcriptional control elements. The vector must be simple and safe enough for clinical use; care must be taken absolutely to avoid generation of replication-competent particles in any viral-based system. Currently, the only clinically applicable system allowing permanent and stable integration of a new gene into the host chromosome involves the use of replication-incompetent retroviruses as gene transfer vehicles. Exposure to retroviral supernatants has not been found to be deleterious to hematopoietic cells, and there has been no

evidence of toxicity in animal models when helper-free viral stocks are use.[3,4]

Animal Models

Efficient retroviral gene transfer into murine hematopoietic stem cells has become routine over the past seven to eight years. The best results have been obtained by treating donor mice 2-4 days prior to harvest with 5-fluorouracil to induce cycling to primitive cells via selective depletion of more mature progenitor cells.[5] After harvest, marrow cells are exposed to viral particles *in vitro* for two to six days in the presence of hematopoietic growth factors, most effectively a combination of several early-acting factors such as IL3, IL6, SCF, IL1, LIF and GM-CSF. Coculture of target cells with the retroviral producer line and the use of producer lines releasing at least 10^5 viral particles/ml have also increased the efficiency of gene transfer.[5] As many as 90-100% of recipient mice are positive in multiple lineages for the transferred gene when they receive marrow transduced under these conditions, and up to 10-90% of their peripheral blood or bone marrow cells carry the provirus long-term, depending on the vector use.[6]

Extension of efficient stem cell gene transfer to larger animal models has been more problematic. Several groups have reported at best 0.1-4% of peripheral blood or bone marrow cells positive for a retrovirally-transferred gene long-term in primate or canine bone marrow transplantation models. Multilineage, long-term presence of the transferred gene has been demonstrated.[7-9]

Human Pre-Clinical Data

Committed human progenitor cells such as CFU-GM or BFU-E can be transduced by retroviral vectors at very high efficiencies (often greater than 50%) in the presence of various combinations of hematopoietic growth factors.[10-13] Many different combinations have been tested; we have found that the highest gene transfer efficiencies to progenitor cells as assayed by PCR of individual CFU-C are achieved using stem cell factor (SCF) in combination with IL3 and IL6. We and other investigators have performed transductions in the presence of primary bone marrow stroma instead of the viral producer line, and have found that even without the addition of soluble hematopoietic growth factors, transduction efficiency of progenitors is very high, 40-50%.[10,14]

We have also explored the use of primitive cells mobilized into the peripheral blood as alternative targets to bone marrow cells for gene

transfer.[11] The use of peripheral blood as a source of target cells is attractive because of the ease of collection and the opportunity for multiple cycles of collection, transduction and reinfusion. Both myeloid and erythroid peripheral blood and bone marrow progenitors from normal volunteers and patients with multiple myeloma could be transduced at high efficiencies with the LNL6 and G1Na.40 retroviral vectors, which contain the neomycin-resistance gene, and the human adenosine deaminase vector G1NSvAd (Genetic Therapy Incorporated).[11] The mobilized peripheral blood CFU-C transduced at an efficiency as high as 70-80%. Cord blood may also turn out to be a desirable target for gene transfer: cord blood has a higher frequency of very immature CD34+/CD38- cells, and one group has shown that colonies grown from single transduced cells from this primitive population contain the retroviral vector at very high frequencies.[15]

More primitive human long-term culture initiating cells (LTCIC) can be transduced at equivalent efficiencies to committed progenitors under similar transduction conditions.[10,13,14,16] Many preclinical studies on human cells employ assessment of LTCIC transduction efficiency as a marker of true "stem cell" transduction, but there is no evidence as yet that this assay is any more predictive than standard progenitor assays of *in vivo* results in primates and humans.

Clinical Studies

Based on the preclinical data summarized above, our group has initiated a genetic marking trial in patients undergoing autologous transplantation for multiple myeloma or breast cancer. The objectives of this protocol include: 1) Determining the feasibility of retrovirally-mediated gene transfer to hematopoietic long-term reconstituting stem cells using a CD34-enriched target cell population, in preparation for gene therapy protocols directed at these and other diseases. 2) Studying the relative gene transfer efficiency into CD34-enriched populations from bone marrow versus chemotherapy and growth factor mobilized peripheral blood cells. 3) If marking is successful, studying the contribution of bone marrow versus peripheral blood cells to short- and long-term reconstitution after autologous transplantation, and the kinetics of autologous reconstitution from these sources. 4) Investigating the source of relapse in these patients, if marked malignant cells are detected at relapse after transplantation.

Patients with chemotherapy-responsive disease in a good partial remission after standard therapy receive cyclophosphamide 4 gm/M^2 and

G-CSF 10ug/kg/day. Peripheral blood cells are collected via apheresis after the white blood count rebounds to >2,000. Three daily collections are performed. Two collections are cryopreserved without further manipulation. One collection is CD34-enriched via column immunoabsorption (CellPro) and transduced for 3 days in the presence of a Neomycin-resistance gene marking vector (either LNL6 or G1Na.40) and hematopoietic growth factors. IL3, IL6, and SCF are used for the breast cancer patients, and IL3 and SCF only are used for the multiple myeloma patients. Viral supernatant and factors are replaced daily. At the end of the transduction period the cells are cryopreserved. The patients then rest for several weeks before receiving 5FU 15 mg/kg/day X 3. Besides possibly improving gene transfer efficiency, this 5FU regimen has been reported to improve platelet recovery after transplantation when it is given as a "priming" agent prior to marrow harvest.[17] Ten days after beginning 5FU, the patients undergo marrow harvest. Two-thirds of the marrow mononuclear cells are cryopreserved without further manipulation, and one-third is CD34-enriched and then transduced with either LNL6 or G1Na.40 (whichever vector was not used on the peripheral blood from that particular patient) under the same conditions as described above. The patient is then ablated and both the tranduced and untransduced bone marrow and peripheral blood cells are thawed and reinfused. Patients have blood and marrow samples collected for analysis periodically after engraftment, and at the time of relapse and any future therapy.

To date, 11 patients (5 multiple myeloma and 4 breast cancer) have been reinfused with gene-marked cells transduced for 72 hours in a supernatant infection in the presence of hematopoietic growth factors. There have been no toxicities related to the CD34-selection and the retroviral transduction procedures. Patients have engrafted on schedule. Nine of eleven patients analyzed to date have had evidence of marked cells in their marrow or peripheral blood, however, levels are only 1-2% at the time of engraftment, and in some cases drop to undetectable levels over the next six months. Four of eight patients analyzed at 6 months or greater still have evidence of vector in peripheral blood or marrow cells, but at levels $\leq 0.1\%$. Two myeloma patients still have stable signals at that level present at 18 months posttransplant. One breast cancer patient that had become negative for the marker gene 3 months after transplantation has again shown a signal at greater than one year after transplantation.

In all patients with positive marker gene signals, both the transduced peripheral blood and marrow contributed to the signal, as determined by a PCR assay that can distinguish the two proviral genomes

(LNL6 versus G1Na.40). The transduced peripheral blood contributed to marking more frequently than the transduced bone marrow. Early after engraftment, granulocyte marking is stronger than mononuclear cell or sorted B or T cell marking, but at 6-9 months, the granulocyte signal diminished in some patients, with relative preservation of the mononuclear signals. The two patients still positive at 18 months have both granulocyte and mononuclear cell marking, with sorted T cells being positive in one patient analyzed after FACS sorting. The persistence of a granulocyte signal derived from the peripheral blood graft over 18 months after transplantation indicates that mobilized peripheral blood is a possible source of true long-term repopulating cells.

None of the myeloma patients have shown disease progression. Three breast cancer patients have relapsed, and metastatic tissue from the liver and skin did not contain detectable marker sequences in these patients.

Retroviral gene transfer to human hematopoietic cells has already produced clinically useful information about the source of relapse after autologous bone marrow transplantation.[18,19] In the autologous transplantation setting, neither normal nor malignant cells derived from the graft can be distinguished posttransplantation from endogenous cells surviving conditioning without the use of marking strategies such as these. The occurrence of marked leukemic cells at relapse in a similar protocol carried out by Dr. Malcolm Brenner at St. Jude Children's Research Hospital has indicated the need for purging of autologous marrow used for transplantation in acute myelogenous leukemia or neuroblastoma.[18,19] Similar results have been reported in CML.[20]

But for therapeutic protocols involving gene transfer to hematopoietic stem cells to be successful, gene transfer efficiency to hematopoietic stem cells must be improved. Dr. Malcolm Brenner has reported much higher efficiency of transfer (5-10% or greater) to normal multilineage progenitor cells in children undergoing autologous transplantation for leukemia or neuroblastoma.[21] His transduction protocol is very simple, involving only one exposure to virus without growth factor supplementation. These pediatric patients may have primitive cell populations more susceptible to retroviral gene transfer due to increased cycling, either due to age or to recent treatment with very high-dose chemotherapy prior to marrow harvest. We plan to compare our transduction protocol to Dr. Brenner's in the myeloma and breast cancer marking protocols, as well as trying transductions in the presence of autologous stroma to try and improve transduction efficiencies.

CONCLUSIONS

The major obstacle to research in this area is the lack of assays which can predict gene transfer to actual human repopulating stem cells: conditions that allow efficient gene transfer in the murine model are at least 10-fold less efficient in larger animal models, and *in vitro* assays assessing gene transfer into progenitor cells or even long-term bone marrow culture initiating cells are also not predictive. Combinations of growth factors acting on earlier cells may be developed, and the use of primary or manipulated stromal cells may be necessary. These approaches will need to be tested in the primate model or in limited human clinical trials. *In vivo* selection, either via strategies such as MDR gene transduction, or in situations where gene-corrected cells would have a competitive advantage over non-corrected cells, such as in genetic disorders such as SCID or Fanconi's anemia, are more likely to be successful given current technologic constraints.[6,22] Use of alternative vector systems, such as adeno-associated virus, may also be successful ultimately, but they all require further preclinical testing and refinement.

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REPETITIVE MARROW TRANSPLANTATION INTO NONMYELOABLATED HOSTS

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INTRODUCTION

It has been assumed for decades that spaces need to be "opened" in order for homing marrow stem cells to engraft. This is partially based upon studies presuming the existence of marrow niches.[1] However, studies in the mid-eighties by Brecher and colleagues[2] and Saxe et al.[3] clearly demonstrated the ability to engraft normal marrow into normal nonmyeloablated hosts using a variety of marker systems. These workers used repetitive injections of relatively large numbers of marrow cells. However, although the interpretation of the studies may have differed, Micklem as far back as 1968 demonstrated the ability of relatively small numbers of marrow cells to engraft into normal hosts.[4] We have used the basic Brecher model and have been able to show extraordinarily high levels of engraftment, at times exceeding 70%, when 40 million male BALB/c cells were injected for 5 days consecutively into normal nonmyeloablated female hosts.[5] Carrying out calculations based on assumptions of total mouse marrow cellularity and total numbers of cells engrafted, along with an additional assumption that all injected stem cells home to marrow (they clearly don't), the maximum expected engraftment would be in the range of 40%; this data suggested that virtually quantitative engraftment of cells occurs when 40 million cells were given over a 5 day injection schedule. This in turn suggests that host marrow cells are actually replaced rather than augmented by infused marrow. In our initial studies engraftment of normal male marrow into nonmyeloablated BALB/c hosts resulted in engraftment percentages running from 15-42% at 21-25 months after transplantation.[5] Engraftment in these studies was determined initially by cytogenetic techniques but subsequently by examining host marrow, thymus or spleen DNA for the presence of Y specific sequences utilizing Southern blot analysis and the PY2 probe which recognizes repetitive sequences in the Y chromosome. Southern blots were also probed with the CDNA for IL-3 as a

single copy loading control so that loading levels could be adjusted. Densitometry was carried out either utilizing an LKB ultrascan XL enhanced densitometer or molecular dynamics phospho-imager. In our studies, the percentage of engraftment in spleen and thymus was generally, although not always, parallel to that seen in marrow.

Five fluorouracil (5-FU) administration to mice has been a technique utilized to enrich for relatively primitive renewal stem cells and also to induce cycling of murine stem cells in retroviral based gene therapy approaches. This maneuver has been found to result in relative concentration of putative stem cells as monitored by the high proliferative potential colony forming cell assay (HPP-CFC) and by short term *in vivo* renewal assays.[6-8] Dr. Harrison and colleagues[9] have studied six day post-5-FU marrow (150 mg/kg) in a competitive myeloablated mice/marrow repopulation assay. These investigators found that post-5-FU marrow competed equally with normal marrow in this myeloablated model and showed no defect in repopulation. Other studies have indicated that post-5-FU marrow progenitor stem cells are rapidly cycling.[10] We have studied the ability of marrow harvested from mice six days after 150 mg/kg 5-FU to engraft into nonmyeloablated animals comparing two tibias and two femurs from post-5-FU or from normal male animals injected into female hosts.[5] We were surprised to find that post-5-FU was markedly defective in engraftment in marrow, spleen and thymus at 1, 2 and 3 months after transplantation. Mean engraftment for six mice receiving normal marrow was 38%, whereas that for six mice receiving post-5-FU marrow was 8%. The defect persisted at 10 and 12 months in marrow, spleen and thymus. Further studies with this model evaluating marrow harvested six days after 5-FU or 35 days after 5-FU reveal that by 35 days the engraftment defect has disappeared and engraftment levels have returned to normal.[11] Thus in this particular model, engraftment of rapidly cycling cells in whole animals was markedly defective. These results suggested that the cycle status of the engrafting cell may be a determinant of engraftment.

Accordingly, we have assessed the cycle status of engrafting stem cells in nonmyeloablated hosts in studies using *in vivo* hydroxyurea suicide. Administration of hydroxyurea (900 mgs/kg) two hours before marrow harvest selectively kills cells passing through S phase. This manipulation had no effect on the capacity of normal and minimal effect on the capacity of 5-FU marrow to engraft into nonmyeloablated hosts indicating that all, or the majority, of cells engrafting in this model are dormant or noncycling.[11]

We have also studied the capacity of a cocktail of individual lympho-hematopoietic cytokines to induce cell cycle progression, to

maintain or expand primitive HPP-CFC or low proliferative potential colony forming cells (LPP-CFC) and to maintain engrafting cells into nonmyeloablated hosts.[12] The cytokines Interleukin-11 (IL-11), Interleukin-3 (IL-3), Interleukin-6 (IL-6) and steel factor were chosen because they all appear to act on relatively primitive dormant stem cells, and because they have been utilized in retroviral transduction protocols. Exposure of male BALB/c marrow to Interleukin-11, Interleukin-3, Interleukin-6 and steel factor in liquid culture for 48 hours resulted in a progressive expansion of the total numbers of colonies formed (LPP-CFC plus HPP-CFC) and a maintenance of 7 factor responsive HPP-CFC. This latter cell responds to the combination of IL-3, IL-1, G-CSF, GM-CSF, CSF-1, steel factor and basic FGF, and appears to represent a very primitive marrow progenitor/stem cell. We assessed the cell cycle status that these cells utilizing high specific activity tritiated thymidine suicide at different times in culture; 6, 24 and 48 hours. We found that there was no killing of either HPP-CFC or LPP-CFC at the zero hour time point indicating that these seven factor responsive stem cells (assayed at 14 days) are dormant. By six hours there was a slight kill of LPP-CFC and there was a progressive increase in the killing effect of tritiated thymidine such that by 48 hours most HPP-CFC and LPP-CFC were killed by tritiated thymidine. These data indicated that dormant primitive cells had been induced to enter cell cycle and that by 48 hours most of these cells were actively proliferating. When these cells were assessed for their ability to engraft into normal nonmyeloablated hosts and compared to an equivalent number of starting normal male cells there was a striking defect in engraftment seen in the cells treated with cytokine *in vitro*. In these experiments animals were injected for three consecutive days with cytokine treated cells or with cells from normal hosts. With this injection schedule, animals receiving the cells from normal hosts without cytokine exposure showed an engraftment in marrow of approximately 21%. The cytokine cultured cells universally failed to engraft in marrow. Although with lower rates of absolute engraftment, similar results were seen in spleen and thymus. These data indicated that cytokine induction of cycling was associated with a major defect in marrow engraftment in a nonmyeloablated transplant model.

Altogether these data indicate very high rates of engraftment of normal marrow into normal hosts; there does not appear to be a need to open spaces or provide any cytoxic therapy for stem cell engraftment to occur. The apparent need for such therapies may be more perceived than real. In the irradiated host a very small number of stem cells will expand to repopulate a host and give oligoclonal hematopoiesis. The same small

number of stem cells would not be detectable with the present techniques in a non-irradiated host on a dilution basis, even if all engrafted. In the nonmyeloablated host there is a direct competition and thus a larger number of cells are needed in order to detect engraftment. Our data would suggest, however, that if looked at from the stem cell engraftment vantage, the irradiated and non-irradiated host probably have relatively similar levels of engraftment and in fact the non-irradiated host may even be superior.

Our data also indicates that induction of cell cycling by either *in vivo* 5-FU exposure or *in vitro* cytokine exposure is associated with the development of a defect in engraftment into nonmyeloablated hosts. One hypothesis here is that the cell cycle status per se may determine the ability of these cells to engraft.

This is of obvious importance in both gene therapy or stem cell expansion approaches. In the latter, cells are typically induced to cycle in order for the retroviral vectors to incorporate into host gene. This type of strategy may preclude adequate engraftment of the retroviral transduced cells and thus impact negatively on gene therapy approaches. In a similar vein, attempts to expand stem cell progenitors have been carried out and most have been judged on the ability of *in vitro* stem cells to be expanded. These cells may actually have acquired an engraftment defect due to their cytokine stimulation and may in fact be poorly engraftable as compared to normal non-cytokine treated cells. If this is the case, then expansion techniques with immediate engraftment of cycling cells may be doomed to failure in a clinical setting. It may be important to devise strategies to return these cells to a dormant or engraftable state prior to transplantation. All of these possibilities are presently under active study.

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INFLUENCE OF DIFFERENT CONDITIONING REGIMENS ON ENGRAFTMENT OF GENETICALLY MARKED HEMATOPOIETIC STEM CELLS

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ABSTRACT

Many of the current gene therapy trials attempt to treat genetic diseases. Conditioning, and conditioning-related toxicities, could be avoided in these patients if transduced hematopoietic stem cells engrafted in sufficient numbers without preceding myeloablation. We have studied the effect of different conditioning regimens on the engraftment of genetically marked hematopoietic stem cells (HSC). Peripheral blood and/or marrow cells collected after treatment with recombinant canine stem cell factor (rcSCF) or cyclophosphamide were transduced in a retrovirus vector containing long-term culture system. Supernatant from three different vector-producing cell lines with similar viral titers were used. Fifteen dogs received either no conditioning (group A, n=5), a sublethal dose of cyclophosphamide 40 mg/kg (group B, n=4), a sublethal dose of 200 or 300 cGy total body irradiation (TBI) (group c, n=3), or an otherwise lethal dose of 920 cGy TBI (group D, n=3) before intravenous infusion of the transduced hematopoietic cells. Dogs were followed for at least 6 months. Peripheral blood granulocytes were obtained every 2 to 3 weeks posttransplant and analyzed by polymerase chain reaction (PCR) for the presence of the transduced gene. The percentages of positive results in dogs more than 4 weeks after transplantation were 0% without conditioning, 18% with sublethal cyclophosphamide, 33% with sublethal TBI, and 17% with otherwise lethal TBI. These data suggest that

myelosuppressive conditioning of the recipient is required for the engraftment of genetically marked hematopoietic stem cells. Engraftment of transduced cells in dogs treated with sublethal TBI or sublethal cyclophosphamide was comparable to engraftment in dogs receiving otherwise lethal TBI, suggesting that complete myeloablation may not be necessary.

INTRODUCTION

Hematopoietic stem cells are attractive targets for retrovirus-mediated gene transfer. A main advantage is the continued presence of the transduced gene for the lifetime of the recipient. Most gene transfer protocols have used conditioning regimens with total body irradiation (TBI) prior to the infusion of transduced cells. We have studied gene transfer in the canine model and showed long-term engraftment of transduced peripheral blood and marrow repopulating cells after an otherwise lethal dose of 920 cGy TBI.[1,2] The toxicity was high and approximately 50% of the animals died early posttransplant secondary to infections. This would not be acceptable in current gene therapy protocols that attempt to treat or cure genetic diseases rather than malignancies. The assumption that conditioning of the host is required is derived from studies in mice showing long-term engraftment of chromosomally marked donor bone marrow in recipient mice that received lethal whole body irradiation.[3] Several investigators have since studied the engraftment of bone marrow cells transfused into normal unirradiated syngeneic mice.[4,5] In subsequent studies the number of donor cells was increased up to ten-fold, which resulted in improved engraftment of donor marrow, suggesting competitive repopulation.[7,8]

Based on the relatively high toxicity associated with TBI, we have studied engraftment of genetically marked hematopoietic stem cells in dogs treated with different conditioning regimens or without pretransplant conditioning.

Our results show that engraftment of transduced stem cells was demonstrated only in dogs receiving either myeloablative or myelosuppressive conditioning regimens, suggesting that some form of myelosuppressive conditioning is required for successful engraftment of transduced stem cells.

MATERIALS AND METHODS

Animals

A total of 15 beagle dogs either raised in an indoor facility approved by the American Association for Accreditation of Laboratory Animal Care at the Fred Hutchinson Cancer Research Center (FHCRC) or obtained from private USDA licensed kennels were used as marrow donors and recipients. Animal holding areas were kept at 70°F ± 2°F with 50% ± 10% relative humidity using at least 15 air changes per hour of 100% conditioned fresh air. The animals were on a 12-hour light/dark full-spectrum lighting cycle with no twilight. They were dewormed, vaccinated for rabies, distemper, leptospirosis, hepatitis, and parvovirus. Studies were carried out according to the "Guide for Laboratory Animal Facilities and Care" prepared by the National Academy of Sciences, National Research Council. The protocols used in this study were approved by the Institutional Animal Care Committee of the FHCRC.

Cell Collection and Transduction Method

Transduction methods of canine hematopoietic cells in long-term marrow culture (LTMC) have been described.[9] Transduction conditions varied slightly in dogs receiving different conditioning regimens (Table 1). Peripheral blood hematopoietic cells (PBHC) were harvested by leukapheresis on 2 consecutive days (days -7 and -6) after mobilization with recombinant canine stem cell factor (rcSCF) (groups A, B and D). After buffy coat separation, white cells were cultured in LTMC at 2-4 x 10^6 MNC/mL on an autologous stroma layer established 2-3 weeks before. After the second leukapheresis, bone marrow was obtained under general anesthesia from both front and rear legs. Buffy coat separated bone marrow cells were cocultivated on irradiated (25 Gy) vector-producing cells in 225 cm^2 canted-neck flasks (Corning, Corning, NY) at 1-2 x 10^8 mononuclear cells per flask in a humidified 7% CO_2 incubator for 24 hours. This cocultivation step was omitted in three dogs in group D. A mixture of growth factors (rhIL-1, rhIL-3, rhIL-6 and rcSCF, at a final concentration of 50 ng/ml each) and polybrene (4 μg/ml) were added on the first day of culture and replaced with every supernatant exchange. After cocultivation, adherent and nonadherent cells were harvested and incubated in LTMC. Medium containing 20% pretested horse serum (HS; GIBCO), RPMI 1640 (M.A. Bioproducts, Walkersville, MD), 1% L-glutamine, 1% nonessential amino acids, 1% penicillin/streptomycin and hydrocortisone at 10^{-7}M was used. Fifty percent of the supernatant of

LTMCs was replaced daily with fresh cell-free vector-containing medium. PBHC were kept for 5 or 6 days and bone marrow cells for 4 days in LTMC. Both adherent and nonadherent cells were harvested, washed twice with PBS, filtered and injected either locally into the humeri of the dogs conditioned with local irradiation, or systemically into the external jugular vein.

Table 1. Number of Mononuclear Cells (MNC) Transplanted, Packaging Cells Used, and Survival of Dogs

Dog Number	No. of MNC Infused (x 10^8)/kg	Packaging Cells/Vectors/ Genes	Survival in Months (cause of death)
Group A			
No conditioning			
D675*	0.6	PA317/MFG/hGC	10 (euth.)
D685	0.5	PA317/MFG/hGC	8 (euth.)
D856	2.1	PA317/LN/*neo*	>6
D857	0.4	PG13/LN/*neo*	>6
D860	0.8	PG13/LN/*neo*	>6
Group B			
Cyclophosphamide (40 mg/kg)			
D495*	0.8	PG13/LN/*neo*	12 (euth.)
D586*	0.9	PG13/LN/*neo*	11 (euth.)
D569*	1.9	PA317/MFG/hGC	12 (euth.)
D628	1.1	PA317/MFG/hGC	10 (euth.)
Group C			
Sublethal TBI (200/300 cGy)			
D407*	0.03	PA317/LN/*neo*	>12
D482*	0.03	PA317/LN/*neo*	13 (euth.)
D411*	0.02	PA317/LN/*neo*	12 (euth.)
Group D			
Otherwise lethal TBI (920 cGy)			
D499*[#]	1.0	PA317/MFG/hGC	>12
D600	1.5	PA317/MFG/hGC	7 (euth.)
D615	1.3	PA317/MFG/hGC	8 (euth.)

Abbreviations: euth., dogs killed with sodium pentobarbital

* Indicates dogs tested for helper-virus. All tested dogs were negative as determined by vector rescue assay.

[#] This dog also received 3.4x10^8/kg unmodified bone marrow cells.

Conditioning Regimens and Posttransplant Care

Prior to the infusion of the genetically marked autologous hematopoietic cells, dogs received one of the following conditioning regimens: no conditioning (group A, 4 dogs), cyclophosphamide 40 mg/kg IV (group B, 4 dogs), TBI at 200 cGy, 2 dogs, or 300 cGy, 1 dog (group C), or 920 cGy TBI (group D, 3 dogs). Group C also included a single dose of cyclophosphamide 30 mg/kg IV 7 days prior to the marrow harvest to eliminate committed progenitor cells and thereby to induce stem cell cycling. TBI was administered as a single dose at a rate of 7 cGy/min from two opposing ^{60}Co sources as described.[10] Posttransplant care included parenteral fluids, systemic antibiotics, and whole blood transfusions which were irradiated with 2000 cGy cesium radiation before transfusion to inactivate hematopoietic repopulating cells and immunocompetent cells. In addition, dogs given TBI or cyclophosphamide received oral nonabsorbable antibiotics (polymyxinB and neomycin sulfate) three times daily starting on day -5 (day 0 being the day of TBI and transplantation) until the absolute neutrophil count (ANC) reached 1500/mm^3 postgrafting. Hematocrit, reticulocyte, leukocyte, platelet and differential counts were obtained before and daily after day 0 until full hematologic recovery. Dogs were either euthanized after completion of the study after 6 months, or kept alive for further observation (Table 1).

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to demonstrate the presence of the *neo* and the human β-glucocerebrosidase cDNA in peripheral blood granulocyes and lymphocytes obtained at least every 3 weeks for a minimum of 6 months. Granulocytes were obtained from the buffy coat fraction after Ficoll-Hypaque gradient separation (density 1077, centrifugation at 1000 g for 30 min) and had a purity of 99% as determined by cytospin.

For the amplification of the *neo* gene, the following oligonucleotide primers were used: sense primer *neo* 350 5' AAG AGA CAG GAT GAA GGA TCG 3', antisense primer *neo* 1150 5' CAG AAG AAC TCG TCA AGA 3', sense primer *neo* 450 5' ACA AAC AGA CAA TCG GCT GCT 3', and antisense primer *neo* 1027 5' GCC AAC GCT ATG TCC TGA TA 3'. Two micrograms of genomic DNA were amplified by using 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Conditions included initial denaturation at 94°C (4 min), followed by 30 cycles of 55°C annealing (1 min), 72°C extension (3 min)

and 94°C denaturation (1 min). Reaction products were electrophoresed on 1.5% agarose gels and transferred onto zeta-probe blotting membranes. Hybridization was performed overnight at 42°C using a ^{32}P-labeled 577-bp internal fragment amplified with primers *neo* 450 and *neoR* 1027 as described.[2]

RESULTS

All dogs had complete hematopoietic reconstitution and survived for at least 6 months (Table 1). Myelotoxicity of the different conditioning treatments was evaluated by monitoring granulocyte and platelet counts posttransplant. Dogs that received otherwise lethal TBI (group D) had a more prolonged granulocyte nadir than dogs treated with sublethal cyclophosphamide (group B) or low dose TBI (group C). Decline in platelet counts and time of thrombocytopenia was similar in dogs given low dose TBI or otherwise lethal TBI. Granulocyte and platelet counts were within normal range posttransplant in dogs given local irradiation and no conditioning.

Peripheral blood granulocytes were obtained every 2 to 3 weeks posttransplant and analyzed by PCR for the presence of the transduced gene. Only PCR results from granulocytes were evaluated for this study because granulocytes, due to their short half-life, represent a cell fraction recently developed from earlier progenitor cells. The results of PCR-positive granulocytes (percentage of positive results of the total number of PCR analyses in each dog) obtained more than 4 weeks after transplantation of transduced hematopoietic cells in dogs without conditioning, sublethal cyclophosphamide, sublethal TBI, and an otherwise lethal dose of TBI were 0%, 18%, 33% and 17%, respectively (Figure 1). Peripheral blood samples of the dogs were free of helper virus and no long-term side effects attributable to the retroviral transduction have been observed during the follow-up.

DISCUSSION

Most of the gene transfer protocols in animals have used conditioning regimens prior to the transfusion of transduced hematopoietic cells. This was based on the assumption that marrow "space" or "niches" are required for engraftment of hematopoietic repopulating cells.[4-6] Several reports in the mouse model have recently suggested that conditioning might not be necessary for engraftment of hematopoietic

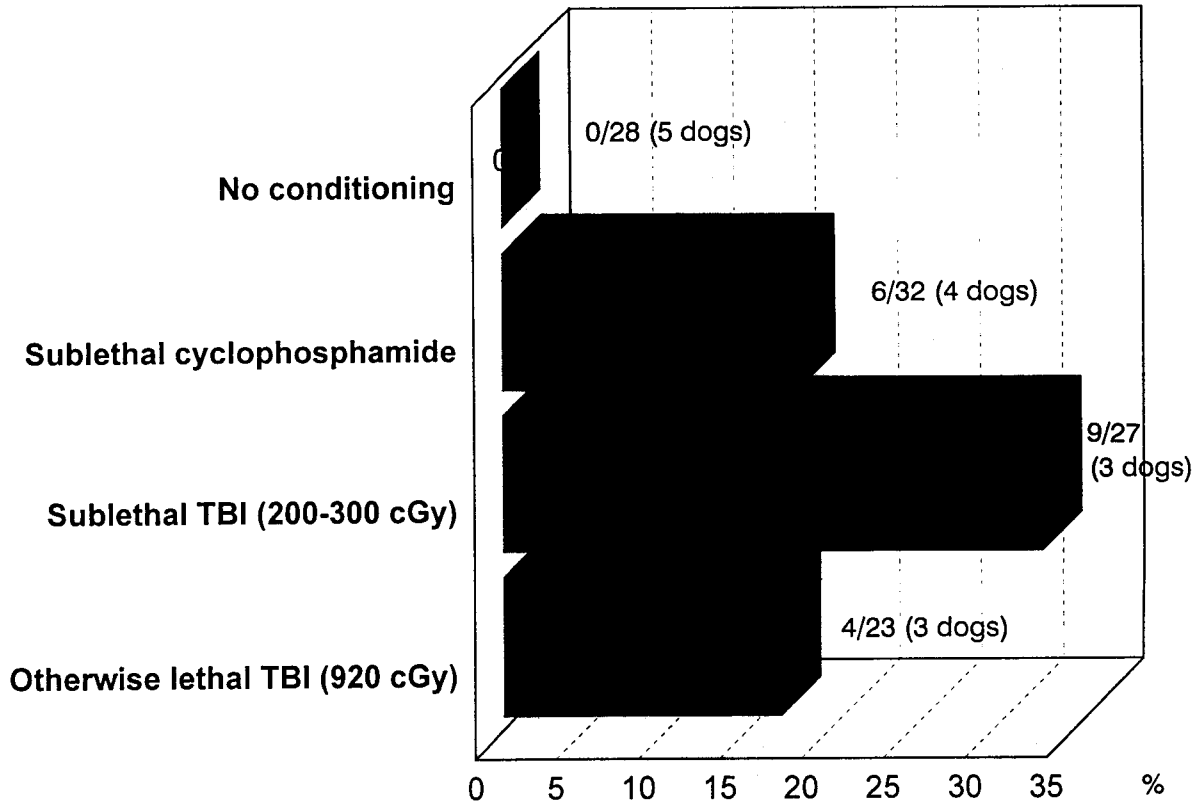

Figure 1. Percentage of PCR-positive samples of peripheral blood granulocytes obtained more than 4 weeks after the transplant of transduced hematopoietic cells.

stem cells.[7,11,12] We have studied this question in our canine model and found improved engraftment of transduced hematopoietic stem cells in dogs that received either otherwise lethal TBI, sublethal TBI or sublethal cyclophosphamide compared to dogs that did not receive conditioning or only local irradiation before the infusion of the transduced stem cells. No transduced granulocytes were detected in dogs not given any conditioning. Our data are in accordance with Tomita et al.[13] who showed that myelosuppressive conditioning is required to achieve engraftment of transplanted murine hematopoietic stem cells. These data confirmed earlier studies by Takada et al.,[5] who showed that 6 months after infusion of the bone marrow cells, 80% of dividing bone marrow cells were of donor origin in irradiated mice, whereas only 2% donor bone marrow was seen in unirradiated mice. Brecher et al.[14] transfused 40 x 10^6 bone marrow cells from male donors to normal unirradiated female recipients on each of 5 consecutive days, 5 to 10 times the number used in the past, and saw 16% to 25% engraftment of donor marrow up to 13 weeks posttransplant. More recently, Stewart et al.[7] reported up to 42% long-term engraftment of male bone marrow transfused into nonmyeloablated female mice for up to 2 years after transplanting high numbers of marrow cells.[15,16] Wu et al.[11] transplanted unirradiated mice with a single infusion of only 20 x 10^6 donor bone marrow cells and found that up to 47% day-12 CFU-S and 10% marrow cells at 8 weeks postinfusion were of donor origin. The follow-up in this study was short and the results therefore cannot be compared to studies with longer follow-up investigating engraftment of pluripotent hematopoietic stem cells.

We conclude that some form of myelosuppressive conditioning is required to reduce the number of competing endogenous HSC and/or to create "space" or "niches" in the bone marrow to allow significant engraftment of the low number of marked stem cells currently available in clinical marrow transplantation. The number of marrow cells used in most of the studies in unirradiated mice was approximately 80 x 10^8/kg. The average number of marrow cells harvested from a conventional bone marrow harvest in a human donor is around 40 times less. Our findings do not exclude the possibility that large numbers of transduced stem cells engraft competitively in an unconditioned host.

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ALLOGENEIC CELL-MEDIATED IMMUNOTHERAPY (ALLO-CMI) USING MATCHED PERIPHERAL BLOOD LYMPHOCYTES FOR PREVENTION AND TREATMENT OF RELAPSE FOLLOWING BONE MARROW TRANSPLANTATION: A new approach for successful eradication of hematological malignancies resistant to conventional chemotherapy and bone marrow transplantation

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ABSTRACT

Background. Mutual donor T lymphocytes following allogeneic bone marrow transplantation (BMT) represent the only effective treatment for hematologic malignancies resistant to conventional doses of chemotherapy. The beneficial effects of BMT derive from a combination of high dose chemoradiotherapy and mostly from the immunologic effects of immunocompetent donor T cells against residual leukemia cells of host origin, known as graft vs leukemia (GVL) effect. Unfortunately, GVL effects are usually associated with graft vs host disease (GVHD). We document a long-term follow-up of the first case successfully treated for chemoradiotherapy-resistant relapsed acute lymphoblastic leukemia by allogeneic cell-mediated immunotherapy (allo-CMI), currently alive and well, free of disease >7 years. Efficacy of allo-CMI was confirmed by successful reversal of relapse in a total of 17 consecutive cases at our center in a variety of malignant hematological diseases. The benefits of allo-CMI was recently confirmed in many transplantation centers, particularly in patients with chronic myeloid leukemia. These studies motivated us to attempt to introduce cell therapy following autologous bone marrow/blood stem cell transplantation (ASCT). We report the first 5 patients who underwent allo-CMI following ASCT, for high risk non-Hodgkin's lymphoma. Overall, our data suggests that antitumor effects inducible by cell-therapy following BMT as well as ASCT should be considered as the treatment of choice for eradication of overt relapse or minimal residual disease, respectively, following high-dose

chemoradiotherapy. Based on our observation in rodents, allo-CMI may be further potentiated following ASCT by additional activation of donor PBL *in vivo* with a short course of well-tolerated rhIL-2 doses on an outpatient basis.

INTRODUCTION

A growing bulk of experimental and clinical data indicates the possible utilization of the immune system in the prevention, control or eradication of malignant hematological disease and certain solid tumors.[1-6] These observations include high incidence of secondary tumors in immunosuppressed patients and spontaneous regression of some following withdrawal of immunosuppressive agents, as well as increased risk of cancer in experimental animals and patients with congenital and acquired immune deficiency. Allogeneic bone marrow transplantation (BMT) seems to provide a most effective mode of immunotherapy for a large number of malignant hematologic diseases, including lymphomas, because immunocompetent donor T cells are capable of mounting effective immune response against residual host tumor cells escaping chemoradiotherapy, a phenomenon known as graft versus leukemia (GVL) or graft versus tumor (GVT) effect. However, although myeloablative high-dose chemoradiotherapy supported by autologous marrow or autologous peripheral blood stem cell transplantation is potentially curative for chemoradiosensitive malignancies, residual tumor cells may account for the quite frequent relapse occurring post ASCT. GVL effects following BMT, an element that is missing following ASCT, represents the only possible modality for cure of chemotherapy resistant disease, thus reinforcing the potential role of passive adoptive transfer of allogeneic lymphocytes in the setting of minimal residual disease for leukemia.[1-5] Recently, it was suggested that similar immune-mediated GVL reactions may also be effective in recipients of marrow allografts for non-Hodgkin's lymphoma (NHL), a phenomenon described as graft vs lymphoma effect.[7-9] Evaluation of large numbers of patients undergoing BMT suggests that graft vs lymphoma effect is more likely to be clinically beneficial for lymphoblastic lymphoma which represents one end of the spectrum of acute lymphoblastic leukemia (ALL).

We have recently managed to improve the efficacy of ASCT in a preclinical animal model by overcoming the lack of GVL effects, introducing posttransplant immunotherapy. Based on successful experiments using a murine model of spontaneous, transplantable

leukemia/lymphoma,[10] we have subsequently initiated a pilot clinical study to test the feasibility of allogeneic cell-mediated immunotherapy (allo-CMI) following ASCT, using allogeneic peripheral blood lymphocytes (PBL) obtained from an HLA-A,B,DR matched sibling posttransplantation, on an outpatient basis. Here we summarize the initial experience in treating relapse following allogeneic BMT,[10-13] recently confirmed by other centers in Europe[14] and in the USA,[15-17] as well as report our most recent innovative experience in applying cell-therapy following ASCT in a cohort of 5 patients with NHL.

MATERIALS AND METHODS

Patients with relapse following allogeneic BMT for leukemia. Starting in early 1987, we have introduced allo-CMI as a new modality for the treatment of relapse post BMT in a patient with pre-B ALL in early 3rd relapse following allogeneic BMT. Sixteen additional cases with a variety of hematologic malignancies were similarly treated at the Hadassah Hospital in Jerusalem. As soon as relapse was documented following BMT, a total of 17 patients treated in Jerusalem received infusions of donor's PBL.

Patients at high risk to relapse following ASCT for NHL. Five patients with NHL at high risk to relapse underwent allo-CMI procedure following ASCT. All of the first 4 patients received a combination of bone marrow cells and G-CSF-mobilized peripheral blood stem cells, purged with anti-CDl9-coated beads due to bone marrow involvement in 2 cases, using immunomagnetic separation (Dynal, Oslo, Norway). One additional patient received autologous bone marrow cells without blood stem cells. Immunotherapy was initiated on an outpatient basis as soon as patients were hematologically reconstituted.

Allo-CMI procedure. PBL were separated by the Baxter CS-3000+ apheresis unit (Deerfield, IL). Two of the 17 patients with bulky disease received non-myeloablative chemotherapy prior to allo-CMI. Whenever indicated and in the absence of GVHD, allo-CMI was further escalated by concomitant administration of recombinant human interleukin-2 (rhIL-2), daily injections of 6×10^6 international units/M^2 given subcutaneously on an outpatient basis, starting concomitantly with donor PBL.

RESULTS

The first patient who gave us the opportunity to prove the efficacy of our new modality late posttransplant allo-CMI, was a 2-½ year old boy who relapsed (3rd relapse) following BMT for aggressive drug resistant acute lymphoblastic leukemia. Patient was treated with his sister's peripheral blood lymphocytes with impressive elimination of all leukemic cells, which included heavy infiltrate, in the marrow and extramedullary disease subcutaneously in several locations. To date, nearly 8 years after, the patient is alive and perfectly well with no evidence of tumor cells (by cytogenetics) or male cells (by PCR and cytogenetics), fully reconstituted by his normal sister's marrow. Thus far, a total of 17 patients have been treated for relapse at our center with a complete response observed in 59% (Table 1). None of these patients would be expected to be cured by any alternative modality except allo-CMI.

Table 1. Treatment of Relapse Post Allogeneic BMT: The Hadassah Experience

	Total	Response
ALL	6	4
AML	3	0
CML	1	5
Burkitt's	1	0
RAEB	1	1
Total	17	10 (59%)

We have recently initiated a clinical trial utilizing allo-CMI using matched sibling PBL following ASCT for prevention and treatment of relapse in leukemia, lymphoma and solid tumors. Our suggested approach for treatment of high risk patients with residual tumor cells in a variety of malignancies is based on a two-step procedure: (a) maximal tumor debulking by high-dose chemoradiotherapy followed by rescue with autologous stem cell transplantation, with no risk of GVHD; (b) subsequent allo-CMI (on an outpatient basis) starting with donor's blood lymphocytes alone, escalating if indicated to PBL activated *in vivo* and/or *in vitro* by rIL-2, provided there are no signs of GVHD.

The outcome of the first 5 patients with NHL who underwent a similar allo-CMI procedure is shown in Table 2. One patient developed marrow aplasia and was successfully rescued with allogeneic BMT from the PBL donor, using no further conditioning on an outpatient basis. He is fully reconstituted with donor cells, alive and well 2.5 years out. All patients with responding disease, except one patient with evidence of disease due to chemo-resistant NHL, are alive and well. One patient (UPN 515) with minimal relapse at a previously involved lymph node site, who did not receive radiation therapy to previously involved field, is being cytoreduced to complete remission, and another course of allo-CMI will be attempted.

DISCUSSION

Allogeneic T lymphocytes can induce effective immune-mediated antitumor effects in experimental animals and man which frequently, but not necessarily, are associated with anti-host responses, which may result in acute and/or chronic graft vs host disease (GVHD).[4] The concept that allogeneic effector cells matched or even mismatched at all major histocompatibility loci may exert antileukemic or antitumor effects independent of GVHD is supported by our studies in mice utilizing a murine model of B cell leukemia/lymphoma (BCL1),[18-20] murine myeloid leukemia[21] and sarcoma[6] featuring induction of GVL by donor T-cells, independently of GVHD. Thus, genetically identical tumor cells with leukemia-associated antigens not recognized by the host, may be recognized by MHC-matched, MiHC-incompatible, donor's allogeneic immune, CD8+ T-cells fully tolerant of host's alloantigens[19,20] or MHC matched donor T cells further activated by recombinant human interleukin 2 (rIL-2).[5] Hence, tumor eradication by donor T-cells may be induced against tumor-specific or tumor-associated antigens, or alternatively, against minor histocompatibility antigens. Data pertaining to specificity of reactive T cell clones against leukemic cells *in vitro* strongly support the possibility of the existence of well-defined T cell clones with antileukemic reactivity separable from anti-host reactivity, in full agreement with the experimental and clinical data.[7] More recently we have shown that it may

Table 2. Characteristics of Patients with non-Hodgkin's Lymphoma (NHL) Receiving Allogeneic Cell-Mediated Immunotherapy Following ASCT Using Matched Sibling Peripheral Blood Lymphocytes (PBL)

UPN[1]	Age	Sex	Diagnosis	ASCT[2]	PBL	PBL + rIL-2[3]	LAK + rIL-2	Outcome
496	39	M	NHL, Intermediate grade, 2nd PR	29.4.92	+	+	0	Bone marrow aplasia, allogeneic BMT, A&W >28 mos
515	38	M	NHL, low grade, 2nd CR	12.7.92	+	+	+	A&W, relapsed,[4] A&W >25 mos
560	21	F	NHL, high grade, 2nd CR	13.12.92	+	+	0	A&W >20 mos
584	36	F	NHL, high grade, 2nd CR	15.11.92	+	+	+	A&W >21 mos
612	22	F	NHL, high grade, resistant relapse	24.5.93	+	+	0	No CR - died

[1] *Unique patient number*
[2] *Autologous stem cell transplantation*
[3] *Allogeneic donor's PBL in vitro activated with rIL-2, followed by in vivo rIL-2*
[4] *Patient had minimal relapse at 20 mos, scheduled for continuous cell therapy following involved radiation which was omitted following BMT*

be possible to administer donor T-cells for effective induction of GVL with non or controlled GVHD by delaying administration of donor T-cells after induction of stable chimerism following T-cell depleted allogeneic BMT.[22-24] The longer the time interval between cell therapy and BMT, the larger proportion of donor T-cells can be safely administered.[4,8,25]

As indicated above, we have recently initiated a pilot clinical trial investigating the feasibility of applying allo-CMI allogeneic cell-mediated cytokine-activated immunotherapy (and allo-CCI) for prevention and/or treatment of relapse following ASCT in more than 25 patients with various malignancies at high risk. Thus far, we have documented that the procedure of cell therapy is well tolerated on an outpatient basis shortly following ASCT as soon as hematopoietic reconstitution is stabilized. Results of the first 5 patients treated for NHL are presented. Once shown to be effective, allo-CMI/CCI may represent a new potential therapeutic modality for patients with a variety of hematologic malignancies and solid tumors who have MHC matched sibling or MHC compatible unrelated donor available, thus representing a new treatment option for nearly 50% of patients with cancer.

As for allo-CMI following ASCT, the first patient described (UPN 496) did not show evidence of engraftment of donor cells immediately after PBL infusion but did engraft following administration of an equal number of PBL with additional *in-vivo* rhIL-2. Unfortunately, activated donor T cells caused severe marrow aplasia in one patient that necessitated allogeneic bone marrow rescue, which was carried out on an outpatient basis uneventfully, because the right interpretation and decision for allogeneic rescue was made early enough. Marrow aplasia induced by donor buffy-coat infusion was also described following allogeneic BMT and has resulted in the death of some patients in several clinical trials involving allo-CMI following allogeneic BMT for CML and acute leukemia.[26,27] Our case of allo-CMI/IL-2 induced aplasia which occurred in one of the first 5 patients with NHL in our ongoing trial suggests that this complication should be diagnosed as early as possible, as it can be completely resolved once recognized.

Taken together and based on established results of immunotherapy following allogeneic BMT, allo-CMI seems most effective and as of this date the only possible rational treatment of chemo-radioresistant leukemia and lymphoma. The use of allo-CMI, and whenever indicated allo-CCI, following ASCT in patients with a matched sibling donor (or possibly matched unrelated donor) represents a novel and potentially effective modality for prevention and/or treatment of relapse.[3,28] However, this

approach should be regarded strictly as an experimental procedure, since a larger number of patients and larger observation periods are required to assess the potential clinical benefits of allo-CMI in the context of minimal residual disease established following ASCT.

Unfortunately, the use of anti-tumor-specific donor T cells is not yet feasible, hence, cell-mediated immunotherapy based on infusion of unselected donor T cells may lead to non-specific anti-host responses such as marrow aplasia and GVHD. Early identification and understanding of the pathophysiology of marrow aplasia may allow immediate and effective treatment.

The feasibility of utilizing cell-therapy for the treatment of a large number of patients with documented minimal residual disease, or at high risk to relapse with a matched sibling available, that may not wish or may have partial or complete contraindication to allogeneic BMT, may open new horizons for eradication of tumor cells escaping chemo-radiotherapy and thus warrant further investigations.

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EXPANSION OF HEMATOPOIETIC STEM AND PROGENITOR CELLS IN PERFUSION CULTURES

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INTRODUCTION

In recent years, we have witnessed the birth of a new discipline that has been called Tissue Engineering.[1] The goal of this endeavor is to reconstitute fully, or partially, functional human tissue *ex vivo*. The reconstitution of a number of tissues has been addressed in this manner, and success has been reported for several human tissues such as liver, skin, the nervous system and bone marrow.

The philosophy that underlies the application of tissue engineering principles to the reconstruction of hematopoietic function *ex vivo* is outlined in Figure 1. The governing hypothesis is that the provision of accurately simulated *in vivo* conditions in an *ex vivo* device will bring out the inherent ability of the cells to reconstitute tissue function. The environment that cells experience can be divided into their immediate environment, or microenvironment, and the larger environment provided by the body as a whole. As depicted in Figure 1, cells experience a microenvironment that is characterized in terms of their neighboring cells, extracellular matrix (ECM), diffusion of nutrients, growth factors and respiratory gases, and in many tissues, by the local geometry. The size scale of this local or microenvironment is typically on the order of 100 microns. Microenvironments on this size scale are uniformly perfused by the micro-circulation. Little difference should exist from one microenvironment to the next in terms of nutrients, respiratory gases and trafficking of growth factors. The micro-circulation is connected with the circulation, and in this way every cell in the body is connected with other major organs, such as kidney for removal of toxic waste, the lungs as a source of oxygen, the liver and intestine as a source of nutrients, and so forth. The challenge in tissue engineering human hematopoiesis *ex vivo* is to provide both the proper microenvironment as well as substitution for the larger or whole body environment. Both conditions can be met by the proper design of perfusion-based bioreactor systems.

The parameters that characterize the microenvironment can loosely be grouped into four categories (Table 1). These are: 1) physical variables, such as the perfusion rate, oxygenation and geometry; 2) materials; primarily the extracellular matrix and any growth factors found therein, but also biocompatible materials used in the construction of perfusion devises; 3) the chemical environment, which refers to the chemical composition of the soluble environment, such as nutrients and soluble growth factors, and 4) the cellular environment, which primarily refers to the cell density, the cell composition and local arrangement of cells.

Table 1: Variables that Characterize the Microenvironment (~100μm)

Physical	Perfusion rate, oxygenation, micro geometry
Materials	Extra-cellular matrix bound growth factors
Chemical	Nutrients, soluble growth factors
Cellular	Cell density, cell composition, cellular arrangement

Much emphasis has been placed on the manipulation of the chemical environment through the addition of soluble growth factors, and toward the cellular environment by cell purification and other methods. These approaches have resulted in partial progress towards the study and reconstitution of hematopoiesis *ex vivo*. Less progress has been reported with the physical variables and material selection. In recent years, the examination of the effects of perfusion rates and oxygenation on bone marrow cultures has resulted in improved reconstitution of human hematopoiesis ex vivo.[2-6] Proper perfusion rate and oxygenation have been found to be critical for the outgrowth of stroma and accessory elements from bone marrow mononuclear cell (BM MNC) inocula. These conditions have led to 10 to 20 fold expansions of CFU-GM and 4 to 10 fold expansions of LTC-IC. It should be noted that these expansions are obtained directly from BM MNCs, and thus no losses of CFU-GM or LTC-IC are experienced due to purification prior to the inoculation of these devices. In the section that follows, we briefly review the performance of these devices and requirements for the successful implementation in a clinical setting.

GROWTH PERFORMANCE IN THE PERFUSION-BASED BIOREACTOR SYSTEMS

Perfusion-based bioreactor systems have been described recently.[4,5] These systems provide for uniform and physiological perfusion rates, simulation of *in vivo* oxygenation rates, and supply of the proper nutrients and growth factors that enable both stroma formation and balanced hematopoiesis to occur.

The growth factors that have been used in these perfusion cultures are stem cell factor (SCF), erythropoetin (Epo), interleukin-3 (IL-3), and granulocyte-macrophage colony stimulating factor (GM-CSF). Figure 2 shows typical growth curves obtained from such perfusion bioreactors.[5] This figure shows the results from three separate experiments. In each case, ten identical bioreactors were inoculated with BM MNC at a density of 300,000 per square centimeter. Over a 14 day cultivation period cells expanded 8 to 10 fold in number to 2.5 to 3.0 million cells per square centimeter.

It should be noted that most of the cell expansion originates from the $CD34^+$ sub-population present in the MNC inoculum. The frequency of $CD34^+$ cells in MNC is on the order of 1%. Therefore, only about 3000 CD34 cells inoculated per cm^2 result in about 2.5 to 3 million total cells. Thus, a ten fold expansion of total cell number corresponds to 800 to 1,000 fold expansion of the $CD34^+$ population contained in the MNC inoculum.

The number of progenitor cells in these cultures is a function of time are shown in Figure 3. The growth curves shown correspond to the experiment of Figure 2. The growth of the CFU-GM population parallels the growth of the total cell population. Frequently, some enrichment (increase in density) of progenitor cells is observed over time, resulting in CFU-GM expansions that are 1 to 3 fold higher than the expansion of total cells.[4,5]

Figure 4 shows the expansion of LTC-IC as assessed by limiting dilution assay in these same experiments. In all cases, the LTC-IC population expanded between 4 and 10 fold. It should be noted that the increase in the number of LTC-ICs is fairly slow. In our experience, the doubling of this cell population is 80 to 100 hours. The cell cycle activity of LTC-ICs, however, may be much higher since only a fraction of the divisions lead to self-renewal. Many divisions will lead to differentiation. Direct time-lapse video observation of highly purified $CD34^+$ cells suggests that the time between the first two doublings of primitive cells is

on the order of 60 hours.[7] Thus, one can expand LTC-IC in culture, but their self-renewal rates are slow.

The endogenous production of growth factors in these cultures has also been monitored.[8] Figure 5 shows the concentration of G-CSF, MIP-Iα, IL-6, and LIF present in the bioreactors. G-CSF and MIP-lα were generated in high quantity during the first week of culture. Their concentration then decreased as a function of time. The concentrations shown were measured in the effluent stream (the spent medium) from the perfusion chamber. The drop in concentration during the second week of culture could either be due to increased consumption of the produced growth factor by other cells in the culture or by cessation of production itself. Based on these data, we cannot distinguish between the two possibilities. IL-6 also was produced early in culture and then declined. The concentration of IL-6 increased again in the second week of culture, presumably in parallel with the growth of stroma. LIF was produced only during week 2 of culture. LIF is known to be stroma-derived, so this production is probably related to the outgrowth of stroma. SCF is not provided in the fresh medium, it is produced in a similar fashion as LIF, except at a 10-fold higher concentration (Figure 6).

Thus, perfusion-based stromal-driven hematopoietic cell cultures are capable of producing a variety of growth factors endogenously. This attractive feature alleviates, or eliminates the need to provide many, but not all, the growth factors in the fresh medium. Further, these systems allow the stroma to produce what could be the physiologically desirable mixture of growth factors to drive the hematopoietic process. We have found that the perfusion-based cultures can expand hematopoietic stem cells from a variety of cell sources.[4,5,9,10] These sources include: 1) whole bone marrow; 2) bone marrow mononuclear cells; 3) CD34-selected cells; 4) 4-HC purged bone marrow; 5) mobilized peripheral blood; and 6) umbilical cord blood. For the first two sources, no performed stroma is necessary. Stroma forms from cells present in the inoculum. However, for sources 3) and 4), performed stroma is needed if LTC-IC expansion is desired.

IMPLEMENTATION IN THE CLINICAL SETTING

The desirable objectives of a clinically useful cell expansion system fall into two categories. In the first category are the requirements of the composition of the cell expansion product, while in the second

category are important issues with regard to implementation in the clinical setting.

In the first category, one needs to require the system to increase the number of hematopoietic stem cells and to produce cytokine-stimulated progenitor cell populations. Such populations derived from mobilized peripheral blood have been shown to lead to more rapid engraftment compared with standard bone marrow transplants.[11] In addition to the production of hematopoietic stem and progenitor cells, perfusion-based hematopoietic cell culture systems may be able to produce transplant-facilitating accessory cells. Lastly, such a system may be able to purge malignant contaminants from the harvested hematopoietic cell populations. Systems described in the previous section naturally purge cells of lymphoid origin, and we have found that tumor cells derived from certain B lymphocytic malignancies, such as chronic lymphocytic leukemia (CLL) decline in number during the cultivation process (Rummel, unpublished observations).

In the second category, there are several requirements that need to be met in order to implement this technology successfully in a clinical setting. These requirements include a closed automated system that can be operated by blood bank personnel and provide GMP cell processing procedures. All of these requirements add up to basic FDA approvable cell production in the blood bank setting.

The concept behind the perfusion-based bioreactor system and its implementation in the clinical setting is schematically shown in Figure 7. A small aspirate is obtained from the patient. This aspirate is inoculated into the bioreactor system where cell expansion takes place over a time period of approximately 12 days. Towards the end of this period, the patient undergoes chemo- and radio-therapy. Following the therapy, the cell expansion product is harvested from the bioreactors and transplanted intravenously at bedside. The procedures from aspiration to transplantation are a closed process carried out with instrumentation that can be installed in any blood bank and operated by blood bank personnel. The fully automated system allows tracking of every step in the process leading to complete documentation capability and thus attainment of GMP cell production procedures.

EX VIVO STEM AND PROGENITOR EXPANSION

Taken together, the material presented above represents a new approach to *ex vivo* cell expansion and bone marrow transplantation. The

system offers the advantage of growth of hematopoietic stem cells, (as measured by the LTC-IC assay), the growth of stroma, and the production of cytokine stimulated progenitor cells (in addition to being carried out in a blood bank setting). An automated stem and progenitor cell system would have significant impact on clinical practice if proven efficacious through clinical trials.

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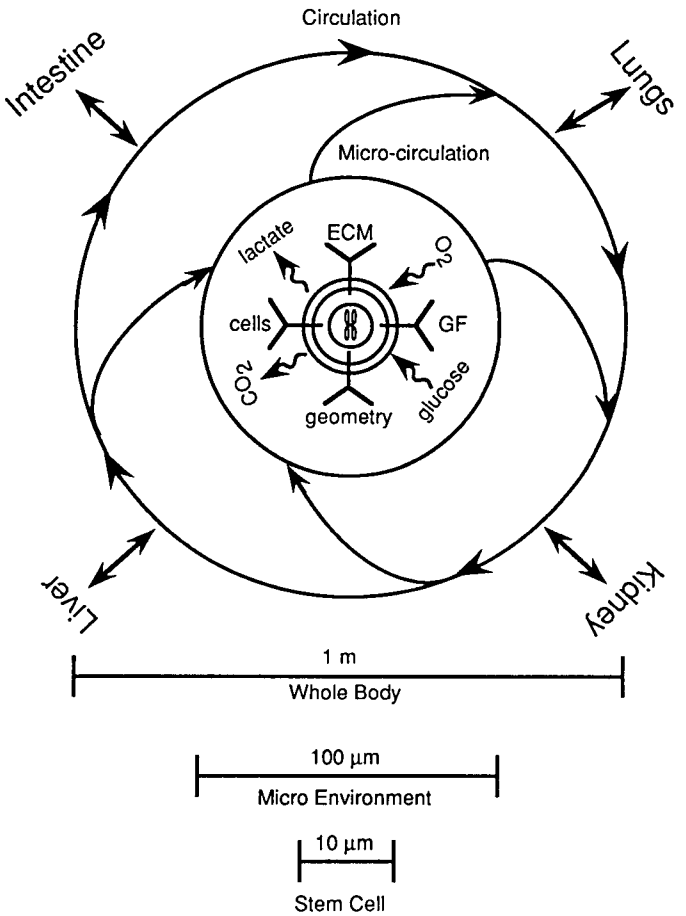

Figure 1. Schematic representation of a stem cell, its communication with its microenvironment, and other organs.

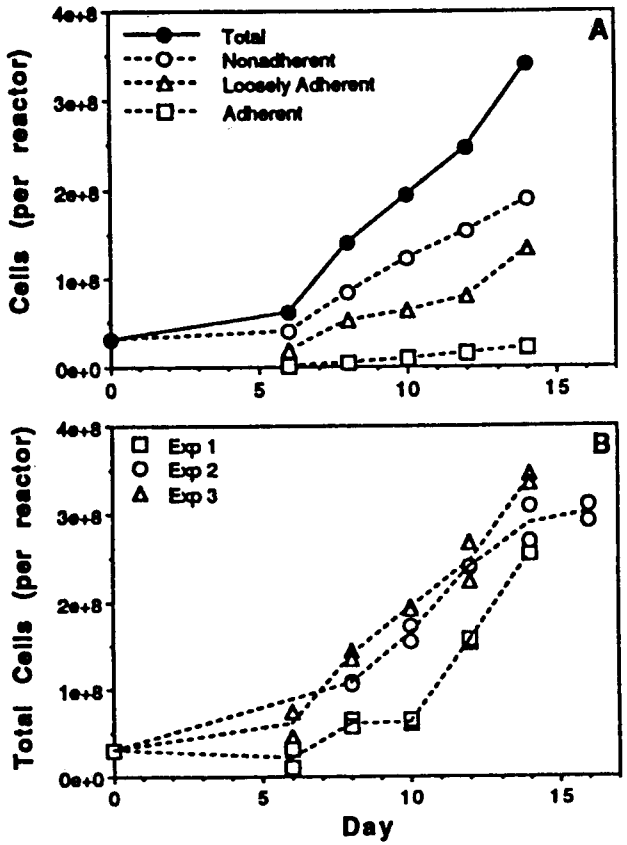

Figure 2. Cell numbers as a function of time in continuously perfused cultures. At each time point, the nonadherent, loosely adherent, and adherent cells were harvested from two bioreactors and MNC were counted. Cell numbers are shown per bioreactor resulting from an inoculum of $3x10^7$ BM MNC per bioreactor (A). Total cell numbers from each bioreactor in the three separate experiments are also shown (B). Source: Koller et al, Blood 82:378, 1993.

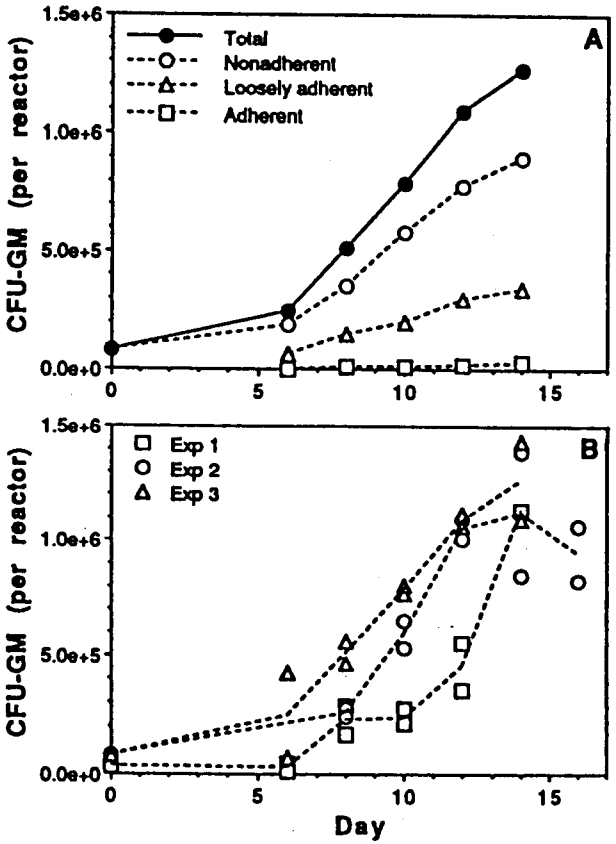

Figure 3. CFU-GM numbers as a function of time in continuously perfused cultures. At each time point, the cells harvested from two bioreactors were plated into methylcellulose colony assays. Nonadherent, loosely adherent, and adherent cells were assayed separately (A). Total CFU-GM numbers from each bioreactor in the three separate experiments are also shown (B).

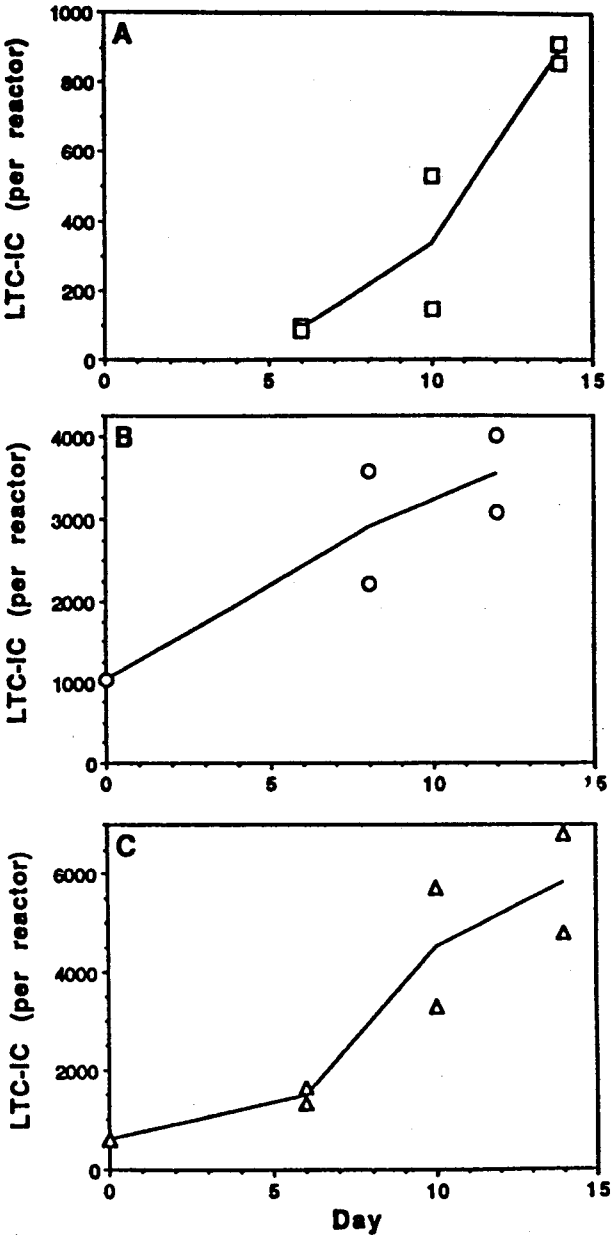

Figure 4. LTC-IC numbers as a function of time in continuously perfused cultures. At various time points in each of three experiments (A through C), the cells harvested from two bioreactors were plated into LTC-IC LDA. For each bioreactor, the number of LTC-IC was determined through an iterative calculation procedure based on the maximum likelihood method.

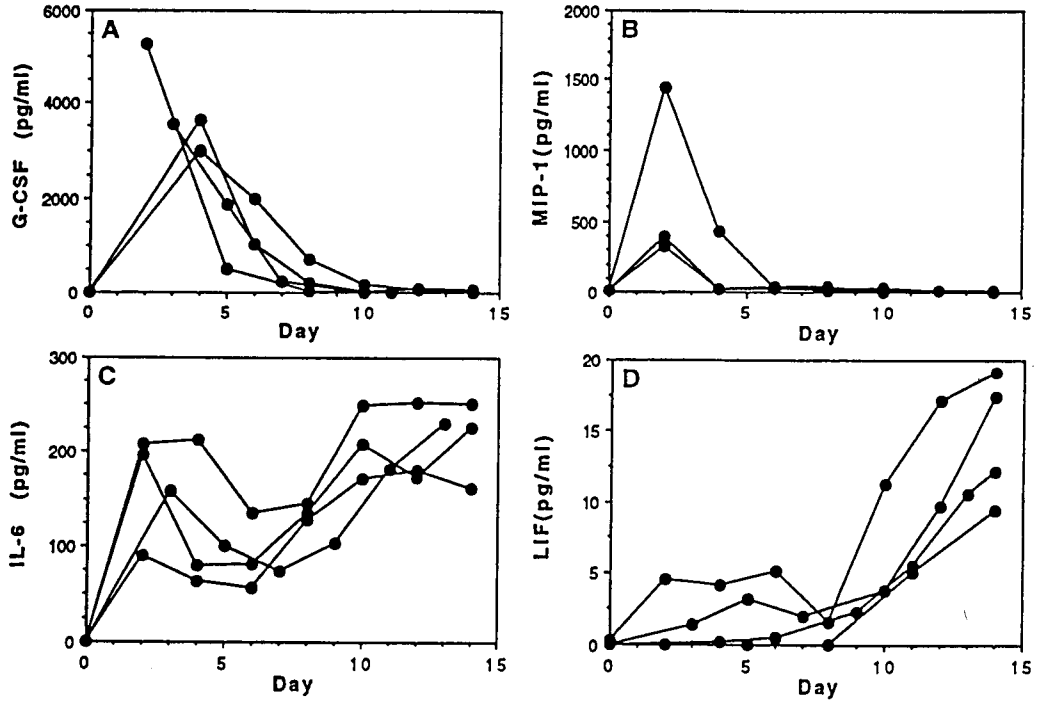

Figure 5. Endogenous production of growth factors in perfusion cultures of human bone marrow mononuclear cells. The exogenously supplied growth factors were SCF, Epo, IL-3 and GM-CSF.

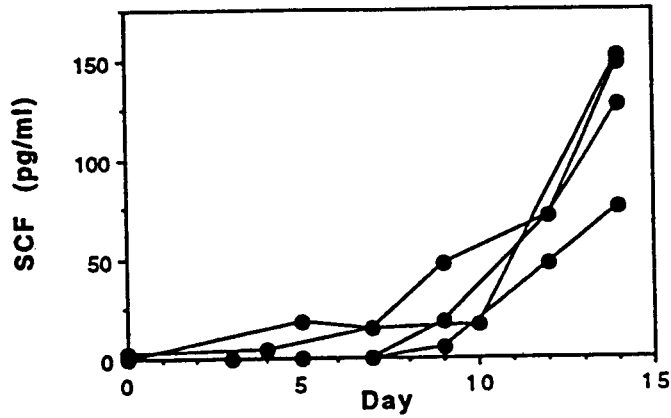

Figure 6. Endogenous production of stem cell factors in perfusion cultures of human bone marrow mononuclear cells. The exogenously supplied growth factors were Epo, IL-3 and GM-CSF.

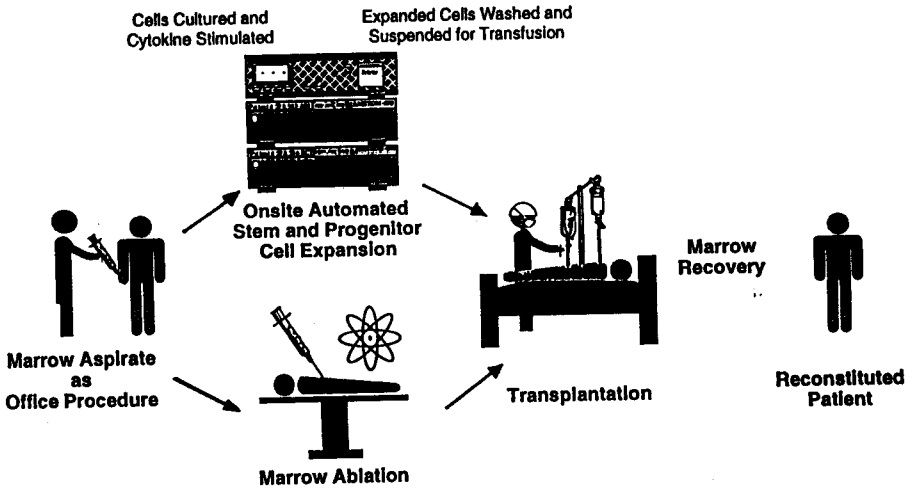

Figure 7. Ex vivo expansion of bone marrow for transplantation. First, a small hip aspirate is obtained from the patient. Mononuclear cells are obtained from the aspirate and inoculated into an automated cell expansion system that is located at the clinical site. The MNCs are grown while the patient undergoes chemo and radiotherapy. The expansion product, containing a transplantable dose of CFU-GM and an expanded number of LTC-ICs, is then transplanted into the patient. The expansion process is fully automated and meets GMP requirements.

EFFECT OF SHORT TERM INCUBATION WITH CYTOKINES ON ENGRAFTMENT FOLLOWING AUTOLOGOUS BONE MARROW TRANSPLANTATION

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INTRODUCTION

Refrigerated marrow has been shown to be equivalent to cryopreserved marrow with regard to time to blood count recovery following dose intensive therapy and autologous marrow transplantation.[1-4] The time to blood count recovery can be further accelerated by using cytokines and peripheral blood progenitor cells.[5-7] *Ex vivo* expansion of marrow cells has been performed in laboratory conditions.[8,9] We decided to evaluate the influence of various cytokines on marrow refrigerated at 4° C. In addition we evaluated the impact on time to blood count recovery following dose intensive, potentially myeloablative therapy, followed by infusion of refrigerated marrow incubated with sargramostim (Leukine®, GM-CSF), along with cryopreserved marrow and peripheral blood progenitors.

MATERIALS AND METHODS

Bone marrow was collected under aseptic conditions from the posterior iliac crest and mixed with heparin, tissue culture medium and ACD-A anti-coagulant solution. Mononuclear cells were isolated using Haemonetics V-50 or COBE CS 5000 cell processors. Aliquots of bone marrow mononuclear cells were incubated with varying concentrations and combinations of the cytokines GM-CSF, PIXY 321, interleukin-3 and c-kit ligand. These aliquots were refrigerated at 4°C and assayed daily for up to 7 days for CFU-GM, BFU-E and CFU-GEMM using semi-solid culture media.

Patients with breast cancer deemed to be at high risk of relapse on account of either supraclavicular or multiple (>10) involved lymph nodes or inflammatory cutaneous involvement had peripheral blood progenitor

cells mobilized using sargramostim collected by apheresis techniques and cryopreserved. Marrow was collected under aseptic conditions in the operating room under general or spinal anesthesia. Patients ere then randomly assigned to have 2.0 x 10^8 mononuclear marrow cells/kg body weight refrigerated following the addition of GM-CSF and have the remainder of the marrow preparation cryopreserved or have the entire preparation cryopreserved. Patients were then treated with ThioTEPA 750 mg/M^2, Mitoxantrone 50 mg/M^2, and Carboplatinum 1000 mg/M^2. To reduce the potential confounding effect of DMSO, a subgroup of patients receiving stimulated refrigerated marrow had this preparation infused before the infusion of cryopreserved products (Group 1) while the other (Group 2) received refrigerated stimulated marrow 24 hours later. All patients received sargramostim in the posttransplant period. Blood counts were checked daily and followed at least until WBC and platelet count recovery. All patients received fluconazole and acyclovir as fungal and viral prophylaxis. Systemic antibiotics were administered for episodes of febrile neutropenia. Platelet transfusions were given for platelet counts below 20,000 cells/mm^3 and red cells were given for blood hemoglobin of <10 G/dl. All patients received irradiated blood products. Granulocyte transfusions were not given either prophylactically or therapeutically. All patients signed written informed consent approved by the Institutional Review Board of New York Medical College.

RESULTS

Effect of Cytokines on Refrigerated Marrow

Various cytokines were able to expand cell populations as measured in terms of nucleated cell counts, cell viability as determined by trypan blue exclusion, and by CFU-GM/BFU-E assays for up to 5-7 days. Interestingly most of the expansion in cell numbers occurred in the first 3-5 days. Cell populations could rarely be maintained beyond numbers seen after cryopreservation ("freezing range") beyond 7-9 days. Combinations of cytokines worked better than individual cytokines. A combination of stem cell factor (c-kit ligand), interleukin-3 and GM-CSF resulted in the greatest expansion. Figures 1 & 2 graph the effects of various cytokines on refrigerated marrow.

Clinical Results

Forty-two patients with breast cancer had marrow and stem cells collected. Of these, 28 received stimulated refrigerated marrow in addition

to cryopreserved marrow and stem cells while seven patients served as controls. Tables 2 and 3 summarize the number of marrow and stem cells infused and the results of progenitor cell assays on aliquots of the product being given. While the total number of mononuclear cells and CFU-GM given to patients was higher in the group receiving stimulated refrigerated marrow, it is interesting to note that the time to blood count recovery and transfusion requirements were identical in both groups (Table 4).

DISCUSSION

Methods of *ex vivo* expansion have been evaluated extensively of late. Various culture systems have yielded several hundred fold expansion of progenitor cells as determined by various assays. It is possible that the expansion noted may be more of a reflection of growth of progenitor cells downstream from true stem cells. It is unlikely that *in vitro* expansion results in expansion of earlier as opposed to late progenitors. It is intuitively unlikely that later progenitors would lead to expansion of the pool of earlier stem cells. Since blood count recovery may be more dependent on earlier progenitors, *in vitro* expansion techniques that expand the pool of earlier progenitors need to be developed. Most of these studies, including ours, have not looked at subgroups of phenotypically CD34-positive cells. It is possible that different subpopulations of progenitor cells may lead to differences in time to engraftment and transfusion requirements. Thus the clinical impact of expansion of certain populations may be advantageous compared to other populations.

A number of *in vitro* expansion techniques are being evaluated. The ideal system has not been defined. It is likely that combinations of earlier and later acting cytokines would be advantageous. Similarly, systems using breathable bags or continuous feeding into the culture system are likely to be useful. While we were able to administer larger quantities of assayable progenitor cells, there did not appear to be a hastening in blood count recovery. It is possible that one may need logarithmically higher quantities of progenitors in order to see clinical impact.

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Table 1.

	Stim/Stem 1 7 day	vs	Stim/Stem 2 8 day
Day 0	Stimulated BM		Cryopreserved BM/PSCH
Day 1	Cryopreserved BM/PSCH		Stimulated BM

Hypothesis: Reduced DMSO toxicity to stimulated BM cells if they are infused 24 hours following infusion of thawed HSC.

	Preincubated/Liquid stored Stim/Stem 1		Control 1	
	n = 19		n = 7	
	Median	(Range)	Median	(Range)
Patient Information				
Age	42	(30-59)	40	(51-21)
Time from Dx to BMT	210	(187-578)	207	(141-666)

	Control 2		Preincubated/Liquid stored Stim/Stem 2	
	n = 7		n = 9	
	Median	(Range)	Median	(Range)
Patient Information				
Age	40	(51-21)	40	(22-46)
Time from Dx to BMT	207	(141-666)	216	(135-648)

Table 2.

	Preincubated/Liquid stored Stim/Stem 1		Control 1	
	n = 19		n = 7	
	Median	(Range)	Median	(Range)
Nucleated Cell Dose/kg				
Liquid Stored/Stimulated	2.00	(1.05-3.00)		
Cryopreserved BM	2.20	(0.86-4.83)	4.64	(2.16-3.60)
Cryopreserved PBSC	5.53	(0.98-10.42)	4.64	(2.20-14.20
Total cell dose/kg	9.55	(5.61-12.65)	7.72	(4.95-17.80)
	Control 2		Preincubated/Liquid stored Stim/Stem 2	
	n = 7		n = 9	
	Median	(Range)	Median	(Range)
Nucleated Cell Dose/kg				
Liquid Stored/Stimulated			2.00	(1.80-2.70)
Cryopreserved BM	2.74	(2.16-3.60)	2.96	(0.30-3.79)
Cryopreserved PBSC	4.64	(2.20-14.20)	5.15	(3.30-21.66)
Total Cell Dose/kg	7.72	(4.95-17.80)	9.91	(8.47-24.36)

Table 3.

	Preincubated/Liquid Stored Stim/Stem 1 n = 19		Control 1 n = 7	
	Median	(Range)	Median	(Range)
Progenitor Cell dose/kg				
Liquid Soted/Stimulated (x1e4)	3.9	(0.14-11.0)		
Cryopreserved Bm (x1e4)	2.4	(0.15-17.0)	1.2	(0.5-54.0)
Cryopreserved PBSC (x1e4)	2.2	(0-35.0)	7.1	(2.8-8.6)
Total CFU-GM dose/kg	9.7	(2.00-41.0)	1.5	(0.5-14.0)

Table 3 (cont'd.)

	Preincubated/Liquid Stored Stim/Stem 1 n = 19		Control 1 n = 7	
	Median	(Range)	Median	(Range)
Recovery Data				
Units of RBC	5	(2-9)	4	(2-14)
Units of Plt	36	(12-146)	41	(15-90)
Days to RBC independence	12	(5-18)	9	(7-45)
Days to Plt independence	15	(9-27)	16	(12-39)
Days to WBC >1000	13	(9-14)	11	(11-19)
Days to Plt >20,000	13	(5-18)	20	(12-41)

	Control 2 n = 7		Preincubated/Liquid Stored Stim/Stem 2 n = 9	
	Median	(Range)	Median	(Range)
Nucleated Cell dose/kg				
Liquid Stored/Stimulated			2.00	(1.80-2.70)
Cryopreserved BM	2.74	(2.16-3.60)	2.96	(0.30-3.79)
Cryopreserved PBSC	4.64	(2.20-14.20)	5.15	(3.30-21.66)
Total Cell dose/kg	7.72	(4.95-17.80)	9.91	(8.47-24.36)

	Control 2 n = 7		Preincubated/Liquid Stored Stim/Stem 2 n = 9	
	Median	(Range	Median	(Range)
Recovery Data				
Units of RBC	4	(2-14)		
Units of Plts	41	(15-90)		
Days to RBC independence	9	(7-45)		
Days to Plt independence	16	(12-39)		
Days to WBC >1000	11	(11-19)		

Table 4.

	Preincubated/Liquid Stored n = 9 Median (Range)	Control n = 8 Median (Range)	p value
Pt. information			
Age	42 (31-51)	40 (52-21)	
Time from Dx to BMT	210 (187-578)	207 (141-666)	
Cell Dose			
Liquid Stored/Stimulated	2.25 (1.25-3.00)	NA (0.00-0.00)	
Cryopreserved BM	2.4 (0.00-4.03)	2.74 (2.16-3.60)	
Cryopreserved PBSC	5.55 (2.78-10.42)	4.64 (2.20-14.20)	
Total cell dose	8.99 (5.90-12.65)	7.72 (4.95-17.80)	0.507
Total Volume	1859 (1331-2308)	1102 (846-2349)	
Recovery data			
Units of RBC	4 (2-9)	4 (2-14)	0.354
Units of Plts	36 (12-146)	41 (15-90)	0.686
Days to RBC independence	15 (5-18)	9 (7-45)	0.479
Days to plt independence	15 (9-27)	16 (10-39)	0.308
Days to WBC >1000	12 (11-14)	11 (9-19)	0.618
Days to plt >20,000	16 (9-27)	20 (10-41)	0.125

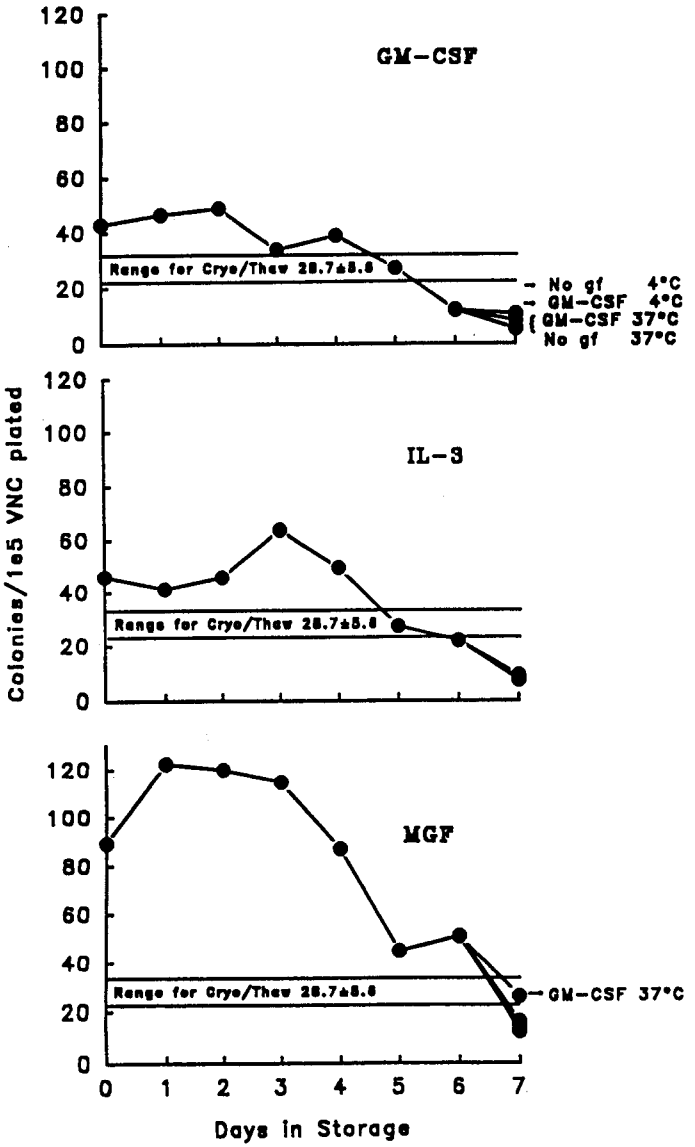
GM-CSF
IL-3
MGF
Range for Crye/Thaw 28.7±8.8
No gf 4°C
GM-CSF 4°C
GM-CSF 37°C
No gf 37°C
Colonies/1e5 VNC plated
Days in Storage
0
1
2
3
4
5
6
7
20
40
60
80
100
120

Figure 1.

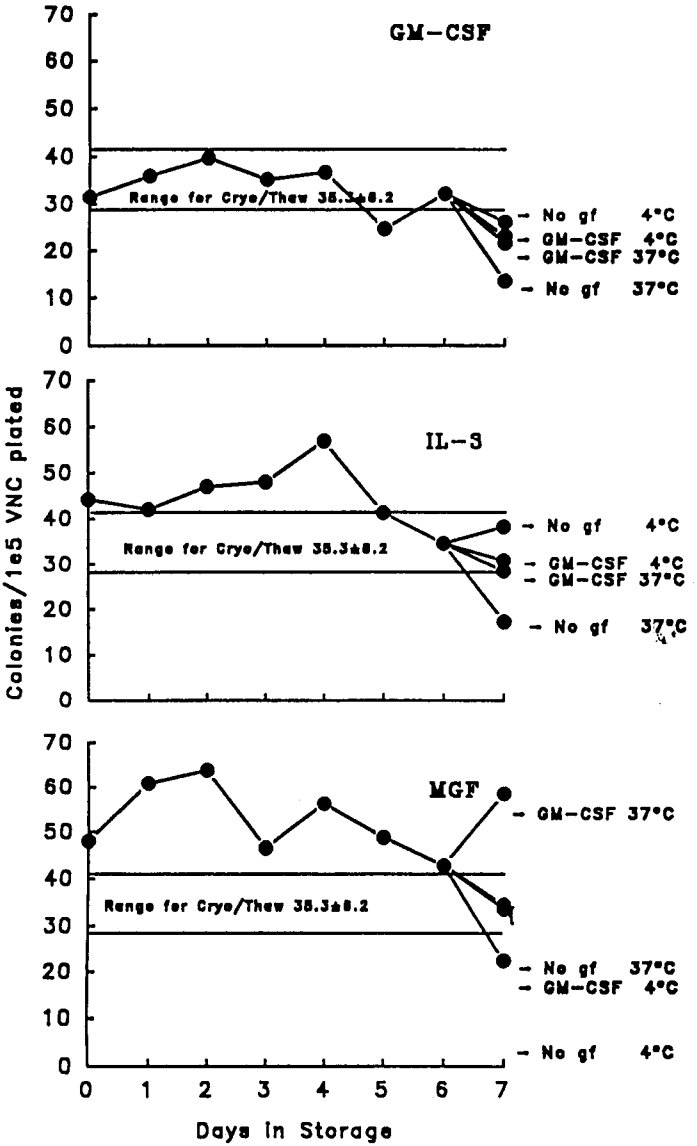
GM-CSF
IL-3
MGF
Range for Crye/Thaw 38.3±8.2
No gf 4°C
GM-CSF 4°C
GM-CSF 37°C
No gf 37°C
Colonies/1e5 VNC plated
Days in Storage
0
1
2
3
4
5
6
7
10
20
30
40
50
60
70

Figure 2.

THE GRAFT-VERSUS-LEUKEMIA (GVL) REACTION: ITS EARLY HISTORY, VALUE IN HUMAN BONE MARROW TRANSPLANTATION AND RECENT DEVELOPMENTS CONCERNING ITS MECHANISM AND INDUCTION

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HISTORY

When James B. Murphy in 1916 at the Rockefeller Institute in New York City inoculated the chorioallantois membrane of developing chicken embryos with fragments of adult chicken organs, among them spleens, he observed rapid enlargement of the splenic implants and spread of splenic tissue in the form of nodules within the membrane and in the embryo itself especially in the fetal spleen while many of the embryos died during the process. He could not give an explanation for this spectacular phenomenon.[1] It befell Morten Simonsen of Denmark[2,3] and Macfarlaine Burnet of Australia[4] some 40 years later to decipher the mechanism and conclude that the histoincompatible immunocompetent graft mounted an immune reaction against its immunoincompetent recipient: a graft-versus-host reaction (GVHR). By then it was known from the works of Billingham, Brent and Medawar[5,6] that homologous lymphoid tissue transplanted into histoincompatible neonatal mice can immunologically attack the recipient and cause an often fatal disease termed later homologous, allogeneic, secondary or runt disease (identical with graft-versus-host disease).

An entirely similar condition ("secondary disease") ensued in leukemic rodents treated with total body irradiation and bone marrow and spleen cell rescue.[7,8] The antileukemic effects of this condition were not immediately recognized even though Murphy in 1916[1] observed that rat tumor implants that could grow on the chorioallantois membrane of otherwise untreated chicken embryos would perish if adult chicken spleen fragments were co-implanted and let spread throughout the embryo (see above); and Woodruff et al[9,10] showed early that solid and ascitic rat tumors receded in animals that were made recipients of homologous lymphoid cells (often derived from the thoracic duct) and thus were

induced to develop homologous graft-versus-host disease (GVHD). For example, when Georges Mathé in Villejuif, France observed GVHD in his leukemic patients treated with whole body irradiation and allogeneic bone marrow transplantation, he exclaimed that a major "stumbling block" in the way of successful therapy had been encountered.[11] Consequentially extraordinary efforts were and still are (see below) initiated to prevent, treat and eliminate GVHD.

Soon thereafter the antileukemic effects of GVHD were clearly documented in mice. The abstract presented at the AACR meeting in 1963 in Toronto is the first English language report describing this phenomenon (see below).[12] The first English report of Mathé et al on the graft-versus-leukemia reaction (GVLR) appeared in 1965.[13] This article gives reference to a previous French publication[14] that probably contains the very first hint at possible antileukemic effect of "secondary disease" in AKR mice but this publication was not known to this author until 1965. Likewise, Mathé et al were not aware of the abstracts presented by this author contemporaneously[12] even though they worked with Friend virus-induced leukemia which is very similar to Rauscher virus-induced leukemia the subject of study by this author. The results of these two independent and contemporaneous studies are entirely similar.

By the late 1960s, the beneficial antileukemic effects of the GVLR were allowed to be exerted in mice but halted short of causing irreversible damages to the host. Boranic[15] and Bortin et al[16,17] used anti-lymphocyte sera and cyclophosphamide to stop GVHD (and inadvertently GVLR). Treatment of GVHD in tumor-bearing mice could result in decreased antitumor effect; untreated GVHD could kill the host. In some systems GVLR continued without GVHD.[18]

A major further development in adoptive immunotherapy with (or without) GVHD was achieved by the use of lymphoid cells derived from donors immunized against the tumor (leukemia-lymphoma) in question or against the causative virus thereof. In Mathé's report[13,19] the median survival of control mice inoculated with Friend leukemia virus was 83 days. Mice with Friend virus-induced leukemia treated with whole body irradiation and isogenic marrow cells survived only 52 days. The median survival of mice with Friend virus-induced leukemia treated with irradiation and isogenic marrow cells derived from Friend virus immune donors survived 163 days. An earlier report of these results was published in French.[19] Alexander et al reported successful adoptive immunotherapy of mouse leukemia with immune spleen cells and sera.[20] Independently at the same time this author treated mice with Moloney or Rauscher virus-

induced leukemias with whole body irradiation and bone marrow and spleen cells of mice actively immunized (with live attenuated or killed leukemia viruses) against these leukemias and succeeded in significantly prolonging the duration of leukemias (delaying death) or rendering some of the leukemic mice leukemia-free (vide infra).[21-23] These data however were not recognized 3 to 7 years later in reports re-establishing the therapeutic value of leukemia-lymphoma-immune lymphoid cells given with or without cyclophosphamide.[24-28]

When in 1962 it had become evident that adult Swiss (TIMCO; Balb/c) white mice were exquisitely susceptible to Rauscher's viral mouse leukemia (MLV), while adult C57 black mice displayed resistance to it, experiments were designed at MD Anderson Hospital to determine if "chimeric" mice could be rendered-resistant to MLV. Newborn Swiss mice inoculated with hemato- and lymphopoietic (He-Ly) cells of young adult C57 mice developed fatal homologous (allogeneic or "runt") disease (RD)[29] and could not be readily tested for leukemogenesis. By reducing the number of inoculated C57 cells, a more chronic form of RD could be induced. In this condition, exact and reproducible retitrations of MLV revealed significantly lower titers in mice with RD (in comparison to healthy controls inoculated with the same dose MLV). Consequently, virally induced leukemias advanced very slowly and leukemic deaths of mice with RD were significantly delayed.[12,22,23] Example: "Chronically runted Balb/c mice succumbed to Rauscher virus leukemia after an average time of 168 days, while control Balb/c mice inoculated with the same dose of Rauscher virus succumbed to leukemia after 61 and 80 days." In another system, tolerance to adult C57 cells was induced in newborn Swiss mice by inoculating them first with disrupted fresh and thereafter with viable He-Ly cells of adult C57 mice. A chronic form of RD resulted with less than 10% death rate over 120 days. In this system small MLV inocula failed to induce leukemia and if leukemia was induced it progressed extremely slowly. These mice were able to produce antibodies neutralizing the MLV. These experiments were reported between 1962-1965 and were reviewed in 1970 and 1976.[30] These data were presented in details in monograph Harris JE & Sinkovics JG, *The Immunology of Malignant Disease*. Mosby, St. Louis, Tables 2.2, 2.7, 2.8; pp 169-171, 209, 211; 1976. The phenomenon was not referred to as "GvL reaction" but it had been emphasized already in 1963 that chronic allogeneic disease was inhibitory to leukemogenesis: "...lymphocytes show hostility toward the leukemic cells. We suspect that the lymphocytes represent the resistant C57 black component of the hematopoietic system

of these mice; the activity of these lymphocytes may consist of an attempted rejection of the susceptible leukemic Balb/c component of the chimeric hematopoietic system."[31] It was not possible to foresee or guess these results in advance: GVHD could have been envisioned as a form of immunodeficiency[32] and accelerated leukemogenesis might as well have been expected: careful experimentation showed it otherwise. On occasions, it was not easy to distinguish the histopathology of GVHD from that of incipient or regressing leukemia but comparative retitrations of the leukemia virus from tissue extracts could be done accurately and survival times of mice could be recorded with precision.

In later experiments with inocula of newborn C57 cells given to newborn Swiss mice; or with inocula of adult C57 cells deriving from donors rendered tolerant to Swiss cells and given to Swiss mice tolerant to C57 cells,[33,34] leukemogenesis (as measured in a spleen focus assay) was reduced in its incidence and delayed in its course but without RD in some of these systems: especially when recipients were tolerant to donor and donors were preimmunized against leukemia. Thus, GVLR without GVHD was also observed and reported at a very early time (1968-1970).[34] In the high leukemia AKR mice, RD was induced by C57 spleen and lymph node cells; survivors of RD showed 4-fold decrease of leukemia incidence.[34]

The purpose of this publication is to direct attention to these pioneering studies, by now entirely forgotten, and to briefly review the value of GVLR in human bone marrow and stem cell transplantation as its mechanism now unfolds thus enabling us to induce GVLR independently from GVHD.

Value in human BMT. The great therapeutic value of GVLR in human bone marrow transplantation (BMT) has now been repeatedly documented. GVHD and within it GVLR or GVLR independently from GVHD are now considered to be an obligatory contribution to sustained remission without relapse.[35-43]

Pathophysiology of GVHD. Disparity between MHC antigens of donor and recipient induces the reaction.[44] CD8 cells of the donor now residing in the recipient will recognize and react to MHC class I and minor histocompatibility antigen discrepancies. CD4 cells of the donated graft will react to MHC class II incompatibilities. Alloactivation of CD4 and 8 cells takes place promptly with rapid clonal expansion of the reactive cells under the influence of IL-2. In the acute phase of GVHR tumor necrosis factor is the dominant circulating cytokine. Its origin (from lymphocytes or macrophages) is not clear. It is also debated if direct donor lymphocyte-

mediated recipient target cell killing occurs and, if it happens, what is the share of IL-2 activated NK cells (LAK cells) and immune T cells in the process. Large granular lymphocytes (NK cells) of donor origin appear to be numerous and active in the recipients of bone marrow transplants. If CD4 & 8 cells initiate it, it appears as if NK cells would carry out GVHD. Human NK cells do not rearrange by somatic gene recombinations their receptors to generate receptor diversity and specificity[45]like classic B or T cells do; instead NK cells possess receptors for and thus recognize Class I MHC antigens. Recipients in turn are able to carry out cytotoxic response to allogeneic lymphocytes and bone marrow cells.

Epidermal damage in the skin is connected with the appearance of another subclass of lymphocytes (thy-1,$^+$ CD2,$^+$ CD4,$^+$ CD8,$^-$ CD3,$^-$ NK 1.1$^-$). The majority of these cells are CD4$^+$ TCR $\alpha\beta$ recognizing HLA-associated antigens.[46] In chronic GvH disease IL-1,2,4, TNF and IFN-γ are elaborated[47]and while exogenous IL-1 exacerbates, IL-1R antagonist alleviates the manifestations of GVHD.[48]

In patients with acute and chronic GVHD, a cytokine cascade dominated by IL-6, TNF and IFN-γ has been recognized in a sequence suggesting an initiator role for IL-6 and IFN-γ and a potentiator role of TNFα. Occasionally these cytokines appeared in the blood without clinical manifestations of GVHD (suggesting the involvement of as yet unrecognized other factors).[47] Antibody production occurs in chronic GVHD: neutralizing antibodies were produced against MLV[23] and an array of autoantibodies are also produced simulating close resemblance of chronic graft-versus-host (GVH) to collagen diseases.[49]

Histologically, selective epithelial cell damage is highly characteristic of GVHD; this occurs with a paucity of mononuclear cell infiltrates. Undifferentiated epithelial cells with embryonic-type membrane structure appear to be targeted the most (intestinal crypts; hair follicles; nail beds; mucosal linings).[44] The same structures appear to be damaged by chemotherapeutic agents. This peculiar preference in tissue damage in GVHD suggests that GVHD induced for the treatment of certain adenocarcinomas may turn out to be useful in the future.

Another function of donated cells is enhancement of engrafting: T cell-depleted bone marrow grafts often fail to take (and when they do they do not cause GVHD). It appears as if among other functions donor T cells exert suppression function in that recipient's lymphocytes and will not reject the graft. The recipient can mobilize T cells cytotoxic to minor histocompatibility antigens of the donor.[50] Removal from the graft of cytotoxic T cells and NK cells does not deplete the graft's ability to

enhance its engraftment. GVHD exerts harmful effect on the hematopoietic stem cell supply measured as colony forming units of both donor and recipient marrow.[51]

Transplantation of syngeneic, or reinfusion of autologous bone marrow can induce GVHD! In this condition, autoreactive T cells directed at MHC class II antigens are generated consequentially to loss of"negative selection" in damaged thymic stroma. Over 20 years, thymic irradiation (van Bekkum's "isologous secondary disease"), nowadays cyclosporin A cause this condition and interferon-α promotes its development. Cyclosporin A is the most prominent inducer of this condition. Interferon-γ promotes the reaction by virtue of upregulating antigen expression; IL-2 amplifies the reaction by effecting expansion of the reactive T cell clone(s).[52] Syngeneic/autologous GVHD induced in patients was observed to exert significant antitumor activity against high grade lymphomas and breast carcinomas.[52,53]

When cytotoxic cells (T lymphocytes, NK cells, monocytes) are removed from donor marrow with the lysosomotropic compound L-leucyl-L-leucine CH_3-ester, the human recipient will be exempted from GVHD.[54] A ricin-coupled rat monoclonal antibody FD441.8 immunotoxin directed at the LFA1α chain (CD11a) inhibits the afferent limb of GVHD in blocking cytotoxic T cells and NK cells by removing these cells from the donated human marrow.[54] In a murine system, $CD4^+$ cells were effective; $CD8^+$ cells were ineffective in inducing GVHD. When given to the recipients within a few days after transplantation, an IgG2b anti-Thy-1 monoclonal antibody prevented GVHD.[55]

Of lymphokines, the IL-1R antagonist prevented GVHD in mice.[48] IL-2 acts according to the timing of its administration to bone marrow recipients. In mice administration of IL-2 delayed one week after transplantation exacerbates GVHD whereas IL-2 given immediately after transplantation prevents GVH.[56] Table 1 summaries those agents that are commonly used to treat recipients of allogeneic bone marrow in order to prevent GVHD.[44,57,58] Transplantation of allogeneic T cell-depleted human marrow after pretransplantation conditioning with CD3 cell depletion and cylosporin without GVHD-induction has been accomplished.[59] In the mouse in one form of GVHD donor T_{86} and T_{88}.1 cells expand but their removal from the graft prevents GVHD.[60]

Selective *ex vivo* depletion of $CD8^+$ T cells from allogeneic human donor marrow (with CT-2 or anti-Leu2 monoclonal antibody and complement) prevented GVHD in the recipients.[61] Bone marrow to be transplanted can be treated with Campath-1 (CDw52) antibodies (IgM and

IgG2b) for depletion of T cells that initiate GvH disease in the recipient.[62,63] Treatment of the recipient with cyclosporin A reduces graft failure. XomaZyme H65-CD5-ricin immunotoxin depleted T cells from the donated marrow *in vitro* and when given *in vivo* posttransplant to recipients it prevented graft rejection.[64] However, recipients displayed immune defects toward viral infections and often succumbed to cytomegaloviral infections.[64]

Table 1. Chemoprevention of GVHD in the Recipient

Drug	Mechanism of action
Glucocorticoids	Induce apoptotic death of reactive lymphocytes. Inhibit IL-1 production by Ag-presenting cells
Methotrexate	Inhibits clonal expansion of reactive T cells
Cyclophosphamide	Inhibits clonal expansion of reactive T cells. Recruits new T cell population sin cell cycle
Cyclosporin A	Antagonizes IL-2 production

Effective treatment of established GVH is very difficult. Corticosteroids and methotrexate often fail and 2-chlorodeoxyadenosine is much more toxic than effective. Antilymphocyte antibodies remain the most promising. Table 2 lists antibodies commonly used for the treatment of GVHD.[65-67] Even IV given commerical gamma globulin may effectively ameliorate GVHD.

Table 2. Antibodies for the Treatment of GVHD

Antibody	Effects Directed To
OKT3, monoclonal	CD3 (CD3-TCR complex)
BMA031 murine IgG2b	TCRαβ
BT563 murine IgG1	IL-2R. Prevents organ rejection
Investigational monoclonal antibodies are being developed.[44]	TNF and TNF-R

The immunosuppressive macrolide FK506 and deoxyspergualin will probably be more effective for prevention than treatment.[57,68] The macrolide lactone FK506 isolated from Streptomyces tsukubaensis blocks calcineurin and through this mechanism inhibits T cell activation to the degree that it allows allografts to take in nonmatched recipients. In addition to inhibition of rejection of transplanted bone marrow, FK506 also inhibited with or without methotrexate GVHD.[68]

The ideal GVLR. Swiss mice actively immunized with MLV vaccines (attenuated or inactivated) developed antibodies neutralizing MLV or lysing with C' leukemic cells. These mice yielded He-Ly cells protecting Swiss mice against induction of leukemia or leukemic Swiss

mice (leukemia induced by Moloney or Rauscher MLV) against death and reinduction of leukemia when given after 800r radiotherapy.[33,69,70] Leukemic cell colonies in these mice showed morphological features of programmed cell death (apoptosis) as shown in pictures published[70] but this was not presented as such at that time (1966) and has just recently been recognized upon review of this material. Separation of GVHD and GVLR was first accomplished in mice with cross-tolerance between donors and recipients; in this system leukemia-immune donor cells gave the highest protection to recipients against viral leukemia.[34]

Apparently immune T cells turned against leukemia-specific antigens carry out GVLR. Host-specific and leukemia-specific cytotoxic T cells can be separated.[71-73] This phenomenon receives extraordinary attention,[39-42] nevertheless the ideal GVLR cannot be accomplished in the human setting: neither cross-tolerization between donor and recipient nor active leukemia-specific preimmunization of the donor is possible. Once human leukemia-specific antigens (viral and others) will be identified and made available in purified form, donors actively immunized with these antigens are expected to yield populations of immune T cells that react specifically with leukemic cells surviving high dose chemotherapy in the recipient. Thus, the ideal GVLR that was induced in mice 30 years ago will be possible to induce also in patients.

Chronic myelogenous leukemia (CML) Ph^{1+} with t(9;22)* could possibly be the first neoplasm to be treated with an "ideal" GVLR. Here the donor could be preimmunized with the *bcr-abl* fusion oncoprotein. Leukemia cell-specific immune T cells thus generated in the donor would then be transferred into the recipient with the graft. In this condition, removal of immunosuppression (for graft acceptance; for induction of GVHD) or cessation of chemotherapy[74,75] and additional posttransplantation infusion of donor leukocytes into the recipient with IL-2 administration[76-80] promptly induces GVH (and GVL) reactions. Also patients with CML can generate autologous leukemia cell-specific T cells.[81] Even though there will be no cross-tolerance between donor and recipient in CML, leukemia-immune T cells exist and their efficacy could possibly be intensified. This principle, if feasible, may gain wider application in the adoptive immunotherapy of malignant tumors.

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*Translocation and fusion of genes between 9q34 (c-*abl*) and 22q11 (*bcr*) creating fusion protein $p210^{BCR/ABL}$ with increased tyrosine kinase and anti-apoptotic activities. This oncoprotein is weakly immunogenic in patients with CML![82]

SUMMARY

Early contributions of our laboratory to the knowledge of GVH and GVL reactions have been recapitulated: 1) observation of the antileukemia effect of GVHD in 1962-3: the GVLR; 2) use of leukemia-immune donor cells to rescue leukemic recipients treated with whole body irradiation in 1964-6; and 3) prevention and treatment of leukemia without GVLR in recipients tolerant to donors which are tolerant to recipients and yield leukemia-immune cells (1968-69).

Here we propose the use of the GVHR against adenocarcinomas basing this recommendation on the strong targeting of rapidly proliferating epithelial cells in GVHD. We propose the ideal GVLR for the treatment of CML: donors preimmunized with the oncoprotein BCR-ABL could yield leukemia cell-immune cytotoxic T cells and this population could be further expanded in the recipient with administration of IL-2.

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SESSION V: LYMPHOMA

ANTI-B CELL MONOCLONAL ANTIBODY TREATED AUTOLOGOUS BONE MARROW TRANSPLANTATION IN PATIENTS WITH LOW GRADE NON-HODGKIN'S LYMPHOMAS

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INTRODUCTION

The overwhelming majority of patients with low grade non-Hodgkin's lymphomas (NHL) are not curable with current employed treatment approaches.[1,2] The rationale for the use of high dose ablative therapy in low grade NHL is based upon the fact that relapsed patients can continue to respond to further conventional treatment and salvage regimens. However, a continuous rate of relapse continues to be observed with salvage regimens. As had been previously demonstrated for relapsed/refractory NHLs, resistant disease could be overcome with dose intensification with high dose therapy, followed by allogeneic and autologous bone marrow transplantation (ABMT).[3] However, in contrast to patients with relapsed intermediate/high grade NHL, this approach has been used in a relatively limited number of patients with low grade NHL.[4-9] These studies suggest that a subset of patients with relapsed low grade NHL may benefit from high dose therapy and bone marrow transplantation. Another application of high dose therapy is for early consolidation of first remission in patients with intermediate/high grade NHL who have a poor prognosis when treated with conventional therapy alone.[10] Since patients with advanced stage low grade NHL are incurable with conventional therapy, the early use of ABMT may lead to improved disease-free survival (DFS) and survival in these patients.

In the present study we report on the results of high dose therapy and anti-B cell monoclonal antibody treated ABMT in patients with low grade B cell NHL. The studies to be summarized will include ABMT for patients with relapsed disease as well as in first remission.

METHODS

Selection of patients and treatment protocol. Patients were eligible for this study if they were less than 65 years of age; had relapsed low grade NHL as defined by the International Working Formulation, after standard chemotherapeutic regimens; and had lymphoma cells that expressed the CD20 (B1) antigen as previously described.[8] In addition, patients with sensitive low grade NHL but who had failed to enter complete remission after one or more standard chemotherapeutic regimens were eligible. For all patients, a minimal disease status had to be attained through chemotherapy, radiotherapy, or both prior to entry. This status was defined as lymph nodal mass less than 2 cm in its greatest diameter and histologic evidence of bone marrow involvement of 20% or less of the intratrabecular space as determined by iliac crest biopsy. Additional criteria for entry included the absence of comorbid disease of the heart, kidney, lung, and liver and a Karnofsky score above 80 percent. Informed consent was obtained from all patients. For the patients undergoing upfront ABMT, they had to be less than age 55, have stage IIIB, IIIE, or bulky stage III (>10 cm) or stage IV follicular small cleaved cell, follicular mixed or small lymphocytic NHL. These patients were uniformly treated with CHOP for 6-8 cycles and if achieving minimal disease state, underwent ABMT as described below.

Preparative therapy consisted of cylophosphamide, 60 mg/kg of body weight, infused on each of two consecutive days before radiotherapy. TBI was administered in fractionated doses (200 cGy) twice daily on three consecutive days (total of 1200 cGy) in all patients. Supportive care was provided as previously described.[8]

Collection, processing, and infusion of marrow. Bone marrow was obtained, treated *in vitro* as previously described.[8]

Evaluation and statistical analysis. Before treatment, all patients were evaluated by physical examination, blood-chemistry profile, complete blood count, chest x-ray, abdominal-pelvic CT scanning (chest CT if indicated), bone marrow aspirate and biopsy, as well as cell surface phenotypic studies of peripheral blood and bone marrow mononuclear cells. Other studies such as gallium scanning were done as needed to determine the extent of disease. Follow-up restaging was carried out every 6 months after transplantation or as clinically indicated.

Complete remission (CR) was defined as the disappearance of all measurable and evaluable disease. Failure were defined as relapse of

disease or toxic death. Disease-free survival (DFS) was calculated from the day of marrow transplantation (day 0).

RESULTS

ABMT in Relapsed Patients. One hundred forty-two patients with low grade B cell NHL in sensitive relapse or who failed to enter remission after multiple chemotherapeutic attempts who attained a minimal disease state underwent ABMT between 3/84 and 3/94 (Table 1). The median age of these patients was 42. The histologies of these patients included 97 with follicular small cleaved cell, 31 with follicular mixed small cleaved and large cell, and 14 with other histologies (mantle cell lymphoma - 10; small lymphocytic lymphoma - 3; monocytoid B cell lymphoma - 1). The majority of patients had a history of marrow involvement (99 patients), 47 patients had extramedullary extranodal disease, and 28 had a history of B symptoms. Thirteen patients had a history of masses greater than 10 cm. Seventy-five patients had a history of never achieving a CR prior to consideration for ABMT. At marrow harvest ony 43 patients were in complete clinical remission (32%), 77 patients had no residual histologic evidence of marrow infiltration (46%) (Table 2).

Table 1. Patient Characteristics

	Relapsed	Upfront
Total	142	84
Sex		
Female	82	37
Male	60	47
Age at BMT (y)		
<35	19	15
35-50	101	63
>50	22	6
Histology		
Follicular small cleaved	97	71
Follicular mixed	31	12
Small lymphocytic	14	1
History of BM involvement	99	69
Extranodal disease (exclusive of BM)	47	21
B symptoms	28	15
Mass >10 cm	13	11
Prior response		
PR	75	
CR	67	

Table 2. Status at ABMT

	Relapsed	Upfront
Total	142	78
CR	43	28
PR	99	50
Histologic BM Involvement		
Undetected	77	42
Involved	65	36

As of August 1994, 36 of these patients have relapsed, and 4 died without relapse (Table 3). Eleven patients relapsed in the marrow and 8 of these patients had marrow involvement at harvest. The DFS for the 142 patients is 51% at 4 years with an overall survival of 77% at 4 years (Figure 1). The majority of relapses were in prior sites of disease, with only 7 patients having relapses in entirely new sites. There was no difference in the DFS between patients who were in CR or PR at ABMT ($p=0.4$) (Figure 1). There were 83 patients who had a rearrangement of bcl-2 gene who had post-marrow purging samples available. Thirty-four patients were PCR negative and 49 were PCR positive. The DFS for the PCR negative patients was significantly better than the PCR positive patients ($p=0.002$) (Figure 1). This finding supports our previous studies of the relationship between marrow purging and outcome following ABMT.[11]

Table 3. Clinical Outcome

	Relapsed	Upfront
Total	142	78
Treatment-associated deaths	4	5
CCR	94	52
Relapse (alive)	36 (24)	21 (18)
Sites of relapse		
Previous site	29	16
Previous and new	5	2
New	2	3
BM relapse	11	9
BM+ @ harvest	8	7
BM- @ harvest	3	2

Upfront ABMT in Low Grade NHL. Eighty-four previously untreated patients with advanced stage low grade B cell NHL were considered for CHOP-induction followed by ABMT in first remission (Table 1). The median age of these patients was 43. The majority (85%) of these patients had follicular small cleaved cell lymphoma, 12 had follicular mixed small cleaved and large cell, and 1 had small lymphocytic lymphoma. Most patients had stage IV disease by virtue of marrow involvement. Extranodal disease was present in 25% of the patients, and a subset of patients had bulky masses (13%) or B symptoms (18%). Seventy-eight patients went to marrow harvest, 3 failed to attain CR or PR, 2 were diagnosed with second tumors and one patient declined. At harvest, only 28 patients were in clinical CR (36%), with histologic marrow involvement in 42 of the patients (54%) (Table 2).

As of August 1994, 21 patients have relapsed, the majority of patients relapsing in previous sites of disease (Table 3). Nine patients relapsed in the marrow, seven of whom had marrow involvement at harvest. The DFS for the 78 patients is 71% at 2 years with an overall survival of 96% at 2 years (Figure 2). Patients who were in CR at ABMT had a significantly better DFS than patients in PR (Figure 2). There were 59 patients who had a rearrangement of bcl-2 gene who had post-marrow purging samples available. Twenty-six patients were PCR negative and 33 were PCR positive. The DFS for the PCR negative patients was not significantly better than the PCR positive patients (Figure 2).

SUMMARY

A subset of patients with relapsed low grade B cell NHL appear to benefit from high dose therapy and ABMT. Retrospective studies suggest that the time to treatment failure for patients undergoing ABMT in second remission is significantly better than patients treated with conventional therapy.[9] However, it remains uncertain if ABMT will lead to prolongation of overall survival. In the present study we have observed a DFS for relapsed patients of 51% at 4 years. Generally, the median DFS for patients with relapsed low grade NHL in second or subsequent remission is between 1-2 years. The overall survival for the patients undergoing ABMT in second or greater remission is 77% at 4 years. As reported by the group at St. Bartholomew's Hospital, longer follow-up will be necessary to suggest if survival following ABMT is significantly better than that observed with conventional treatment.[9]

A major unresolved question is the timing of transplant for patients with low grade NHL. Analogous to ABMT in intermediate and high grade NHLs, patients with low grade NHL who have failed multiple regimens and have developed resistant disease are less likely to benefit from ABMT than patients with sensitive disease who can achieve clinical CR. We have begun to investigate the role of ABMT for patients with advanced stage low grade NHL in first remission. In those studies the DFS is 71% at 2 years. With the limited follow-up at present, it is unknown if ABMT in first remission will yield a higher DFS than in second remission.

In an early report from our institution we observed that patients in CR at ABMT had a significantly better DFS than patients in PR.[8] With longer follow-up, there is no significant difference in DFS among patients in CR or PR. The patients in PR are failing earlier, while those in CR fail later. In the patients transplanted in first remission, presently remission status has a significant impact on DFS. For these reasons, our current and future studies are directed toward a higher CR rate prior to ABMT.

We have previously observed that the presence of minimal residual disease detected in the autologous purged marrow is associated with a significantly lower DFS following ABMT.[11] This study strongly suggests that residual lymphoma cells may contribute to relapse and supports a role for bone marrow purging for ABMT in patients wih NHL. In the present study, patients with relapsed low grade NHL whose marrow was PCR negative for the t(14;18) following *ex vivo* purging had a significantly better DFS than patients who had residual PCR detectable cells present in the reinfused marrow. In contrast, the presence of PCR detectable disease does not correlate with DFS in the upfront ABMT patients. This suggests that with the present limited follow-up, the major problem is recurrence of endogenous disease. The impact of purging may be of greater significance with longer follow-up in these studies, and with better control of endogenous disease.

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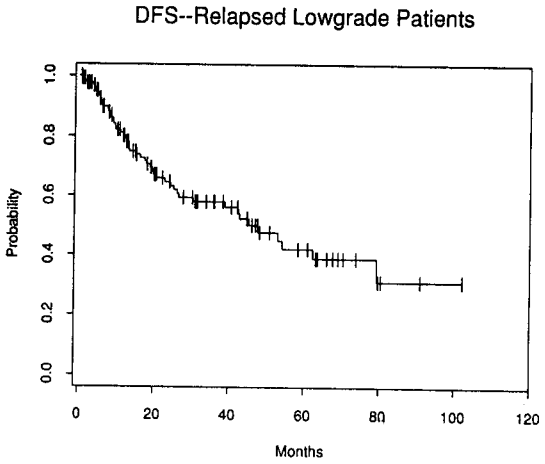

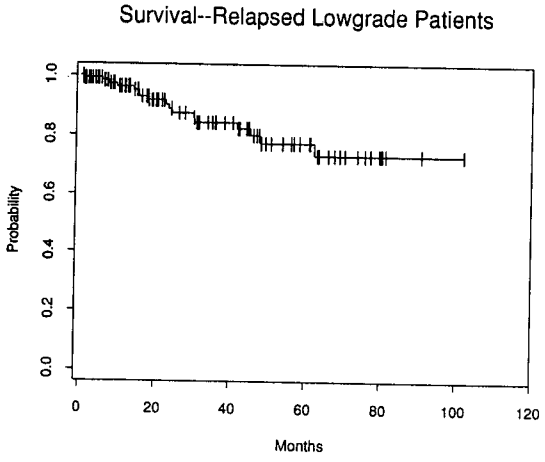

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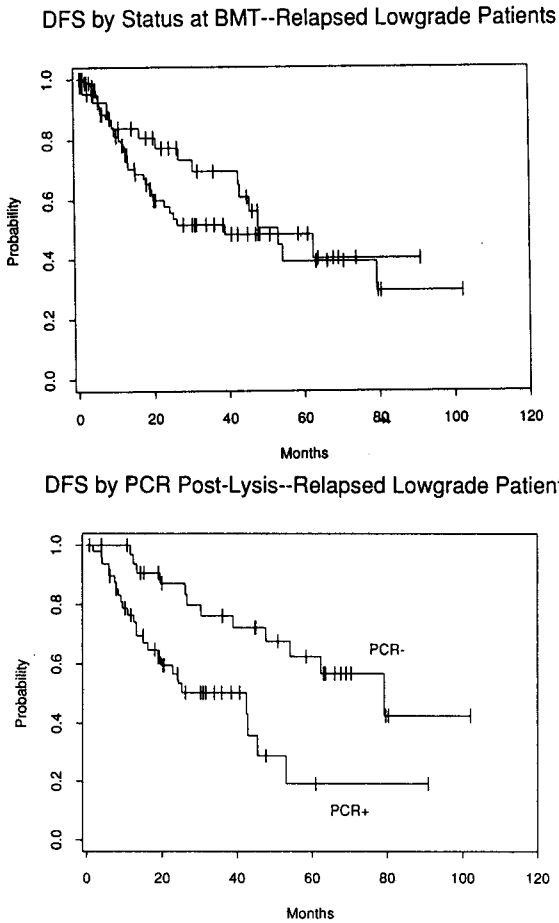
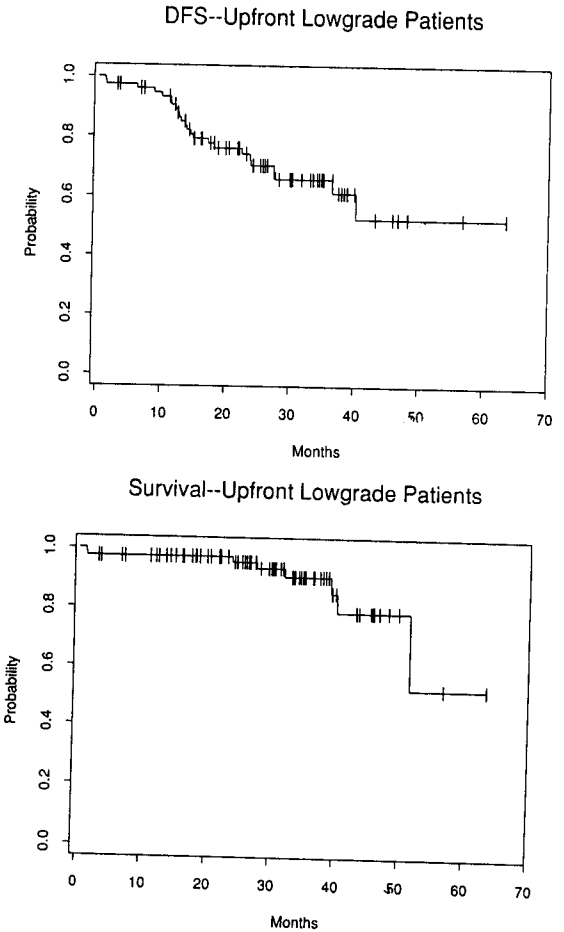


Figure 1. *DFS, overall survival, DFS by remission status at ABMT, and DFS by post-lysis PCR status of 142 patients with relapsed low grade NHL undergoing ABMT.*



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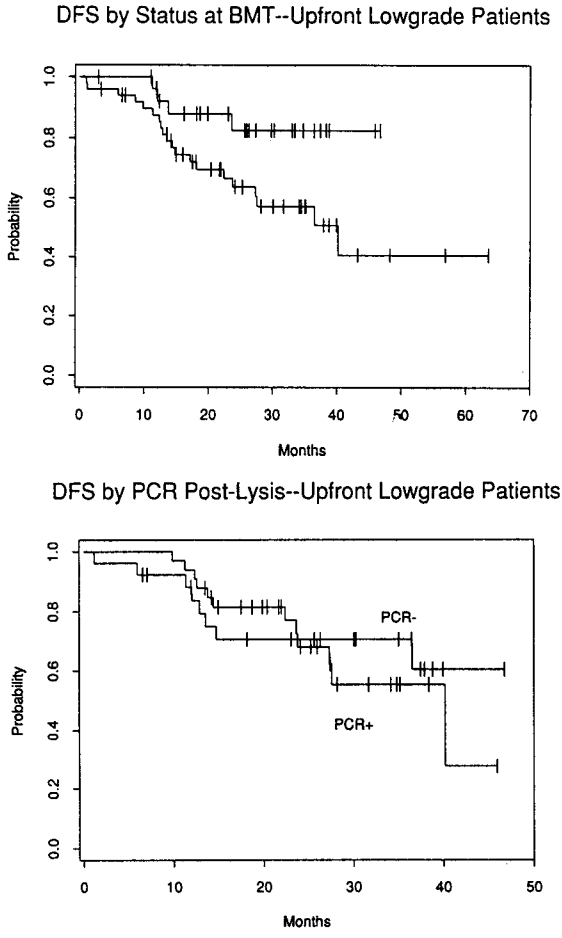

Figure 2. *DFS, overall survival, DFS by remission status at ABMT, and DFS by post-lysis PCR status of 78 patients low grade NHL undergoing ABMT in first remission.*

EUROPEAN CUP TRIAL: A RANDOMIZED TRIAL COMPARING THE EFFICACY OF CHEMOTHERAPY WITH PURGED OR UNPURGED AUTOLOGOUS BONE MARROW TRANSPLANTATION IN ADULTS WITH POOR RISK RELAPSED FOLLICULAR NHL. AN EBMT WORKING PARTY TRIAL

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Follicular non-Hodgkin's lymphomas (NHL) consist .of the follicular centroblastic-centrocytic lymphomas (Kiel classification) and of the follicular small cleaved (FSC), follicular mixed (FM), and follicular large cell (FLC) lymphomas (International Working Formulation, IWF). The FSC and FM subtypes forming the majority of follicular lymphoma are considered to be of low-grade malignancy whereas the FLC subtype is classified as intermediate. About one third of all the patients with NHL have a follicular histology with a median age of over 50 years.

The majority of patients have generalized disease at presentation. About 10-20% of all patients will remain in stage I-II after appropriate staging. Localized follicular NHL can be cured with radiation therapy, but disseminated follicular NHL is rarely curable. Although complete remission can be achieved with intensive cytotoxic therapy, eventually almost all patients will relapse. Survival curves show no evidence of a cure plateau, although the median survival is between 4 and 10 years.

In stage III follicular lymphoma some success has been seen with radiotherapy.[1,2] Approximately 75% of patients survive longer than 5 years following total nodal irradiation and 50-65% more than 10 years.

Recent evidence suggests that high dose therapy followed by autologous bone marrow transplantation in relapsed progressive diffuse NHL is effective and can result in cure.[3,4] Long-term disease-free survival (DFS) may be possible in probably more than 50% of patients depending on disease status at the time of transplantation and response to previous chemotherapy. However, only a comparatively small number of patients with relapsed follicular NHL have been treated with high dose therapy and ABMT.

Bone Marrow Purging. Although the real efficacy of purging procedures in ABMT has yet to be established, neoplastic cells which remain in the marrow harvest may represent an important problem for ABMT in follicular NHL since bone marrow infiltration is common at the time of diagnosis. Moreover, marrow infiltration is frequently the last residual site of the disease following chemotherapy histology-negative bone marrow may still be be infiltrated by lymphoma cells as judged by molecular biology criteria.

The t(14;18) translocation can be demonstrated in approximately 85% of patients with follicular lymphomas.[5] By means of the polymerase chain reaction (PCR) as few as one lymphoma cell bearing the bcl-2 translocation in 10^5 normal cells can be detected.[6] In a recent study, patients in remission after conventional chemotherapy have been shown to have marrow involvement by PCR for bcl-2.[7]

Several methods for eliminating clonogenic cells from autologous marrow have been developed, such as active cyclophosphamide derivatives, monoclonal antibodies, photodynamic dyes and immunomagnetic procedures.[8,9]

Although the real clinical efficacy of cleansing the marrow is still under judgment, recent results support the view that purging may be important in follicular NHL.[10] This study showed that DFS of patients with no evidence of lymphoma in their marrow after immunomagnetic purging was significantly better as compared to those in which the PCR remained positive after cleansing the marrow. However the detection of bcl-2 by PCR does not necessarily reflect the presence of clonogenic tumor cells since lysed cells may release intact DNA sequences from the (14;18) breakpoint.

Although the validity of an in vitro purging in reducing lymphoma cell contamination is convincing, the clinical value of marrow cleansing in lymphoma patients is yet to be established. The fact that the majority of relapses after autologous bone marrow transplantation occur at the site of previous bulk disease, suggests that relapse is mainly due to endogenous minimal residual lymphoma cells which are resistant to high dose therapy rather than from marrow contamination. The role, therefore, of purging in ABMT remains to be established.

Immunomagnetic Bead Purging. Immunomagnetic purging with anti-B cell monoclonal antibodies has been established for clinical use in ABMT of patients with B-cell lymphoma.[10] The immunomagnetic purging has several advantages compared with purging techniques using antibody plus complement.

- Immunomagnetic purging is 10-100 times more efficient than target cell lysis with complement activating antibodies and rabbit complement; with immunomagnetic purging, a reduction of clonogenic cells by 4-5 orders of magnitude can be achieved.
- Immunomagnetic purging can be more easily standardized. In addition, this method involves the least expenditure of time.
- For the analysis of the efficacy of the purging method, immunomagnetic bead purging has some more advantage: first, there is no problem with false positive results as in the case in purging with antibody plus complement. In the latter method an underestimation of the log-kill may result from amplification of contaminating DNA carrying breakpoint sequences liberated by the lysed tumor cells. Second, false negative results can also be avoided by using immunomagnetic techniques, because DNA from removed cells which are enriched in the "bead fraction" can be examined for specific breakpoint sequences by PCR.

Because of the large heterogeneity of neoplastic cells with respect to antigen-density, simultaneous use of several antibodies to the differentiation antigens (antibody cocktail) is necessary for optimal purging.[11]

Rationale for the trial. An international randomized phase III trial for the treatment of relapsed follicular NHL was started in Europe in 1993. Studies referred to the above suggest that ABMT might be an effective therapy for patients with relapsed follicular lymphoma, but it has not been proven to be superior to conventional salvage therapy. Neither is there evidence that ABMT with purged marrow is better than ABMT with unpurged marrow. The high sensitivity to irradiation of follicular NHL would indicate that TBI followed by ABMT could be a curative therapy. Relatively long survival is, however, possible in patients with follicular NHL with only modest treatment. We therefore wish to establish whether patients with adverse prognostic factors, such as a short progression-free interval will benefit from the long established combination CY-TBI and ABMT.[12] Therefore, the objectives of this protocol are:

- to compare in a randomized phase III trial the efficacy of chemotherapy versus HDT followed by either purged or unpurged ABMT in patients with poor risk follicular NHL (IWF Groups: B, C and D).

- to determine the clinical usefulness of immunomagnetic ex vivo purging of autologous bone marrow with regard to progression-free survival and/or survival.

This trial involes treating adult patients with poor risk follicular NHL with 3 cycles of chemotherapy. Patients who achieve either a CR or PR and who have limited bone marrow infiltration (≤20% B-lymphocytes) will be randomized to either three further cycles of chemotherapy or HDT followed by unpurged or purged ABMT.

Trial Design. Adult patients, age 15-65 years, with relapsed follicular NHL, after a first or subsequent remission receive 3 cycles of chemotherapy. Patients who achieve either a CR or PR and who have limited bone marrow infiltration (<20% B-lymphocytes) will be randomized to either 3 further cycles of chemotherapy or high dose therapy and unpurged ABMT or high dose therapy and purged ABMT (Table 1).

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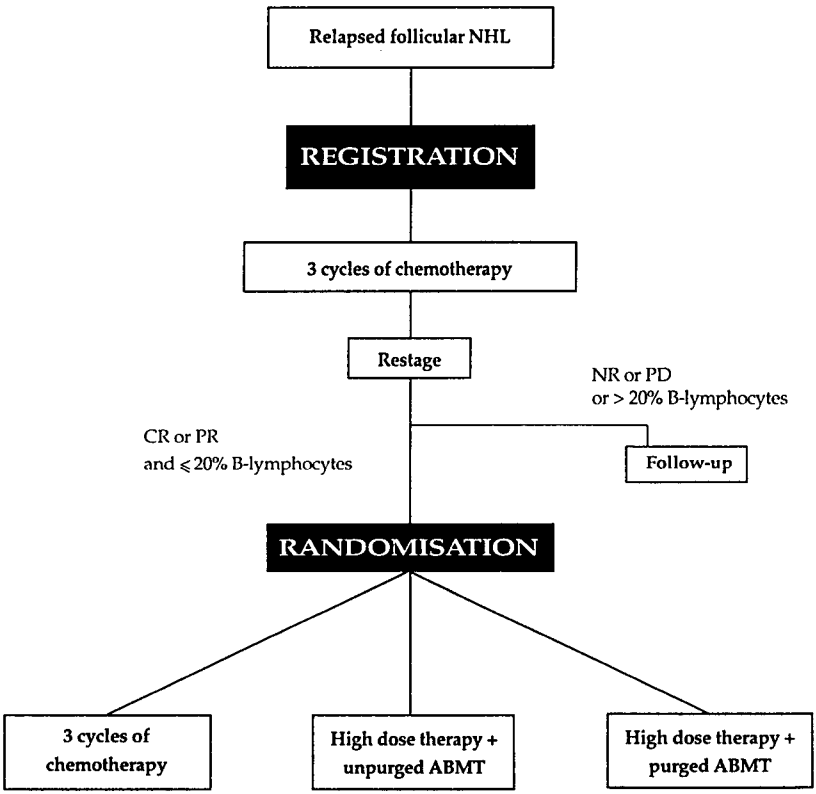

Figure 1. *European CUP Trial: Trial Summary*

AUTOLOGOUS BONE MARROW AND BLOOD CELL TRANSPLANTATION WITH ETOPOSIDE AND MELPHALAN FOR POOR PROGNOSIS NON-HODGKIN'S LYMPHOMA: THE IMPORTANCE OF DISEASE STATUS AT TRANSPLANT

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INTRODUCTION

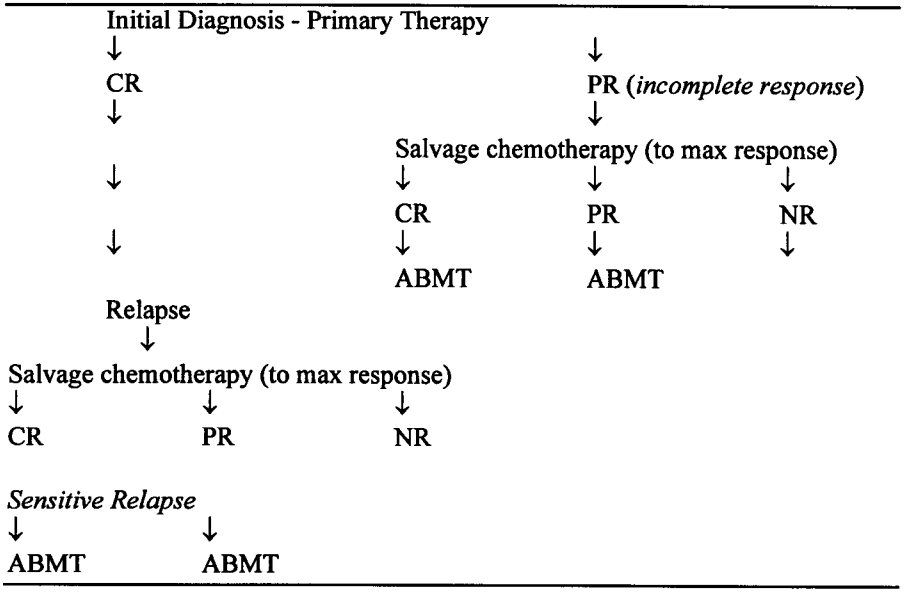
Intensive therapy with autologous bone marrow transplantation (ABMT) and/or blood cell transplantation (BCT) for poor prognosis non-Hodgkin's lymphoma (NHL) gives best results in patients with chemotherapy sensitive disease.[1-14] Most intensive therapy programs include a limited number of cycles of conventional salvage regimens to determine chemotherapy sensitivity prior to transplant. The Parma study will help answer the question of intensive therapy versus conventional dose salvage chemotherapy for patients with chemotherapy sensitive disease but will not directly address the influence of residual tumour bulk at the time of transplant. Indeed, as tumour growth models suggest, the presence or absence of residual tumour may be of critical importance since the persistence of drug-resistant tumour cell clones is directly dependent on tumour bulk rather than on initial chemotherapy sensitivity.[15,16] If this were the case clinically, the extent of residual tumour at transplant might predict for outcome after transplant suggesting that the relapse rate might be reduced by prior maximal tumour bulk reduction. The possible adverse consequence of this strategy is the development of chemotherapy resistant clones.[15,16]

As a first step in addressing this issue, we report our experience with 86 patients with chemotherapy sensitive advanced intermediate and transformed NHL who were treated with salvage therapy to maximum response prior to transplant. We previously demonstrated acceptable nonhematologic toxicity with the same intensive regimen of high-dose etoposide and melphalan followed by ABMT in patients with advanced hematologic malignancies, including NHL.[17,18] Multivariate analysis of disease- and patient-related variables were performed to predict for long-term disease-free survival following ABMT and/or BCT.

METHODS AND RESULTS

Table 1 outlines the treatment strategy for patients with biopsy-proven intermediate or transformed (from low to intermediate grade) NHL referred to the University of Toronto ABMT program. Patients with immunoblastic lymphoma were treated in the same fashion as those with intermediate grade NHL. Patients with *sensitive relapse* were defined as those relapsing after obtaining an initial complete remission (CR) to anthracycline-containing primary therapy regimens who then achieved a further 50% or more reduction in tumour size after conventional dose salvage therapy. Similarly, patients with an *incomplete response* to primary therapy required a further >75% tumour size reduction with conventional dose salvage therapy. Complete restaging was performed immediately prior to ABMT. Complete remission was defined as the absence of all clinical, radiological and biochemical evidence of disease. Partial remission (PR) was defined as those who did not achieve a CR with salvage therapy but met the above response criteria. Patients who did not achieve PR were considered nonresponsive (NR). Other eligibility criteria included age 18-61 years, absence of serious underlying medical illness, negative serology to HIV-1 and normal values for creatinine clearance, pulmonary function, left ventricular ejection fraction and liver enzymes.

Table 1. Treatment Schema



Bone marrow (BM) was obtained, processed and stored as previously described.[19] Patients with morphologically evident lymphoma in the marrow had blood cells collected after mobilization with cyclophosphamide followed by interleukin-3 (2.5 μg/kg/d) and GM-CSF (5 μg/kg/d), or with G-CSF (5 μg/kg/d) alone. Purging of the BM or BC was not performed.

Intensive therapy comprised etoposide (60 mg/kg) as a 5- or 32-hour infusion and melphalan (160 or 180 mg/M^2IV). In addition, patients with transformed or any NHL with T cell phenotype, received fractionated total body irradiation to a total of 1200 gCy followed by autograft infusion. Patients received GM-CSF (5 μg/kg/d) from day 1 until the abolute neutrophil count (ANC) was >1 x 10^9/L for three consecutive days and were supported as previously described.[20]

Patients with a minimum of three months follow-up from the time of transplantation are included. Patients transplanted in CR who remained in CR and those transplanted in PR who showed improvement or no change in residual radiologic abnormalities without other evidence of disease for a minimum of six months were reclassified as being disease-free. Both survival and disease-free survival (DFS) were analyzed and compared using log-rank analysis. A Cox proportional hazards model was used to evaluate a number of transplant variables for influence on DFS. The variables included age, grade (transformed and intermediate with immunoblastic grouped with the latter), stage at diagnosis, phenotype, extranodal disease at diagnosis, prior BM involvement, bulk at diagnosis (>10 cm), bulk at relapse (>5 cm), relapse in a previous radiation field, and remission status immediately prior to transplant.

Characteristics of all 86 patients are described in Table 2. There were 53 males and 33 females with a mean age of 44 years (range: 19-61). The median time from referral to our centre to transplantation was 6 months (range: 1-19) with a median time from last salvage treatment to transplant of 2 months (range: 1-14). The salvage chemotherapy regimens are summarized in Table 3. Patients achieving CR required a mean and median number of salvage regimens of 2 (range: 0-5) and those patients whose maximal response was a PR, required a mean and median of 3 (range: 0-8). Fifty-one patients entered transplant in CR, 30 in PR while five had nonresponsive disease (Table 4).

Tables 2 & 3. Patient Characteristics

	Total	Intermediate	Transformed
Age-mean (years): (range 19-61)	44	43	47
Number of Patients:	86	72	14
Male	53	44	9
Female	33	28	5
Stage at Diagnosis:			
I	7	5	2
II	18	15	3
III	21	16	5
IV	40	36	4
Subtype:			
Follicular large cell[45]		8	
Diffuse small cleaved		6	
Diffuse mixed		14	
Diffuse large cell		36	
Immunoblastic		8	
Phenotype:			
B	71	57	14
T	12	12	0
unknown	3	3	0
BM (+) at diagnosis (dx):	20	19	1
Bulk (>10 cm) at dx:	32	27	5
Extranodal at dx:			
Patients	38	34	4
Sites	46	41	5
CNS	2	2	0
Bone	6	6	0
Liver	6	4	2
Lung	6	5	1
Other	26	24	2

Table 3. Salvage Characteristics

	Patient Number		
Number Rx per patient:	Total	CR	PR
Median	2	2	3
Mean	2.4	2.2	2.7
Range	0-8	0-5	0-8
Chemo alone			49
Chemo + Rt			31
RT alone			2
No salvage treatment			4
Salvage chemotherapy			
DHAP[29]			42
Mini BEAM[43]			30
CHOP			7
DICE[31,36]			2
Other			8

Table 4. Remission Status at Transplant

	Total	Intermediate	Transformed
CR	51	39	12
CR1*	9	8	1
CR2	37	29	8
CR>2	5	2	3
PR	30	28	2
NR	5	5	0

**Patients with an incomplete response to primary therapy who achieved CR with salvage therapy*

The total treatment related mortality was 10% with six deaths due to sepsis and single cases due to hemorrhage, interstitial pneumonitis and venoocclusive disease. The median time to ANC >0.5 x 10^9/L was 15 days and for platelets >20 x 10^9/L was 35 days. The predicted four-year survival and DFS for the entire cohort was 48% and 45%, respectively (Figure 1).

Univariate analysis identified two factors predictive of outcome, remission status at time of ABMT (p=0.0001) and relapse in a previous radiation field (p=0.049). By multivariate analysis, however, only remission status at transplant remained significant (p=0.0001).

For patients with sensitive relapse who were in CR at the time of transplant, the predicted four-year DFS and survival was 62% (95% CI; 51-79%) and 67%, respectively and the median DFS was not reached. In patients entering transplant in PR, median DFS was six months with a four-year predicted DFS of 14% (95% CI; 7-39%) (Figure 2). Similarly, for those with an incomplete response to primary chemotherapy who achieved CR after salvage treatment, the median DFS was 9 months with a predicted four-year DFS of 76% (95% CI; 51-91%). For the PR group, the median survival was 6 months with a predicted four-year DFS of 12% (95% CI; 6-40%) (p=0.02) (Figure 3). Disease and patient-related characteristics for PR versus CR cases were equivalent (results not shown).

Of 21 patients who had tumour bulk (≥5 cm) at relapse, 11 achieved CR, 7 achieved PR and three were nonresponders prior to ABMT. There are 10 long-term disease-free survivors from this group. Notably, three of these 21 patients received radiation to further reduce tumour size but only one achieved CR pretransplant and remains the only long-term disease-free survivor at 56 months. There was no relationship between tumour size and risk of relapse in the PR group at transplant (p>0.10, Fischer's exact test). The risk of relapse for both the partially sensitive and sensitive relapse groups was the same irrespective of whether residual disease was greater or less than two centimeters (Table 5).

Table 5. Effect of Tumour Size in Patients with Intermediate or Transformed NHL with a Partial Response at the Time of Transplant

	Number of PR Patients	>2 cm	≤2 cm
Sensitive Relapse			
Total	16	5	11
Relapsed Post ABMT	9	1	8
Partially Responsive			
Total	14	3	11
Relapsed Post ABMT	9	3	6
Relapse Rate	60%	50%	63%

There was no correlation between the two most common pretransplant salvage regimens, DHAP (dexamethasone, cytosine arabinoside and cisplatin) and mini-BEAM (BCNU, etoposide, cytosine arabinoside and melphalan) (Table 3) and outcome posttransplant ($p>0.10$, Fischer's exact test).

DISCUSSION

Our results confirm that intensive therapy with high-dose etoposide and melphalan followed by autotransplant is an effective regimen for the treatment of patients with chemotherapy sensitive intermediate and transformed NHL. Our intensive chemotherapy regimen was well tolerated. Bone marrow engraftment[21-24] and regimen associated mortality (10%) were similar to those reported using other published protocols.[2,3,5,10,11,13,14,25-28]

Patients with relapsed disease. Patients relapsing after obtaining an initial CR treated with conventional dose salvage chemotherapy alone have a prolonged DFS only if they achieve a CR. Complete remission rates of 23-37% are obtained with the median duration of CR ranging from 4 to 24 months and a predicted survival of 5-42% at 3 years[7,29-42] (Table 6). In comparison, our cohort achieving CR immediately prior to ABMT, appear to have a superior outcome with a four-year DFS of 62%.

Table 6. Results of Conventional Salvage Chemotherapy

Regimen	Pt #	CR Rate	Median Duration of CR (mos)	% Survival (mos)	Survival CR	Survival PR	Median Survival Duration (mos)
DHAP[29]	90	31%	24	25%(24)	71%(12)	<10%(24)	7
DHAP[7]	48	25%	-	-	-	-	-
DHAP[30]	40	23%	-	50%(36)	-	-	36
MIME[31]	191	32%	15	37%(15+)	42%(36)	<10%(36)	9
MIME[32]	123	32%	18	25%(24)	42%(36)	<10%(36)	9
ESHAP[33]	88	37%	20	31%(36)	28%(40)	<10%(40)	14
CEPP(B)[34]	75	34%	7	16%(24)	<5%(36)	<5%(36)	12
IMVP16[35]	38	37%	12	25%(24)	37%(12)	0%(10)	15
DICE[36,37]	34	23%	23+	22%(57)	25%(12)	18%(12)	7
"GELA" group[38]	44	23%	4	10%(36)	<10%(36)	<10% (36)	4

Sensitive relapse patients who fail to achieve a complete remission with conventional dose salvage chemotherapy alone have a very poor

outcome with less than five percent being long-term survivors.[7,29-40,42,43] (Table 6). Our results suggest improvement may be achieved with further intensive therapy and autotransplant since 14% achieved a four-year DFS, although confidence intervals are wide. Our data, however appear inferior to results of other ABMT studies which predict between 36-62% long-term survival[2,6,25,26] (Table 7). Gulati et al[25] report a 62% four-year DFS for their PR group treated with TBI, cyclophosphamide, etoposide and radiotherapy to sites of bulky disease prior to transplant. A possible explanation for these differences in outcome relates to our protocol of aggressive administration of salvage therapy to maximum response such that some subjects staged as PR cases in other studies would have achieved CR at our center. Another factor is that unlike our series, some studies included patients with low grade disease.

Table 7. ABMT Results According to Remission Status at the Time of Transplant

Regimen/Author	% DFS for Total	% DFS for CR	% DFS for PR	Median Follow-up (mos)	Median DFS Total
McMillan et al[26*]	40	50	38	36	24
Gulati et al[25**]	57	80	62	42	NR
Santini et al[2]	-	54	16	60	-
Takvorian[3]	58	[65]		11	NR
Philip et al[4++]	36	40	36	36	11
Bosly et al[38]	-	38	35	36	8

*NR = not reached, *overall survival, **11 with low grade disease, [+]includes 6 primary refractory patients, [#]good PR and CR patients considered together, [++]of total 100 patients treated*

Resistant relapse portends an abysmal prognosis with salvage chemotherapy alone and although dose intensification protocols have achieved a 12-14% three-year DFS[13,26] our results and those of others predict a worse prognosis with no long-term DFS.[13,14,25-27]

Patients with an incomplete response to primary chemotherapy. Patients with no response to primary therapy (refractory NHL) treated with conventional salvage therapy have a <5% long-term DFS. The best results for ABMT report a 14% progression-free survival at 3 years[26] but most studies have not been able to achieve these results[2,8,9,14,25,26,38] and therefore all but five patients in our study who failed primary chemotherapy were transplanted after achieving CR or PR with salvage

therapy. Patients who demonstrate some, albeit incomplete response to their primary anthracycline-containing induction regimen have a similarly poor prognosis with conventional salvage regimens. Several studies including the largest study of salvage chemotherapy using the MIME protocol[31,32] demonstrate that the likelihood of achieving a CR with salvage chemotherapy after failing primary therapy is low and dependent on the quality of response to the frontline regimen. Indeed, nonresponding patients, incomplete responding patients and those who initially respond to primary chemotherapy but then progress, achieve CR rates of 10%, 12% and 19%, respectively. Long-term DFS is <10% for all groups.[31,32]

Early ABMT studies in patients who achieved some response to initial therapy, demonstrated substantially higher CR rates (40-100%) compared to conventional dose salvage therapy. The long-term survival varies from 20-80% at two years[6,11-14,26,30] (Table 8) but the influence of remission status at transplant has previously not been investigated. Our results demonstrate in this group also, the major influence of remission status at ABMT: 4-year DFS for patients in CR at ABMT of 72% versus only 12% for those entering transplant in PR.

Table 8. Results of ABMT for Patients with an Incomplete Response to Primary Therapy

Regimen/Author	Number Patients	Salvage Prior	% CR Post ABMT	Survival Predicted (mo)	Survival Mean (mo)	% DFS Predicted (mo)	Mean Duration DFS (mo)
Philip et al[12]	17*	no	88	75%(24)	NR	-	-
Philip et al[14]	7	no	86	80%(24)	NR	80%(24)	NR
Philips et al[13]	25	no	-	-	-	20%(36)	6
Colombat et al[6]	5#	no	100	100%(36)	NR	82%(36)	NR
Tura et al[30]	22	no	14	74%(40)	-	-	-
Gribben et al[11]	15+	yes	40	50%(24+)	24	-	-
McMillan et al[26]	73++	yes/no	-	60%(48)	NR	-	-

Our strategy of maximal tumour bulk reduction prior to transplant with conventional salvage chemotherapy has: 1) allowed the identification of patients with chemotherapy sensitive tumours, 2) achieved a major reduction in tumour bulk, and 3) ensured that all patients received minimum standard conventional dose salvage therapy. With this approach, the only independent predictor of relapse was remission status immediately prior to ABMT. Although our data suggest a survival benefit for the chemotherapy sensitive PR transplant group over those who receive conventional salvage chemotherapy, the improvement appears to be small.

Nonetheless, the excellent overall DFS results for our whole group and the similar interval from transplant to relapse for CR or PR cases suggest that multidrug resistance was not induced with repeated cycles of salvage chemotherapy. Indeed, previous ABMT studies in which maximum pretransplant tumour bulk reduction was not attempted, show similar survival and DFS[5-9,11,14,30,44] (Table 9).

Table 9. Results of ABMT Independent of Pre-Transplant Remission Status

Regimen/Author	Number Patients	Prior Salvage?	Survival Predicted (mos)	Survival Median (mos)	DFS Predicted (mos)	DFS Median (mo)
Freedman et al[5]	100	yes	50%(38)	38	-	-
Colombat et al[6]	46*	yes	-	NR	60%(36)	NR
Philip et al[14]	19**	yes	55%(7)	NR	38%(7)	5
Philip et al[7]	48	yes	-	-	40%(24)	12
Philip et al[30]	44	yes	50%(36)	36	-	-
Peterson et al[8]	101†	yes	42%(60)	26	28%(60)	8
Vose et al[9]	57	yes	-	-	38%(36)	14
Gribben et al[11]	20††	yes	50%(24)	24	-	-

*NR = not reached, * 21 intermediate grade NHL, ** 4 with high grade, † 51 with Hodgkin's, low and high grade, †† responding group only*

In summary, we have shown that clinical evidence of disease at time of ABMT is a powerful predictor of outcome posttransplant. The issue remains that those in CR may merely represent a group with favorable tumour characteristics. This can be addressed by a randomized study of chemotherapy sensitive patients comparing ABMT early after the determination of chemotherapy sensitivity versus ABMT after maximal tumour reduction. Alternatively, one could utilize a regimen with higher CR rates and compare the posttransplant outcomes. Our strategy has also identified the PR group prior to transplant as having a very poor prognosis. Although ABMT appears to be superior to conventional salvage treatment for this group, additional novel strategies are needed to improve long-term survival.

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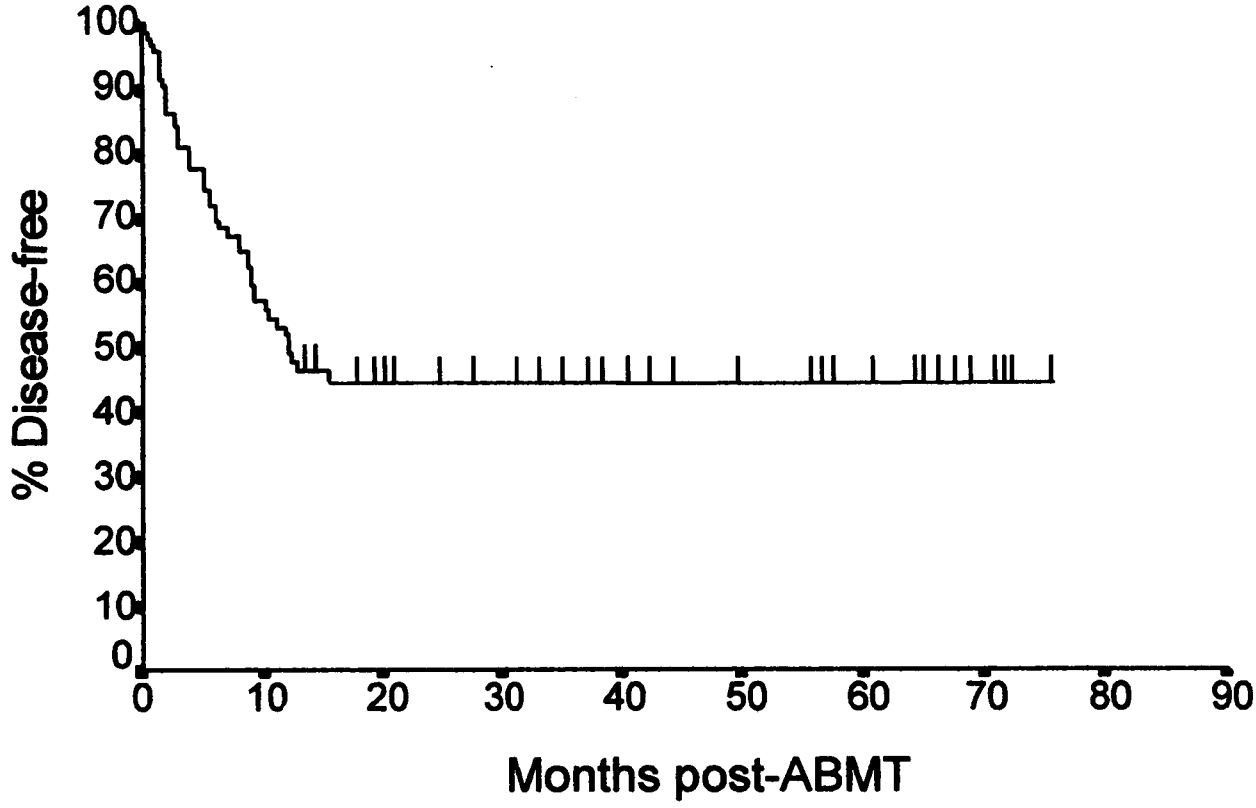

Figure 1. *Kaplan-Meier estimate of survival probability for the entire cohort of poor-prognosis intermediate and transformed NHL.*

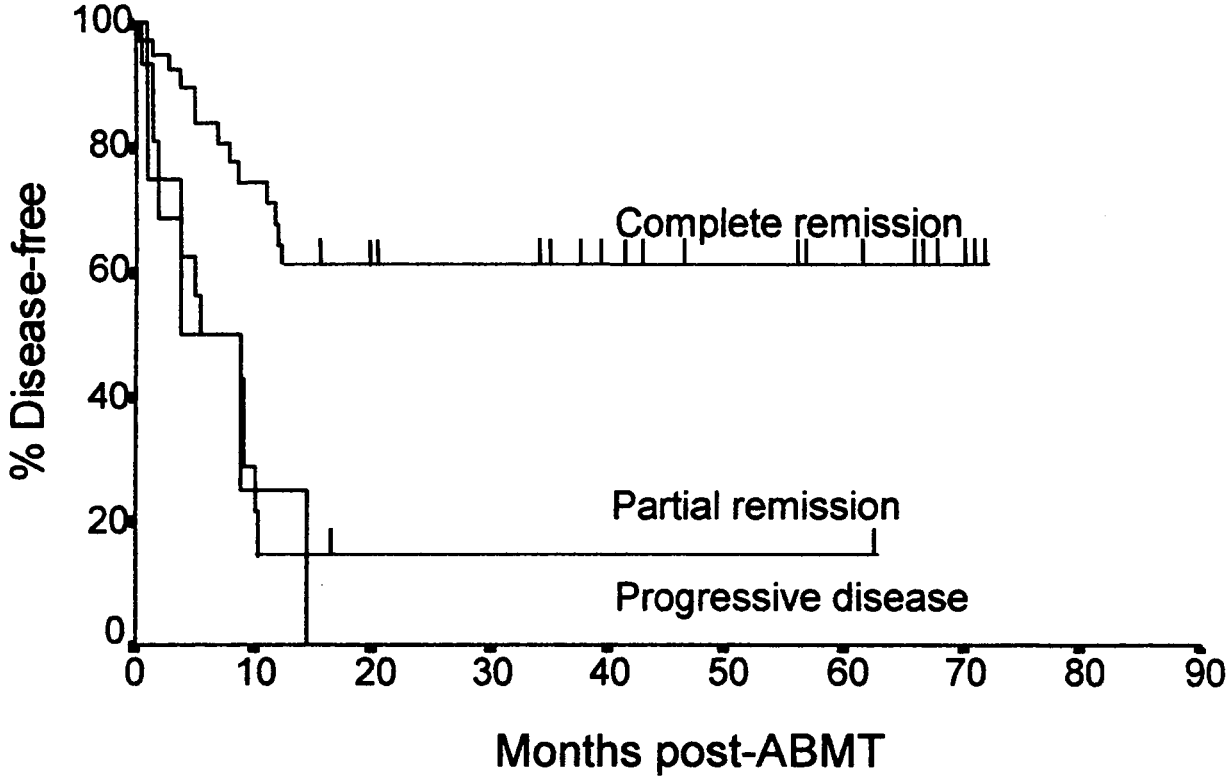

Figure 2. *Kaplan-Meier estimate of DFS of intermediate and transformed non-Hodgkin's lymphoma in sensitive relapse according to remission status at ABMT.*

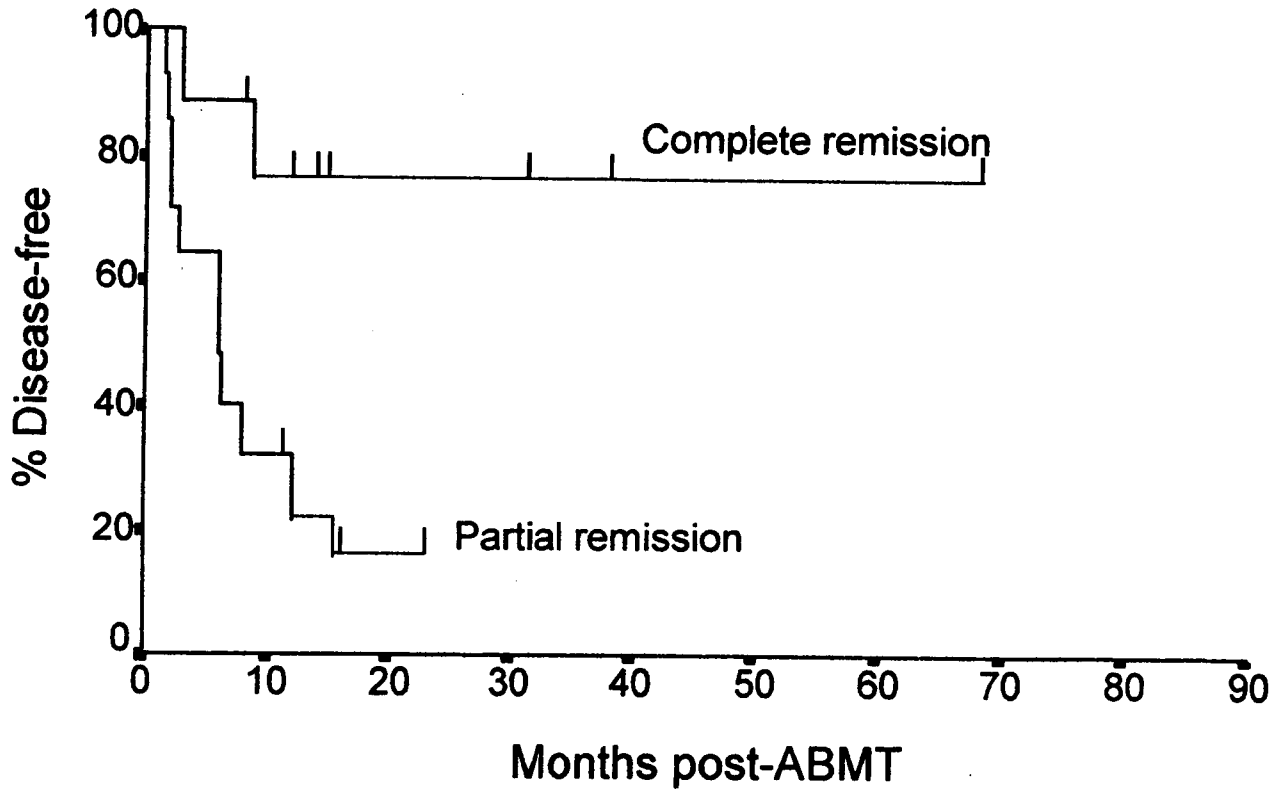

Figure 3. *Kaplan-Meier estimate of DFS of intermediate and transformed NHL patients who only achieved a PR with primary therapy (incomplete response). All patients were transplanted after obtaining either CR or PR with conventional dose salvage chemotherapy.*

HIGH DOSE THERAPY AND AUTOLOGOUS STEM CELL TRANSPLANTATION (ASCT) FOR LYMPHOBLASTIC LYMPHOMA IN ADULTS: RESULTS FROM THE EUROPEAN GROUP FOR BONE MARROW TRANSPLANTATION (EBMT)

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INTRODUCTION

Lymphoblastic lymphoma (LBL) is a rare disease in adults, accounting for about 4% of all adult cases of non-Hodgkin's lymphoma (NHL).[1] Early studies of the treatment of LBL in children and adults, used first and second generation chemotherapy regimens originally devised for the treatment of intermediate grade NHL. Results with these regimens were disappointing, with long-term disease-free survival (DFS) rates of only 15% to 30%.[2,3] Subsequent series were reported using more intensive chemoradiotherapy protocols similar to those used for ALL. Long-term DFS and overall survival rates of 40% to 60% were reported for these studies.[4,5] High dose therapy and autologous bone marrow or peripheral blood progenitor cell transplantation has been widely used in the treatment of NHL, and several previous reports of its use in LBL have been published, both as salvage therapy, and for consolidation of first remission.[6,7]

Two hundred and eighty-seven adult patients with LBL have now been reported to the EBMT lymphoma registry, of whom 147 have undergone autologous stem cell transplantation (ASCT) with bone marrow or peripheral blood in first complete remission. Previously reported data[8] from this group are updated below, and provide a clear rationale for the ongoing EBMT/UK Lymphoma Group randomized study of first remission ASCT in this disease.

PATIENTS AND METHODS

Between January 1981 and March 1994, 287 adult patients with a diagnosis of LBL have been reported to the EBMT. The presenting characteristics of this group are summarized in Table 1, and their status at the time of ASCT is shown in Table 2. One hundred and forty-seven patients underwent ASCT in first complete remission (CR).

Table 1. Patient Characteristics at Presentation

Characteristic	Entire Group n (%)	First Remission Group n (%)
Total	287 (100)	147 (100)
Male	202 (70)	103 (70)
Female	85 (30)	44 (30)
Phenotype		
B cell	72 (25)	29 (20)
T cell	144 (50)	88 (60)
Null	4 (1)	2 (1)
Unclassified*	67 (23)	28 (19)
Stage at diagnosis†		
I	18 (6)	5 (3)
II	52 (18)	24 (16)
III	30 (10)	10 (7)
IV	149 (52)	92 (63)
Unknown	38 (13)	16 (11)
B symptoms	94 (33)	44 (30)
CNS+	14 (5)	8 (5)
Bone marrow+	78 (27)	48 (33)
Bulky (>10cm) disease	81 (28)	48 (33)
Elevated LDH‡	49 (58)	33 (67)

* *Immunophenotyping not performed or data unavailable*
† *Ann Arbor stage*
‡ *LDH values available for only 84 patients*

Table 2. Disease Status at Time of ASCT

Status	n (%)
First CR	147 (51)
Second CR	41 (14)
> second CR	3 (1)
Sensitive relapse	20 (7)
Resistant relapse/primary refractory	30 (10)
Untreated relapse	10 (3)

The median age at the time of transplantation was 29 years 3 months for the entire group (range 16y 6m - 57y 10m) and 27 years 3 months for the group transplanted in first CR (range, 16y 6m - 56y 7m). Several combination chemotherapy and combined modality regimens were used as initial therapy in these patients. Most (45%) were treated with regimens similar to those used for acute lymphoblastic leukaemia (ALL), including the LSA_2L_2 regimen.[4] Regimens related to CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone), and 'third generation' regimens for NHL were also commonly used.

Second- and third-line chemotherapy protocols for patients relapsing after, or refractory to first line therapy included ALL-type regimens and cisplatin based therapy such as DHAP (dexamethasone, cytosine arabinoside, cisplatin) or ESHAP (etoposide, methylprednisone, cytosine arabinoside, cisplatin).

Of the entire group, 51% of patients received a high dose regimen including total body irradiation (TBI), most commonly cyclophosphamide/TBI. For patients undergoing ASCT in first CR, 63% received a TBI-based regimen. TBI was given according to several fractionation protocols. The most commonly used chemotherapy-only high dose regimen was BEAM (carmustine, etoposide, cytosine arabinoside, melphalan).

Bone marrow was used as the only source of stem cells in 257 patients. Twenty-five received peripheral blood progenitor cells (PBPCs) only, and 5 patients received both. Bone marrow and PBPC harvesting was performed according to active protocols at each centre. Bone marrow purging was performed in 25% of patients undergoing ABMT, most commonly with anti-T cell antibodies or cytotoxic drugs. Supportive care of patients undergoing ASCT was performed according to standard protocols at each participating institution.

RESULTS

Results were analyzed for the entire group, and separately for patients undergoing ASCT in first CR. All patients who suffered early deaths are excluded from the response data, but included in survival analyses.

Response to high dose therapy is summarized in Table 3. With a median follow-up duration of 25 months, the 5-year actuarial overall survival (OS) for the entire group is 47.3%. Disease status at the time of ASCT was the major determinant of outcome (Figure 1). The actuarial OS

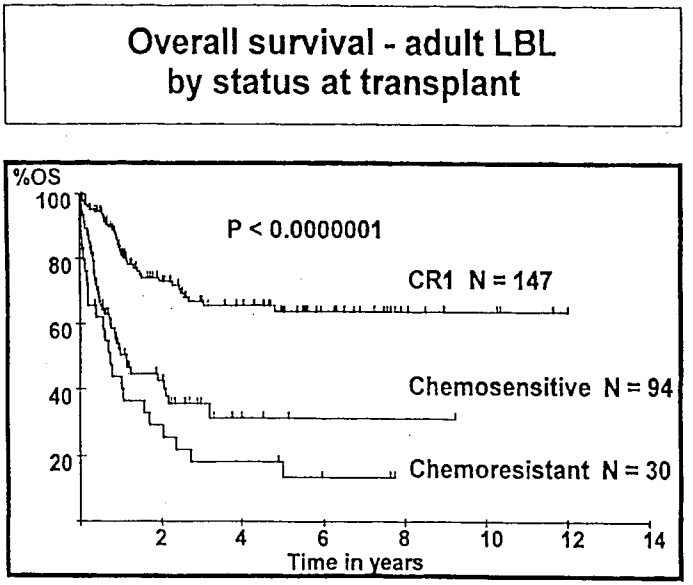
at 5 years was 64% for patients in first CR at the time of ASCT, compared with 31% for those with chemosensitive disease (i.e., in second or subsequent CR or partial remission), and 18% for those with resistant disease (i.e, primary refractory disease, or disease refractory to second- or third-line chemotherapy) (p=<0.0000001).

Table 3. Response to High Dose Therapy and ASCT

Response	n (%)
CR	216 (75)
PR	26 (9)
NR	8 (3)
PD	4 (1)
Early death	33 (11)

Procedure related deaths have occurred in 33 patients(11%). Causes of death included bacterial (n=8) and systemic fungal (n=3) infections and interstitial pneumonitis (n=8).

Figure 1



ASCT in First CR. Of the 147 patients in this group, 135 (92%) maintained CR 3 months after ASCT. With a median follow-up of 30 months, the 5-year actuarial OS for this group is 64%. The median time to relapse was 6 months (range, 0.4 to 38 months). As in our initial report, no presenting features could be identified in these patients which predicted for outcome after transplantation. Although there was a trend for poorer outcome in patients with bone marrow or central nervous system (CNS) involvement at the time of presentation, this did not achieve statistical significance.

DISCUSSION

These results represent an update of the recently reported series from the EBMT,[8] including patients reported to the lymphoma registry up to March 1994. The results are very similar to the original report, confirming that the major factor which predicts outcome in these patients is the disease status at the time of ASCT. Patients with chemosensitive disease at the time of ASCT had a significantly superior outcome to those with chemoresistant disease.

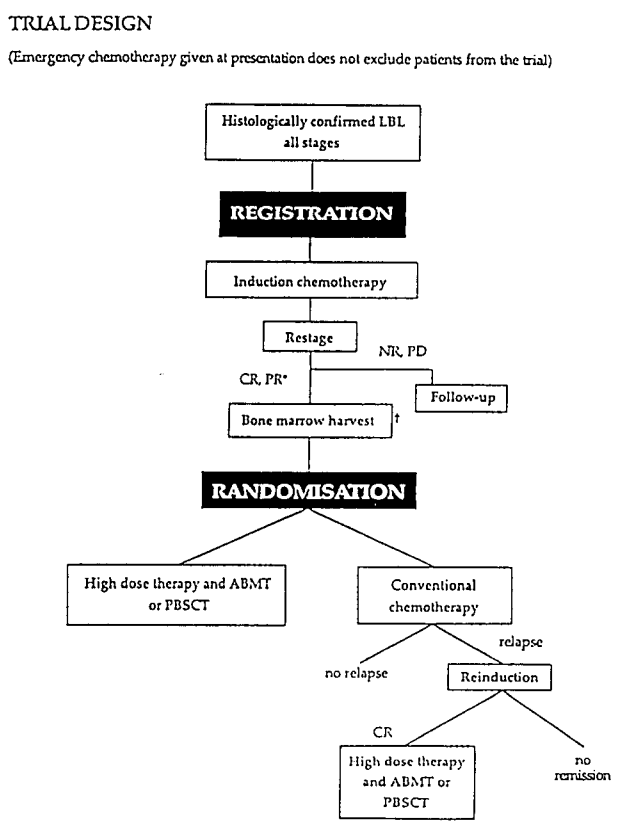
The 5-year actuarial OS of 18% in patients with chemoresistant relapse is superior to results reported for conventional dose salvage therapies in this disease,[5,9] although it is difficult to make direct comparisons. However, these results suggest that ASCT should be considered in this group, whose prognosis is otherwise very poor.

The results for ASCT in first complete remission are very similar to the original report, although the precise role of high dose therapy in first CR remains unclear. This series is retrospective, and the selection criteria for first remission ASCT are likely to have been variable over time and between centres. It is not clear whether first remission ASCT was considered only for 'poor risk' patients in certain centres. Furthermore, the definition of 'poor risk' patients with this disease remains unclear. The most widely accepted risk factors are those previously reported from Stanford University, namely Ann Arbor stage IV disease with marrow or CNS involvement, and elevated serum lactate dehydrogenase (LDH).[10] However, other series have identified different risk factors.

Although the results for patients in first CR in our series are encouraging, at least two previous studies have identified attainment of CR to conventional dose induction therapy as an important favorable factor, with long-term DFS in 75% of patients.[5,9]

Thus, the selection of patients for ASCT in first CR remains unclear. These results are clearly superior to those reported for 'poor risk' patients receiving conventional dose therapy only. In the Stanford series, only 19% of poor risk patients achieved long-term DFS. However, in the same study, 94% of good risk patients survived long-term, and there is clearly no role for ASCT in this group. In order to clarify the uncertainties in the role of first remission ASCT in this disease, the EBMT and United Kingdom Lymphoma Group are undertaking a randomized study comparing first remission ASCT (with bone marrow or PBPCs) with conventional dose consolidation/maintenance treatment for adult patients with LBL. The study design is summarized in Figure 2.

Figure 2



All patients with LBL, irrespective of risk factors, are eigible for the trial. The trial design is 'permissive'in terms of induction chemotherapy and high dose therapy, although 'standard' protocols for each must be used.

To date, 43 patients from several European centres have been entered on this trial. A total of 200 patients will be required to demonstrate a difference of 30% in a 5-year survival with a significance level of 5% and 95% power.

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HIGH DOSE CHEMOTHERAPY WITH STEM CELL RESCUE IN PATIENTS WITH LOW GRADE LYMPHOMA

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ABSTRACT

Patients with advanced low grade lymphoma (LGL) are considered incurable with conventional chemotherapy. In an effort to achieve prolonged remissions, high dose chemotherapy with stem cell rescue (HDCSCR) has been performed in patients with LGL. Our purpose was to determine the peritransplant mortality, response rate (RR) and disease-free survival obtained with HDCSCR in LGL. We retrospectively analyzed our transplants (Tx) from March 1990 until June 1993. Twenty-four patients (18 males, 6 females) aged 36-67 (median, 50) were Tx. Twelve patients had follicular small cleaved, 6 nodular mixed, 5 small lymphocytic and 1 mantle cell lymphoma. The median time from diagnosis to Tx was 32 months (range 6-107). All patients had recurrent or refractory LGL and had received from 1 to 8 regimens (median 3) prior to Tx. Fifteen patients (63%) had extra-nodal disease. A third of the patients progressed to a higher grade lymphoma prior to Tx. Four patients were Tx in complete remission (CR), 7 in sensitive relapse, 9 in resistant relapse or primary refractory, 4 undetermined. Fourteen patients underwent autologous BMT, 7 peripheral blood stem cell Tx (all due to BM involvement), 2 syngeneic BMT and 1 PBSCT + BMT. No stem cell purging was performed. Peritransplant mortality was 8% (2/24). The RR could be evaluated in 16 patients (4 patients were Tx in CR, 2 early deaths, 2 undetermined). The CR rate after HDCSCR was 75% (12/16). Sixteen of 24 patients (66%) remain alive 12-50 months post Tx (median 24). Thirty-eight percent of patients (9/24) remain in CR 13-50 months post Tx. We conclude that HDCSCR can be done with acceptable mortality in patients with LGL and that durable remissions can be achieved in patients with poor prognosis.

INTRODUCTION

Low grade lymphomas are exquisitely sensitive to chemotherapy and radiotherapy.[1] Nevertheless, the long-term survival rate of patients with low grade lymphoma is lower than in patients with intermediate grade lymphoma. This in part, is due to the fact that a slow but continuous pattern of relapse is observed over time. In contrast to the intermediate grade lymphomas, there is no plateau phase of the survival curve. It is widely accepted that patients with advanced (≥ stage II) low grade lymphoma are incurable with standard chemotherapy.[2] The median survival of patients with low grade lymphomas remains in the 6- to 10-year range and has not changed significantly despite the use of combination chemotherapy or radiotherapy. Based on the natural history of the disease, the lack of curative therapy and the advanced age of many patients, many investigators have adopted a "watch and wait" approach in which therapy is deferred until disease progression.[3] Nevertheless it has been reported that patients who fail to attain a complete remission and patients with less than one year response period have poor survival after relapse.[4] Not only do patients eventually relapse, but a significant number of patients progress to a higher grade lymphoma making death from lymphoma virtually inevitable.[5]

High dose chemotherapy with autologous stem cell transplantation is potentially curative therapy for refractory or recurrent Hodgkin's and intermediate grade non-Hodgkin's lymphoma (NHL). In an effort to achieve durable remissions or cure, high-dose chemotherapy with stem cell rescue has been performed in selected cases of low grade lymphoma.

The purpose of this study was to determine the peritransplant mortality, response rate and disease-free survival in patients with low grade lymphoma treated with high-dose chemotherapy and autologous stem cell rescue. We summarize here our experience using this type of therapy in patients with recurrent or resistant low grade lymphoma treated at our institutions.

PATIENTS AND METHODS

We retrospectively analyzed all autologous stem cell transplants performed at our institutions for low grade NHL from March 1990 until June 1993. All patients had low grade lymphoma as the presenting diagnosis. Patients that presented with intermediate or high grade NHL and were later found to have other lymph node or tissue biopsy consistent

with low grade NHL were not included. Nevertheless, patients that progressed to an intermediate or high grade NHL after an initial diagnosis of low grade lymphoma were included in our study.

The patients received a multitude of chemotherapy regimens prior to transplantation at the discretion of the referring physicians. Response to high-dose chemotherapy and stem cell rescue was evaluated by physical examination, CT scans and bone marrow biopsies. Bone marrow transplant mortality was defined as all deaths within seven weeks of transplantation, unrelated to disease progression. Disease-free and overall survival were calculated from the day of bone marrow infusion using Kaplan-Meier analysis.

RESULTS

Patient Demographics. Twenty-four patients (18 males, 6 females), aged 36 to 67 years of age (median 50) were transplanted (Table 1). All patients had recurrent or refractory low grade NHL. Twelve patients had follicular small cleaved cell, 6 nodular mixed, 5 small lymphocytic and 1 had mantle cell lymphoma (initially diagnosed as follicular small cleaved cell).

Table 1. Patient Demographics and Clinical Characteristics

Patients (Male:Female)	24 (18:6)
Age, range in years (median)	36-67 (50)
Histology	
Follicular small cleaved	12
Nodular mixed	6
Small lymphocytic	5
Mantle cell lymphoma*	1
Time from Dx to Tx in months, median (range)	32 (6-107)
Chemotherapy regimens prior to Tx, median (range)	3 (1-8)
Patients that progressed to a higher grade NHL	8 (33%)
Patients with extranodal disease at Tx	15 (63%)
Tumor status at time of Tx	
Complete remission	4
Sensitive relapse	7
Primary refractory or resistant relapse	9
Undetermined	4

* *Initially diagnosed as small lymphocytic*

Extranodal Disease. Fifteen patients (63%) had one or more areas of extranodal lymphomatous involvement. Extranodal areas of involvement included bone marrow, central nervous system, pleura, liver, spleen, breast, skin, lung, lacrimal glands, oral mucosa and stomach. Of these fifteen patients, only three had bone marrow as the only site of extranodal lymphomatous involvement.

Treatment prior to BMT. The median time from initial diagnosis to transplantation was 32 months (range 6-107). Patients received from 1 to 8 different chemotherapy regimens (median 3) prior to transplantation. The number of chemotherapy drugs (including interferon alfa) received prior to transplantation ranged from 2 to 11 (median 8). A multitude of chemotherapy regimens were used, since most patients were treated outside our institution. Most patients were treated with multidrug chemotherapy that included alkylating agents, steroids and an anthracycline.

Disease Status at the Time of Transplantation. A third of the patients (8/24) had progressed to a higher grade lymphoma prior to transplantation. At the time of transplantation, four patients were in complete remission, 7 in sensitive relapse, 9 in resistant relapse or had primary refractory disease and in 4, the sensitivity of their disease was undetermined.

Stem Cell Source. Fourteen patients underwent autologous bone marrow transplantation. Seven patients with bone marrow involvement in all cases underwent peripheral blood stem cell transplantation. Two patients underwent syngeneic bone marrow transplants and one patient received peripheral blood stem cells in addition to autologous bone marrow because of low bone marrow cellularity. No stem cell purging was performed.

Peritransplant Mortality. Two of 24 patients died within seven weeks posttransplantation to give a peritransplant mortality rate of 8%.

Response Rate. The response rate was evaluable in 16 patients since 4 patients were in complete remission at the time of transplantation, two patients experienced death early posttransplant and in two other patients we were unable to determine disease status at transplant. Twelve of 16 patients (75%) had a complete response after high-dose chemotherapy and stem cell rescue. The other four patients experienced only partial remissions.

Overall Survival and Disease-Free Survival. Sixteen of 24 patients (66%) remain alive 12 to 50 months post-transplantation (median 24 months). Thirty-eight percent of patients (9/24) remain free of disease

with the same follow-up. Actuarial overall survival at 24 months is 73% (SE±9) and actuarial disease-free survival at 24 months is 68% (SE±12).

DISCUSSION

High-dose chemotherapy with autologous stem cell rescue is known to be curative in refractory or recurrent Hodgkin's and intermediate grade NHL as well as in other refractory hematologic malignancies.[6] Limited information exists regarding the outcome of high-dose chemotherapy with autologous stem cell rescue in patients with low grade NHL.[7] Despite the fact that advanced stage low grade lymphomas are known to be incurable with standard chemotherapy, only a relatively small number of patients have been treated with high-dose chemotherapy and stem cell rescue. This, in part, relates to the fact that many patients are old and have co-morbid conditions making bone marrow transplantation difficult. The fact that bone marrow involvement is frequently present at diagnosis has also discouraged many investigators from pursuing this therapeutic approach. In addition, these patients have a relatively prolonged median survival when compared with recurrent or relapsed aggressive lymphomas and Hodgkin's disease. Nevertheless, it has been documented that patients who fail to attain a complete remission and those patients with less than one year remission duration have a poor survival rate.[4]

The purpose of this investigation was to determine the outcome of high-dose chemotherapy and autologous stem cell transplantation in patients with low grade NHL. We performed a retrospective analysis of patients transplanted at our institutions for recurrent or resistant low grade lymphoma. We only included in our series patients that presented initially with low grade lymphoma. Patients that presented with intermediate or high grade NHL and were later found to have other lymph node or tissue consistent with low grade lymphoma were not included in our series. Patients that progressed from a low grade to an intermediate or high grade lymphoma were included in our study.

All of our patients had recurrent or refractory low grade lymphoma. Sixty-three percent of patients had at least one area of extranodal disease including bone marrow, central nervous system, pleura, liver and skin among other sites. Only three of those patients had bone marrow as the only site of extranodal involvement, a reflection of the advanced stage of their disease.

Our population of patients was heavily pretreated. They received a multitude of chemotherapy regimens since most were initially treated outside our institution. The median time from initial diagnosis to transplantation was 32 months with a range of 6 to 107 months. Most patients were treated with combination chemotheapy that included alkylating agents, steroids and an anthracycline. The fact that our patients received a median of three chemotherapy regimens prior to transplantation and that half the patients received eight or more drugs before the transplant reflects the extent of pretreatment. More important, almost forty percent of our patients had either resistant relapse or primary refractory disease at the time of transplantation. In addition, thirty percent of our patients underwent peripheral blood stem cell rescue because of bone marrow involvement at the time of transplantation.

Eight percent of our patients died due to transplant complications within seven weeks of transplantation. We believe this peritransplant mortality rate is acceptable given the fact that our population of patients were heavily pretreated and that many patients had very advanced disease at the time of transplantation. In addition, half of our population were older than fifty years of age, reflecting the older average age of low grade lymphoma patients.

The response rate seen in our study was 75%. This compares favorably with results of high-dose chemotherapy and stem cell rescue in other refractory lymphomas[6] and in a recently reported series of patients with low grade lymphoma treated with myeloablative therapy and autologous bone marrow transplantation.[8] Two-thirds of our patients remain alive 12-50 months post-transplantation. Thirty-eight percent of patients remain free of disease with the same follow-up. The actuarial overall survival and disease-free survival at 24 months are 73 and 68% respectively (Figures 1 and 2).

Our results should be interpreted with caution. Although it is encouraging that a significant number of patients remain free of disease, our follow-up is short. Patients with low grade lymphoma have longer median survival when compared to other non-Hodgkin's lymphomas and a more variable behavior. Nevertheless, it has to be kept in mind that all of our patients had recurrent or refractory disease at the time of transplantation and that forty percent of our patients had resistant or primary refractory disease, features association with poor prognosis and the predictive of short survival.[6]

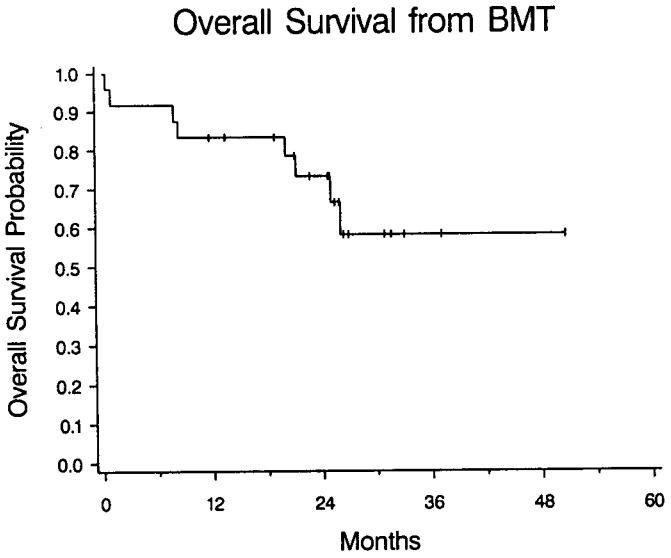

Figure 1.

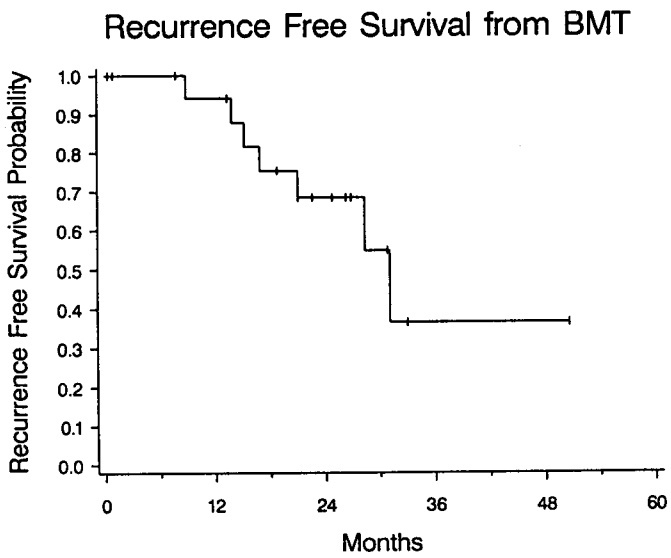

Figure 2.

In summary, our data suggest that HDCSCR can be performed with acceptable mortality in patients with low grade NHL and that durable remissions can be achieved in patients with poor prognosis. Larger number of patients with longer follow-up will be necessary to better ascertain the role of high-dose chemotherapy and stem cell transplantation and its curative potential in patients with low grade NHL.

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UPDATE ON HIGH-DOSE CYCLOPHOSPHAMIDE, CARMUSTINE, AND ETOPOSIDE (CBV), FOLLOWED BY AUTOLOGOUS HEMATOPOIETIC RESCUE FOR REFRACTORY HODGKIN'S DISEASE

P. Bierman, J. Vose, S. Jagannath, G. Spitzer, K. Dicke, J. Armitage

High-dose therapy followed by autologous bone marrow (ABMT) or peripheral stem cell (PSCT) transplantation is being used with increasing frequency for patients with relapsed or refractory Hodgkin's disease. Since 1989, approximately 2000 transplants for Hodgkin's disease have been registered with the North American Autologous Bone Marrow Transplant Registry.

Investigators at M.D. Anderson Cancer Center (MDAH) were among the first to report results of ABMT for Hodgkin's disease.[1] The high-dose chemotherapy regimen in this publication contained cyclophosphamide, carmustine, and etoposide (CBV) (Figure 1).

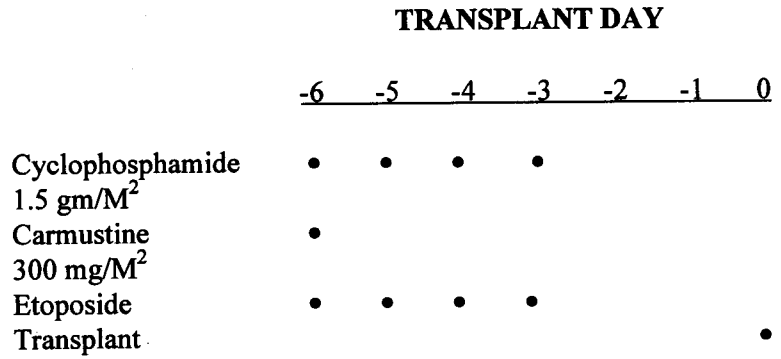

Figure 1. *Original CBV regimen.*

These agents were highly active against Hodgkin's disease. In addition, the use of autologous bone marrow rescue allowed significant dose escalation. A large cohort of Hodgkin's disease patients at MDAH and the University of Nebraska Medical Center (UNMC) have been transplanted with the CBV regimen and reported at periodic intervals.[2-6] This report will update results of the CBV regimen with autologous transplantation for Hodgkin's disease.

RESULTS

The latest update of the combined UNMC and MDAH experience included 128 patients with relapsed or refractory Hodgkin's disease that received CBV followed by ABMT or PSCT.[5] These patients received a CBV regimen similar to that displayed in Figure 1, except that etoposide was administered at a dose of 100-150 mg/M^2 every 12 hours for 6 doses from day -6 to day -4. This regimen has come to be known as "standard" CBV. A unique aspect of this report was the long median follow-up of 77 months, with a minimum follow-up of 48 months, for these patients.

Overall survival and failure-free survival for this cohort were projected to be 45% and 25% at 48 months (Figure 2). Multivariate analysis revealed performance status and the number of failed chemotherapy regimens to be most predictive for failure-free survival. The failure-free survival at 48 months was projected to be 53% for good performance status patients (ECOG 0) who had failed only one chemotherapy regimen prior to transplantation.

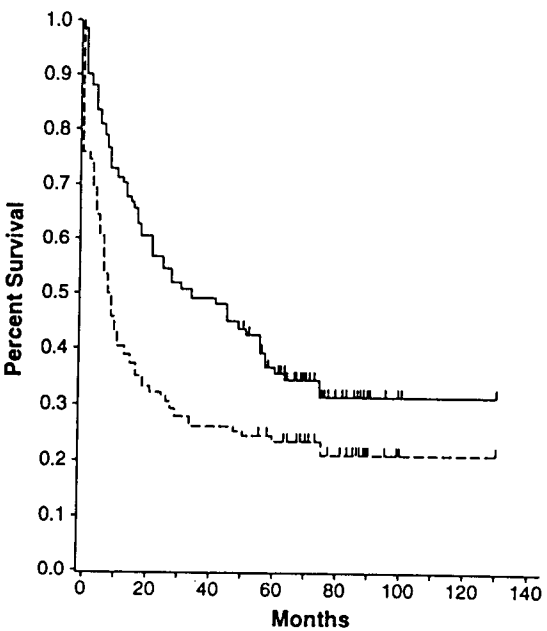

Figure 2. *Overall survival (solid line) and failure-free survival (dashed line) for 128 Hodgkin's disease patients transplanted with CBV at UNMC and MDAH.*[5]

A number of late events were noted with continued follow-up of this cohort. Late relapses (more than 24 months after transplant) were observed in 11 patients. The latest relapse occurred 74 months following transplantation. We also observed five patients who developed a myelodysplastic syndrome (MDS) following transplantation. The latest occurrence of MDS was at 79 months after transplantation. All patients died within one year of diagnosis of MDS. We also observed pregnancies in three women following transplantation. Two of these pregnancies, including one from a donated ovum, resulted in normal deliveries.

More recently, we have reported results of 254 Hodgkin's disease patients transplanted at UNMC between 1983 and 1993 following treatment with "standard" CBV.[6] This series includes 68 patients from the previous report.[5] At five years, overall survival was projected to be 38% and failure-free survival was projected to be 26%. A continuous pattern of treatment failures was observed, and failure-free survival at nine years was estimated to be 19%. Multivariate analysis showed that patients with normal LDH levels and those who had received only one chemotherapy regimen prior to transplant had significantly better failure-free survival. Also, patients with initial chemotherapy remissions longer than three years had superior outcomes.

Among the 186 UNMC patients who were not included in the combined UNMC/MDAH series, 11 more patients were identified who relapsed more than two years following transplantation. The latest relapse occurred 68 months following transplantation. In addition, four more patients with MDS were identified. These occurred between 37-43 months after transplantation. Two additional pregnancies were noted, including one from a patient in the combined series who had suffered a miscarriage. Other late events included one case each of acute myelogenous leukemia, non-Hodgkin's lymphoma, and cervical carcinoma.

A remarkable finding has been the safety of transplantation with the "standard" CBV regimen. At UNMC, 149 patients were transplanted between 1983 and 1989. Fifteen (10%) early deaths (defined as death during the initial transplant hospitalization) were observed. In contrast, we have performed 105 transplants with this regimen between 1990 and 1993 without any early mortality. These results are undoubtedly due to increased experience, better patient selection, and the routine use of hematopoietic growth factors. Although we have not administered the CBV regimen to outpatients, we now routinely discharge patients after the CBV regimen and perform the actual marrow or peripheral stem cell infusion, as well as subsequent care, on an outpatient basis.

OTHER CBV REGIMENS

A number of other high-dose chemotherapy regimens are used in which higher doses of cyclophosphamide, carmustine, and etoposide than those in "standard" CBV are administered.[7-11] Table 1 shows some of these higher dose CBV regimens. The complete remission rates and mortality rates show wide variations. It is difficult to compare results from various institutions due to differences in patient selection and prognostic characteristics.

Table 1. ABMT/PSCT for Hodgkin's Disease: Results with Various CBV Regimens

Ref No.	Cyclophosphamide (mg/M^2)	Carmustine (mg/M^2)	Etoposide (mg/M^2)	% Complete Remission	% Early Death
5	6000	300	600-900	45	9
7	6000	600-800	600-1000	48	4
8	4500-7200	450-600	1200-2000	43	17
9	7200	600	2400	79	21
10	(35 mg/kg)	450-600	1500-2000	44	9
11*	7200	300-600	2400	ns	32

** includes other lymphoid malignancies; ns = not stated*

In our experience, no differences in outcome were noted at total doses of etoposide between 600-900 mg/M^2. Results from Table 1 show that the highest mortality rates have been seen when higher dose CBV regimens have been used.

COMPARISON WITH OTHER REGIMENS

Differences in patient characteristics make it difficult to compare the results of CBV with other conditioning regimens for Hodgkin's disease. The best results of conventional salvage chemotherapy have been seen in patients who have relapsed from their first chemotherapy-induced remission, particularly when the initial remission duration exceeds one year.[12-13] An examination of results of transplantation in patients who have relapsed from their first remission may provide a way to compare results of transplantation with those of conventional chemotherapy. This might also provide a more uniform group of patients to compare various transplant regimens.

At UNMC, we have examined results in 85 patients who were transplanted with "standard" CBV following relapse from their first chemotherapy-induced complete remission.[14] Overall survival and failure-

free survival at four years were estimated at 65% and 43%, respectively. Failure-free survival was 35% for patients who relapsed less than 18 months after initial diagnosis (initial remission duration of less than one year), compared with 57% for patients who relapsed 18 or more months after diagnosis. Results of transplantation after first relapse have also been reported by University College Hospital (UCH) in London,[15] as well as investigators in Vancouver (Table 2).[16]

Table 2. ABMT/PSCT for Hodgkin's Disease in First Relapse

	Drug (mg/M^2)						Failure-Free Survival		
Ref	C	B	V	A	M	P	Overall	CR <yr	CR ≥yr
5	6000	300	600-900				43%	35%	57%
15		300	1600	1600	140		47%	41%	57%
16	7200	500	2400			150	64%	48%	85%

C = cyclophosphamide; B = carmustine; V = etoposide;
A = cytarabine; M = melphalan; P = cisplatin

The UCH group has used the BEAM regimen which contains carmustine at the same dose used in CBV. The etoposide dose is higher, and cytarabine and melphalan are used instead of cyclophosphamide. The Vancouver group has used a high-dose CBV regimen, along with cisplatin in some patients.

DISCUSSION

Hodgkin's disease patients can achieve prolonged failure-free survival with "standard" CBV, followed by autologous hematopoietic rescue. Peripheral stem cell transplantation is frequently being used in place of ABMT. Results from UNMC have shown that results of transplantation with peripheral stem cells are equivalent to those achieved with autologous bone marrow.[17] An obvious advantage of PSCT is that high-dose therapy is possible in patients who would otherwise be ineligible due to inability to harvest bone marrow.

The best candidates for transplantation are those with good performance status, who have not received extensive prior therapy. In addition, we have shown that patients with prolonged initial remissions and those with normal LDH at the time of transplant have the best outcome. These results, support early transplantation before chemotherapy resistance develops. The increasing ease, safety, and decreasing costs of transplantation also support early transplantation for Hodgkin's disease.

With continued observation, we have identified late events following autologous transplantation for Hodgkin's disease. Relapses as late as 68 and 72 months following "standard" CBV have occurred. Relapses more than two years following ABMT for Hodgkin's disease have been noted by other investigators.[9,15,18,19] Chopra et al described a relapse 67 months after ABMT with BEAM, but noted no other relapses beyond 33 months.[15] It is unclear whether our large number of late relapses are unique, or whether they reflect our long follow-up on a large cohort of patients. These observations mandate prolonged follow-up and updates on Hodgkin's disease transplant patients to determine the ultimate value of this form of therapy.

The development of MDS is also a worrisome observation. Other series have previously noted small numbers of patients who have developed MDS or acute leukemia following ABMT for Hodgkin's disease.[5,8,15] Several large series of MDS following autologous transplantation for non-Hodgkin's lymphoma, as well as Hodgkin's disease, have recently been reported.[20,21] The actuarial risk of developing MDS may be as high as 30% for patients who survive five years following transplantation.[21] At our institution, all the recorded cytogenetic abnormalities in patients with posttransplant MDS have involved chromosomes 5 and 7. This suggests that prior therapy with alkylating agents, rather than CBV, is responsible for MDS. All patients had received nitrogen mustard, vincristine, procarbazine, and prednisone (MOPP) prior to transplantation. These results provide additional support for early transplantation, before the extensive use of conventional salvage chemotherapy. These results emphasize again the need for prolonged follow-up of patients who have undergone ABMT or PSCT for Hodgkin's disease.

It is unclear whether regimens that contain higher doses of the agents used in CBV are more effective. If we examine patients treated after relapse from their first chemotherapy-induced remission (Table 2), there is some evidence that higher doses of cyclophosphamide, carmustine, and etoposide may be more effective than doses used in "standard" CBV.[16] Retrospective evidence from the European Bone Marrow Transplant Registry indicates that Hodgkin's disease patients transplanted with BEAM have a better outcome than with CBV.[22] A randomized trial would likely be necessary to evaluate the superiority of a particular high-dose chemotherapy regimen.

A possible benefit of increasing the doses of the individual agents in CBV must be balanced against increases in toxicity (Table 1). Wheeler

et al performed a dose escalation study of CBV for Hodgkin's disease and non-Hodgkin's lymphoma.[8] Fatal regimen-related toxicity occurred in 2 of 40 patients at a carmustine dose of 450 mg/M^2 in contrast to 4 of 18 patients when the carmustine dose was increased to 600 mg/M^2. The incidence of interstitial pneumonitis was also significantly increased at the higher dose, and also in patients with a history of chest irradiation. Ahmed et al also noted less pulmonary toxicity in patients who received less than 600 mg/M^2 carmustine.[10] Weaver et al noted increasing toxicity with 600 mg/M^2 carmustine, compared with 300 mg/M^2.[11] Grade 3 or 4 pulmonary toxicity occurred in 23% of patients at the higher carmustine dose, compared with 0% at the lower dose. Similarly, nonrelapse mortality was 47% at the higher dose, compared with 14% at the lower dose. Toxicity was more frequent in patients with prior mediastinal irradiation. No decrease in relapse rates were seen with the higher carmustine dose.

It is possible, however, to administer higher dose CBV regimens with low mortality (Table 1). It must be noted that many reports include patients transplanted before institutional experience matured, and prior to the use of hematopoietic growth factors and mobilized peripheral blood stem cells. These conditions are certainly responsible for the marked decline in mortality that we and others have observed.[6,16]

SUMMARY

High-dose chemotherapy with "standard" CBV results in long-term failure-free survival in patients with relapsed or refractory Hodgkin's disease. However, only a relatively small percentage of patients are cured with this regimen. The doses of individual agents in this regimen, particularly etoposide, can be significantly escalated. There is some evidence that outcome in these higher dose CBV regimens may be improved, although a randomized trial would be required to confirm this observation. Continued follow-up of patients treated with these regimens is necessary to identify late events such as relapse and secondary leukemia.

Continued efforts are necessary to improve the results of autologous transplantation for Hodgkin's disease. Such efforts might include the use of higher dose preparative regimens, as discussed above. Alternatively, additional radiation therapy or immunotherapy might be used after the transplant to decrease relapse rates.

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HIGH-DOSE CYCLOPHOSPHAMIDE, CARMUSTINE, ETOPOSIDE + CISPLATIN (CBV+P) AND AUTOLOGOUS STEM CELL TRANSPLANTATION (ASCT) FOR HODGKIN'S DISEASE (HD) IN FIRST RELAPSE AFTER CHEMOTHERAPY.

D. E. Reece

INTRODUCTION

In 1985, we began a policy of offering intensive therapy with CBV+P and ASCT for patients with HD progressive after combination chemotherapy. Between 1985 and 1992 we autografted 100 consecutive HD patients using CBV+P and ASCT. Fifty-eight of these patients were entered onto transplant protocols at the time of their first relapse after a complete remission (CR) induced by combination chemotherapy. The initial results of transplantation in this subset of HD patients have previously been published.[1] This discussion will focus on the updated analysis of these patients, all of whom have been transplanted at least 2 years ago.

PATIENTS AND METHODS

Patients were required to have clear evidence of relapsed Hodgkin's disease either on tissue biopsy or by unequivocal radiographic progression of previous lesions. Other requirements for entry onto transplant studies included age < 60 years, no other serious medical illnesses, presumed incurability with further radiation therapy alone and an adequate source of hematopoietic stem cells for transplantation. Fifty-seven patients with normal bone marrow histology underwent marrow harvesting, while one individual with active marrow involvement underwent steady-state peripheral blood stem cell (PBSC) collections.

Although patients were entered on the study at the time of a first relapse, prior to any salvage attempts, subsequent protocol conventional cytoreduction therapy was given before CBV+P and ASCT in the majority of patients. Specifically, patients with at least a 3 month interval from prior chemotherapy received a median of 2 cycles of MVPP (mechlorethamine, vinblastine, procarbazine and prednisone). During the second cycle of MVPP, patients with bulky disease or disease easily encompassed within a single radiation field received 2000 - 3000 cGy of radiotherapy (RT). These cytoreductive therapies were not used as a

means of chemosensitivity testing to select responding patients for transplantation, but were given to try to achieve a reduction in tumor burden pending the availability of a transplant bed. Patients were not restaged following this conventional cytoreductive therapy, and 100% receiving such therapy were subsequently transplanted. Two conditioning regimens were utilized during this 7 year period. Initially, patients received an "augmented" CBV regimen consisting of cyclophosphamide (total dose 7.2 g/M^2), etoposide (2.4 g/M^2 given in six divided doses) and carmustine (0.5 g/M^2). In 1988, the protocol was modified in an attempt to reduce toxicity. In the CBVP regimen, the same total dose of etoposide was given as a 34-hour continuous intravenous infusion. The dose of carmustine was lowered slightly to 0.5 g/M^2. The dose of cyclophosphamide remained the same and cisplatin was added in conventional doses (total 150 mg/M^2). Hematopoietic growth factors were given after ASCT in 18 patients as part of other protocols.

PATIENT CHARACTERISTICS

Table 1 summarizes the characteristics of the 58 patients in first relapse. After entry onto the transplant protocol, 52 patients received conventional cytoreductive therapies, consisting of MVPP alone (25 patients), MVPP and local RT (24 patients) and local RT alone (3 patients). Fourteen patients were conditioned with CBV while 44 received CBVP. After ASCT, 16 patients received GM-CSF, 1 received G-CSF and 1 was given PIXY 321.

RESULTS

Non-relapse mortality (NRM)

At the time of our initial analysis, with a median follow-up of 2.3 years, 3 pts had died due to non-relapse causes. Two of these deaths occurred early post-transplant and were due to myopericarditis on day 0 and pulmonary hemorrhage on day +16 after transplantation. One late death was observed two years post transplant due to pulmonary fibrosis. In the updated analysis, with a median follow-up of 3.6 years (range 0.6-8.2), two more non-relapse deaths have been seen related to secondary malignancies. One patient has died of a glioblastoma 3 years posttransplant while a second has succumbed to bowel carcinoma 4.6 years after transplantation. No cases of secondary acute myelogenous

leukemia have developed.[2] The actuarial probability of NRM at 7 years is 16% (95% confidence interval [C.I.] 6-40%) (Figure 1).

Table 1. Patient Characteristics

Age in yrs, median (range)	29
Sex, male : female	35 : 23
Histology	
Nodular sclerosing	49
Mixed cellularity	9
Initial stage	
II/III/IV	18/27/13
A : B	25 : 33
Extranodal disease at diagnosis	19
Initial chemotherapy	
MOPP/ABV(D) - like regimens	51
MOPP	3
ABVD	3
Other	1
Prior radiotherapy	20
History of positive marrow	6
Initial CR duration <1 yr	35
"B" symptoms at relapse	16
Extranodal disease at relapse	21

Progressive Disease

A total of 15 patients have experienced progressive HD at a median of 8 months (1-42 months) post-ASCT for a probability of relapse of 28% (95% C.I. 17-43%) (Figure 1). These patients progressed a median of 8 months (range 1-42 months) post-ASCT. Four patients have relapsed > 2 years after transplantation, at 2.2, 2.2., 2.5 and 3.5 years. Three of these had nodular sclerosing and one had mixed cellularity HD.

Progression-free Survival

Thirty-nine patients are alive and continuously without evidence of progressive HD. All of these patients have a normal performance status. The actuarial progression-free survival (PFS) is 61% (95% C.I. 43-74%). (Figure 1). Two additional patients who relapsed post-transplant and received local RT remain in a third CR 4.7 and 7.2 years after their post-ASCT relapse.

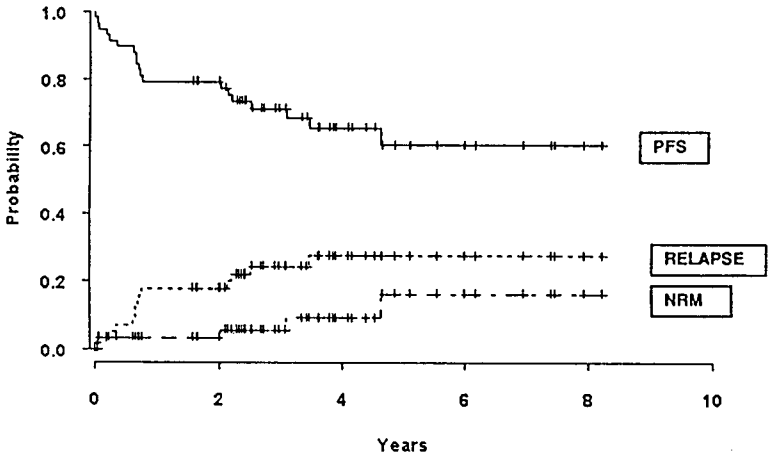

Figure 1. The actuarial curves for probability of PFS, relapse and NRM in 58 pts treated with CBV±P and autotransplantation for HD in first relapse.

Identification of Risk Factors

In our initial analysis, the multivariate analysis identified 3 independent prognostic factors associated with an unfavorable PFS: "B" symptoms at relapse, initial complete remission duration < 1 year and the presence of extranodal disease at relapse. Since these factors had similar relative risks, a simple prognostic model based on a number of adverse factors at the time of first relapse was constructed. In the initial analysis, the PFS for the 11 pts with 0 adverse factors was 100%, compared with 81% in the 26 with 1 adverse factor, 41% in the 17 with 2 adverse factors and 0% in the four with all 3 factors.[1] After an additional 1.3 years of follow-up, one more relapse in the 1 adverse factor group and two secondary malignancies in the 0 and 1 factor group, respectively, have occurred. The updated probabilities of PFS for these four prognostic groups are shown in Table 2. The survival differences remain highly significant ($p < 0.001$). The number of adverse factors also correlated with the probability of disease progression as shown in Table 2.

SUMMARY AND CONCLUSION

In our experience, the use of intensive therapy and ASCT is feasible in the majority of patients with HD whose disease recurs after a

Table 2. The actuarial progression-free survival and probability of disease progression in 58 patients based on the number of adverse prognostic factors ("B" symptoms, extranodal disease, initial complete remission duration <1 year) at first relapse.

		Progression-free Survival			Disease Progression		
No. factors	No. Patients	Probability	95% C.I.	Relative Risk	Probability	95% C.I.	Relative Risk*
0	11	89%	43-98%	1.0	0%	-	
1	26	69%	40-86%	2.81	14%	5-39%	
2	17	48%	21-71%	6.78	52%	29-79%	
3	6	0%	-	69.8	100%	-	
		p<0.001			p<0.001		

*Could not be calculated since no patients with 0 risk factors had disease progression.

complete remission induced by combination chemotherapy. No patients with HD in first relapse seen in our center were excluded from transplantation due to medical contraindications, and although not discussed previously, only 2 patients in first relapse had marrow involvement at this point in their disease course; one received PBSCs and the other allogeneic marrow to support high-dose therapy. Moreover, early posttransplant NRM with this approach was low, i.e. <5% at 1 year post-ASCT. However, these patients remain at risk for fatal late complications which will likely lower the cure rate seen with transplantation. Since late fatal non-relapse events are also seen after nontransplant salvage approaches for HD patients in first relapse[3], prolonged follow-up of these patients will be required to better define these risks and to allow adequate comparison between salvage approaches.

The number of certain adverse biological features present at the time of first relapse ("B" symptoms, extranodal disease and a short duration of CR remission) defines prognostic groups for PFS and relapse after intensive therapy and ASCT. Patients with 2 or 3 risk factors have high rates of tumor progression after transplantation and are candidates for more intensive or innovative therapies. On the other hand, patients with 0 or 1 factors have a low probability of relapse and, at least arguably, may be candidates for less intensive therapy. Possible approaches in the latter patients include the use of lower doses of drugs in the conditioning regimen or the use of repetitive courses of moderately-intensive chemotherapy with growth factor and/or PBSC support.

While our transplant results do not definitively answer the question of the optimal timing of intensive therapy and ASCT in HD, they illustrate that such therapy is capable of curing a high proportion of HD patients in a first relapse and also highlight the importance of prognostic factors among this group of patients which may be useful in further comparative studies. The high PFS rates and low early mortality argue for the application of ASCT in first relapse patients rather than deferring ASCT to more advanced disease states. In our experience, both NRM and disease progression are significantly higher in patients transplanted in a second or greater relapse, or in a relapse resistant to chemotherapy.[4] As in other malignant diseases, the results of intensive therapy and transplantation are anticipated to be optimal early in the disease course.

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OUTCOME OF PATIENTS WITH POOR RISK HIGH GRADE NON-HODGKIN'S LYMPHOMA TREATED WITH AUTOLOGOUS BONE MARROW TRANSPLANTATION

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ABSTRACT

Patients with resistant or recurrent high grade non-Hodgkin's lymphoma (HGNHL) are incurable with conventional chemotherapy. Clinical and laboratory parameters at diagnosis (DX) have helped identify patients with poor prognosis and include age, increased lactic dehydrogenase (LDH), extranodal involvement, response to initial therapy and advanced stage. To improve the outcome of patients with HGNHL, autologous bone marrow transplant (ABMT) have been performed in patients with relapsed HGNHL and in patients in first complete remission (CR) with poor prognostic features. We did a retrospective analysis to determine the peritransplant mortality, response rate (RR) and disease-free survival (DFS) of patients transplanted for HGNHL. Eighteen patients (13 male, 5 females) aged 22-61 (median, 37) were treated. Nine patients had immunoblastic, 6 lymphoblastic, and 3 small non-cleaved lymphoma. All patients had at least one adverse prognostic factor. Three patients had stage I, 4 stage II, 4 stage III and 7 stage IV disease. Four patients had central nervous system (CNS) and 5 bone marrow (BM) involvement sometime during their disease. Seven patients had high LDH, 6 bulky disease. Nine pts had B symptoms. At ABMT 5 patients were in first CR, 4 had sensitive relapse, 7 primary refractory or resistant relapse and 2 had untested relapses. Conditioning regimens consisted of CBV$\pm$DTIC in 12 patients, Cy/TBI in 5 and Cy/VP-16/TBI in 2. Peritransplant mortality was 11% (2/18). The RR was evaluable in 12 patients and was 41%. Sixty-six percent (12/18) of patients are alive 2-37 months (mos) post ABMT. The DFS is 55% (10/18) with a median follow-up of 14 mos (2-37). We conclude that ABMT can be performed with acceptable mortality in patients with HGNHL and that durable remissions can be achieved in a significant proportion of patients with poor prognosis HGNHL.

INTRODUCTION

Combination chemotherapy achieves a complete remission rate (CR) in 60 to 80% of patients with aggressive non-Hodgkin's lymphomas (NHL).[1] Nevertheless, the long-term disease-free survival (DFS) at 5 years is 30 to 50%[2] and patients that relapse eventually die of their disease. High-dose chemotherapy with stem cell rescue provides long-term DFS in 20-25% of patients with relapsed NHL.[3] Prognostic factors have identified patients with NHL at high risk for relapse after induction chemotherapy. The International Prognostic Factors Index for NHL defined low, intermediate and high risk patients based on age, Ann Arbor stage, extranodal disease, performance status, and lactic dehydrogenase levels.[4] Patients in the high risk index had a long-term DFS of 26%.

High grade NHL are rapidly growing tumors with a propensity to spread to the bone marrow (BM) and central nervous sytem (CNS). Patients with high grade lymphomas that have adverse prognostic factors such as bulky disease, CNS or bone marrow involvement or high LDH, have a very poor outcome. Furthermore, patients with refractory high grade NHL are incurable with conventional chemotherapy and eventually die of their disease. Owing to the relatively small number of adult patients with high grade non-Hodgkin's lymphoma, few studies evaluating the role of high dose chemotherapy and stem cell rescue are available.[5,6] We report here the clinical characteristics, toxicities and outcome of patients with poor risk high grade NHL according to the Working Formulation that were treated with high dose chemotherapy and autologous bone marrow transplantation at our institutions.

PATIENTS AND METHODS

Between January 1991 and May 1994, 18 patients with poor risk, high grade NHL underwent autologous bone marrow transplantation at our institution. Patients included those with at least one of the following high risk features: high LDH (>250), extranodal disease (BM or CNS), bulky disease, relapse or failure to achieve CR after induction chemotherapy. Myeloablative therapy included TBI and non-TBI preparative regimens. Response to high dose chemotherapy and BMT was evaluated by a physical examination, CT scans, bone marrow biopsies and CSF analysis if initially abnormal. Bone marrow transplant mortality was defined as all deaths within the first 100 days of the transplant unrelated to diseased progression.

RESULTS

Patient characteristics (Table 1): The age range of our patients was 23 to 61 with a mean of 37 years. Of the 18 patients, 13 were male and 5 were female. Histologic subgroups included 9 immunoblastic, 6 lymphoblastic and 3 small non-cleaved NHL. By immunophenotypic analysis 8 were B-cell and 5 were T-cell. Adverse prognostic factors included 4 events of CNS involvement, 5 events of BM involvement, 5 with bulky mediastinal disease and 7 patients with high LDH.

Treatment prior to BMT: Patients were treated with various regimens that consisted of multiagent chemotherapy including an anthracycline drug. All patients with central nervous system involvement received intrathecal chemotherapy prior to BMT. Twelve out of 18 patients (66%) attained a complete remission (CR). Status at BMT included: 4 patients in first CR, 5 in sensitive relapse, 7 patients had resistant relapse or failed to achieve a CR after induction therapy, and 2 patients had untested relapse.

Conditioning regimen (Table 2): Several conditioning regimens were used and included: cyclophosphamide 1.8 mg/M^2/d x 4 days, VP-16 800 mg/M^2/d x 3 and BCNU 450-600 mg/M^2 for 1 day (CBV) in 6 patients. Six additional patients received the same regimen plus DTIC 5000 mg/M^2/d x 1 day (CBVD). Two patients received cyclophosphamide 100 mg/kg/d x 1 day, VP-16 60 mg/kg/d x 1 day and TBI 1200 cGy. Four patients received cyclophosphamide 60 mg/kg/d x 2 days, TBI: 1200 cGy.

Peritransplant mortality: Two out of 18 patients (11%) died before day 100. Both patients died of sepsis on day 22 posttransplantation.

Response rate: Six patients were in CR at the time of transplantation. The remaining 12 patients were evaluable for response: 5 of these (41%) attained CR. Four patients experienced progressive disease, one had a partial remission and two patients died too early for reevaluation.

Survival and disease-free survival: Twelve out of 18 or 66% of patients in this study remain alive 2-37 months (median 14) posttransplantation. Ten patients (55%) are in continuous complete remission with the same follow-up. Patients with sensitive disease (1st CR and sensitive relapse) at the time of transplantation had a DFS of 75%

Table 1. Patient Characteristics at Diagnosis, Initial Therapy and Outcome

No.	Sex	Age	Bulky Disease	CNS	BM	LDH	Histology (Immunophenotype)	Stage at Diagnosis	Initial Therapy	Outcome
1	M	49	-	-	+	8396	Immunoblastic	IV-B	ABVMT	PR
2	M	23	+	+	-	546	Lymphoblastic	IV-A	Linker	CR
3	M	26	+	-	+	N/A	Lymphoblastic	IV-B	Linker	CR
4	M	28	+	+	+	2280	Lymphoblastic	IV-B	DCVLa	CR
5	F	23	+	-	-	132	Lymphoblastic	II-B	Linker	CR
6	M	17	+	-	-	356	Lymphoblastic	III-A	DVPLa	CR
7	F	29	-	-	-	289	Lymphoblastic	IV-B	DVPM	CR
8	F	25	-	-	-	280	Small Noncleaved	IV	CHOP	CR
9	M	27	+	-	+	N/A	Immunoblastic	IV-B	VACOP-B	PR
10	M	22	+	-	+	195	Immunoblastic	III-B	CHOP	NR
11	M	55	+	-	-	393	Immunoblastic	II-A	CHOP	CR
12	F	45	+	-	-	173	Small Noncleaved	II-B	CHOP	CR
13	M	42	-	-	-	N/A	Immunoblastic	I-A	MCOP	CR
14	F	61	-	+		149	Immunoblastic	III	Promace-Cytabom	CR
15	M	59	-	+	-	173	Small Noncleaved	I-A	XRT	NR
16	M	59	+	-	-	N/A	Immunoblastic	II-A	CHOP	PR
17	M	59	-		-	N/A	Immunoblastic	I-A	CHOP	CR
18	M	40	+	-	-	N/A	Immunoblastic	III-B	Promace-Cytabom	NR

B= Bleomycin, M=Methotrexate, P=Prednisone, D=Daunorubicin, A,H=Doxorubicin, La=Asparaginase, O,V=Vincristine, Linker=Linker induction: D, V, La, P

Table 2. Pre-Transplant Characteristics, Conditioning Regimen and Outcome

No.	Interval from Dx to BMT	Status at BMT	Conditioning Regimen	Outcome of BMT
1	10	1st PR	CBVD	CCR 12+ months
2	5	1st CR	CY/TBI	CCR 4+ months
3	38	1st CR	CBV	CCR 9+ months
4	9	2nd PR	CY/TBI	PD died 3 months post BMT
5	33	2nd relapse	CY, VP-16/TBI	CR (relapsed 6 months post BMT)
6	10	1st CR	CY/TBI	CCR 37+ months
7	7	1st CR	CY/TBI	CCR 19+ months
8	6	2nd PR	CBV	CCR 20+ months
9	4	1st PR	CBV	CCR 22+ months
10	8	PD	CY, VP-16/TBI	PD died d+ 22 of sepsis
11	8	PD	CBVD	PD died d+ 39 of BMT
12	9	1st CR	CBVD	CCR 16+ months
13	9	1st relapse	CBVD	PR died d+ 79 of BMT
14	36	2nd PR	CBV	CR relapsed 6 months post BMT
15	28	PD	CBV	CCR 2 months post BMT
16	7	1st PR	CBV	Died of sepsis d 22+ BMT
17	19	2nd CR	CBVD	CCR 7+ months
18	6	PD	CBVD	PD died 4 months post BMT

CY, C=Cyclophosphamide, B=BCNU, D=Dacarbazine, TBI=Total Body Irradiation, V, VP-16=Etoposide

(9/12). Patients with resistant or untested relapse had a DFS of 16% (1/6). Complete remissions were obtained in all three histologic subgroups of HGNHL (lymphoblastic = 4/6, immunoblastic 3/9, and small non-cleaved cell = 3/3).

DISCUSSION

High grade NHL have a high cure rate in the pediatric population.[6] Outcome with combination chemotherapy in adults yield poorer results;[7] especially in patients with bulky disease, CNS or BM involvement, advanced stage, or high LDH. For these reasons, patients with poor prognostic factors have been considered candidates for transplantation.[5,7] There is limited experience using high dose chemotherapy and stem cell rescue in patients with high grade NHL. In the present study we evaluated the peritransplant mortality, DFS and overall survival of poor risk, high grade NHL patients that underwent ABMT. All patients had at least one adverse prognostic factor and were treated prior to BMT with chemotherapy that included an anthracycline and treatment to the CNS (XRT or intrathecal chemotherapy) if CNS involvement was present at diagnosis.

Our peritransplant mortality was 11% (2/18). Both patients died of sepsis early during the transplant process. Since six patients were transplanted while in complete remission, 12 patients were evaluable for response after transplantation. Five out of 12 patients attained a CR posttransplantation for a CR rate of 41%. Sixty-six percent of our patients (12/18) remain alive 2-37 months posttransplantation (median 14). Ten patients (55%) remain alive and free of disease with the same median follow-up. Similar to the experience with patients undergoing ABMT for intermediate grade NHL, the DFS rate was higher in patients with sensitive relapse compared to patients with primary refractory or resistant relapse (50% vs 16%). Nevertheless, it has to be noted that two patients who failed to achieve CR with induction chemotherapy are in continuous complete remission (CCR) 12 and 22 months post ABMT. One additional patient with progressive disease remains in CCR very early after ABMT. It is of interest that complete remissions were obtained in all histologic subtypes of high grade non-Hodgkin's lymphoma.

Infusion of malignant cells in the BM graft remains a major concern in high grade non-Hodgkin's lymphoma patients treated with high dose chemotherapy and autologous stem cell rescue. This is especially true in patients with prior bone marrow involvement.[8] In our analysis, 5

patients had prior bone marrow involvement. Three of them remain in CCR at 9, 12, and 22 months posttransplantation, suggesting that durable remissions can be obtained even in patients with prior bone marrow involvement.

In summary, our experience suggests that autologous BMT can be performed in patients with high grade NHL with acceptable peritransplant mortality. A high percentage of patients remain in CCR, even some patients with primary refractory disease and patients in resistant relapse. Prospective randomized studies and longer follow-up are needed to better define the role of auto BMT in high risk patients with HGNHL.

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SESSION VI: MYELOMA

TRANSPLANTS FOR MULTIPLE MYELOMA

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INTRODUCTION

Multiple myeloma (MM) is characterized by marked resistance to standard doses of therapy effecting complete responses (CR) in no more than 5% of patients, possibly due to the marked genetic alterations apparent on cytogenetic examination.[1] Permutations of the gold standard of therapy, melphalan and prednisone (MP), unfortunately, have not improved survival[2]. In addition to searching for more effective new drugs, MM investigators began evaluating high dose therapy, requiring transplants, in the mid-1980s.[3-6] More marked tumor cytoreduction can be achieved with autotransplant approaches resulting in CR rates of up to 50%.[7] Utilizing peripheral blood stem cells (PBSC) with hematopoietic growth factors, the duration of marrow aplasia is exceedingly short so that transplant-related mortality is below 5%.[8] Many issues and controversies remain to be addressed with regard to myeloablative therapy, which are summarized in Table 1. This report provides a brief synopsis of the areas of (1) standard therapy; (2) increase in transplant activity for MM; (3) results of a single investigator team initially at the University of Texas M.D. Anderson Cancer Center and later at the University of Arkansas for Medical Sciences (UAMS); (4) transplant results worldwide; (5) and future research directions.

Table 1. Problems with MM Therapy

- Consensus
  - Marked drug resistance to standard dose therapy, low CR rate, no cures
  - Greater tumor cytoreduction with high dose therapy→ 50% CR, <5% mortality with auto Tx

- Issues
  - Do higher CR rates translate into more durable remissions and longer survival; cure fraction?
  - Auto Tx
    - √ Optimal regimen; greater dose intensity (2 Tx)
    - √ PBSC vs ABMT: supportive care, tumor reseeding
    - √ Tumor cell depletion of autografts
    - √ Post Tx maintenance
  - Allo Tx vs auto Tx
    - √ Is greater risk justified by sustained remissions (GVM effect?)

STANDARD THERAPY TRIALS

Under the auspices of the National Cancer Institute, the Southwest Oncology Group (SWOG) has conducted standard dose trials in over 2,300 patients between 1974 and 1990, not revealing a significant improvement in overall survival (Figure 1).

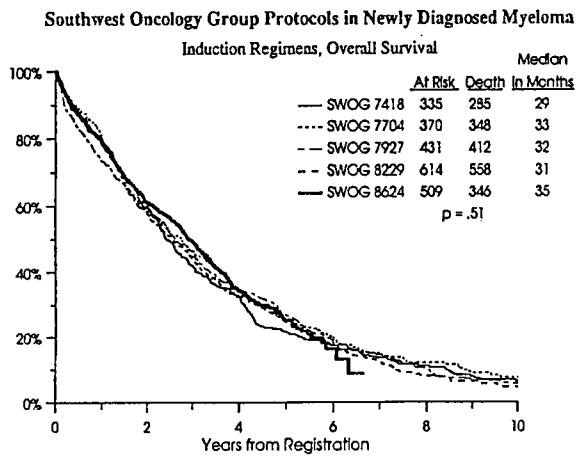

Figure 1.

TRANSPLANT REGISTRATIONS TO NAABMTR/IBMTR

As of January, 1994, 369 autotransplants and 190 allotransplants have been reported by 48 and 51 teams, respectively, to the NAABMTR and IBMTR. There has been a doubling of registrations in both autologous and allogeneic transplants over the past 3 years. The 2-year survival is approximately 35% to 40% for all 559 patients reported.

UNIVERSITY OF ARKANSAS FOR MEDICAL SCIENCES (UAMS) DATA

Two hundred eighty-seven patients have undergone autotransplants at UAMS and M.D. Anderson. Prior to 1990, ABMT was used (with up to 30% plasma cells) in support of TBI 850 cGy with either melphalan 140 mg/M^2 (MEL 140) or thiotepa 850 mg/M^2 (THIO 850). Since 1990, both PBSC plus bone marrow were collected following cyclophosphamide 6 g/M^2 plus GM-CSF with the intent to perform 2 successive autotransplants 3-6 months apart. All patients received MEL 200 for the first and MEL 200 or MEL plus TBI with the second transplant, according to responsiveness to the first transplant (Figure 2). The median age was 50 years, one-third had stage III at diagnosis, over 50% had been treated for more than one year, and 40% were refractory to standard therapy. For all 287 patients, early mortality was 3%, 27% achieved CR; median durations of event-free survival (EFS) and overall survival (OS) were 23 and 40 months, respectively, after transplant. Six prognostic factors were identified which affected both EFS and OS markedly; duration of prior therapy, beta-2-microglobulin (B2M) prior to transplant and immunoglobulin isotype all retained independent prognostic significance on multivariate regression analysis. The combination of these 3 parameters provided excellent separation of risk groups in terms of CR rates, EFS and OS durations. In order to discern the effect of the second transplant on outcome, a six month landmark analysis was conducted for the surviving 230 patients. In addition to the aforementioned pre-transplant parameters, completion of 2 transplants emerged as an independently significant favorable variable for both EFS and OS.

Current practice in the investigators' program (UAMS) utilizes the "Total Therapy" program, employing a series of mutually non-cross resistant induction regimens (VAD x 3, HDCTX, EDAP) followed by 2 autotransplants (or allotransplant after one autotransplant in case an HLA-matched sibling donor is available). Interferon maintenance was offered to all autotransplant recipients.[9] Approximately 75 to 80% of the patients

completed the intended second transplant. Among 66 evaluable patients, 50% achieved CR after 2 transplants whereas, on an intent-to-treat basis, the CR rate was 35%. At 30 months, EFS and OS are projected at 65% and 80%, respectively. The durations of neutropenia <500/mL and thrombocytopenia <50,000/mL with HDCTX, EDAP and both transplants did not exceed a median of one week.

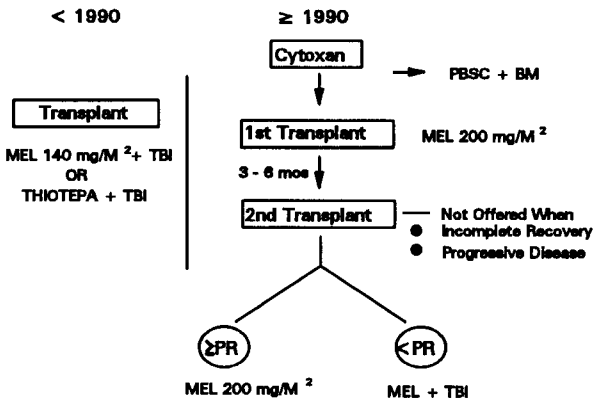

Figure 2. *Treatment plan.*

WORLDWIDE RESULTS

Results of 6 large autotransplant studies for recently diagnosed MM are summarized in Table 2 (mostly < 1 year from diagnosis).[10-15] Among these 571 patients with a median follow-up of approximately 2 years, early mortality (< 2 months) was under 5% and approximately 40% achieved CR. EFS was 30 months and OS had a median of 4 to 5 years post-transplant. Similar data for 5 allotransplant trials are summarized in Table 3.[10,16-19] Among 268 patients, the median age was 43 years, the median time interval from diagnosis to allotransplant was 2 years, 40% suffered early mortality (usually defined as deaths within 100 days post-transplant), 35% achieved CR and apparent plateaus emerged for both EFS and OS at about 35 to 40%. Detailed information, however, is lacking with regard to true myeloma-free survival.

Investigators from 8 different institutions (Table 4) kindly provided detailed clinical characteristics and outcome of 765 patients (Table 5). Results are depicted separatelv for autologous and allogeneic transplant in

Table 2: AUTOTRANSPLANTS FOR RECENTLY DIAGNOSED MM

GROUP	N	F/U MOS	Tx REGIMEN	GRAFT	AGE	MOS TO Tx	SENS	%ED	%CR	EFS	OS	PROGNOSIS
Anderson	32	24	MEL or Cy TBI 1200	ABMT (P)	45	22	+	3	34	24 mos	70% at 48 mos	
Attal	38	20	MEL 140 TBI 800	ABMT	57	2	+	3	25	80% at 30 mos	83%	
Cunningham	53	31	MEL 200	ABMT	52	7	+	2	75	25 mos	63% at 54 mos	
	84	37	MEL 200	ABMT ±IFN	52	6	+	0	77	39 mos IFN 27 mos ⊖	87% at 48 mos 74% at 48 mos	
Fermand	63	44	MEL + TBI 1000 -1200	PBSC	44	4	±	11	26	43 mos	59 mos	B2M at Dx
Harousseau	168	24	MEL-TBI	ABMT (102)	52	130 ≤ 6mos 38 > 6mos		5	35	32% at 36 mos	41 mos	B2M at Tx
Jagannath ≤ 12 mos	14	57	MEL-TBI 850	ABMT	44	7	±	0	33	24 mos	66 mos	B2M at Tx
	119	15	MEL 200	PBSC	49	8	±	2	31	37 mos	68% at 43 mos	
Total	571	~24 mos			50			(< 5)	[~42]	30 mos	4-5 yrs post Tx	

Table 3: ALLOTRANSPLANTS FOR MM

GROUP	N	Tx REGIMEN	%SENS	AGE	MOS TO Tx	%ED	%CR	EFS	OS
EBMT	162	TBI	~50	43	19	~40	46	37%-4 yr	30%-5 yr
Bensinger	60	BuCy	35	43	6 mos - 9.2 yr	40	37	48%-2 yr*	68%-2 yr*
Vesole	35	BuCy	19	43	24	26	34	22%-2 yr	43%-2 yr
Cavo	33	BuCy (19) TBI (14)	48	43	21	64	33	26 mos	36%-19 mos
Anderson	18	TBI 1200	100	44	25	36?	44	50%-2.5 yr	60%-2.5 yr
Total	268	TBI or BuCy	50	43	24	(40)	[35]	35%	35-40%

* CR's only, detailed information not available

Figure 1. With autotransplants, using univariate and multivariate regression analyses, the combination of duration of prior therapy, patient age and Ig isotype emerged as independently important variables permitting definition of good, intermediate and poor risk groups (Figure 3). With allotransplants, patient age and sensitivity to standard therapy were the only important pre-transplant variables with significant differences in EFS and OS (Figure 4).

Table 4. Auto and Allotransplants in MM

Group	Auto	Allo
Dana-Farber Cancer Institute, USA	32	18
Hopital du Hasenrain, France	12	0
Hopital du Haut Leveque, France	50	0
Mayo Clinic, USA	20	0
Royal Marsden, United Kingdom	223	0
UCLA, USA	33	5
University of Arkansas, USA	287	35
Vancouver General Hospital, Canada	29	21
Total	686	79

Table 5. Patient Characteristics (N=765)

Parameter	Percent of Patients		P
	Auto (N=686)	Allo (N=79)	
Age >50	49	13	.0001
Male	69	66	.5
IgG	60	44 }	
IgA	17	14 }	.02
Other	23	42 }	
Stage III	55	61	.4
>12 mos from Dx	48	67	.0001
Resistant*	33	42	.2
B2M pre Tx 2.5*	44	51	.3

** Available in 75% of patients*

SUMMARY AND PERSPECTIVES

The clinical trial results of the "first phase" of transplant activity for MM are gradually maturing and permit the following conclusions: true biochemical and hematologic CR can be obtained in about 40%; early

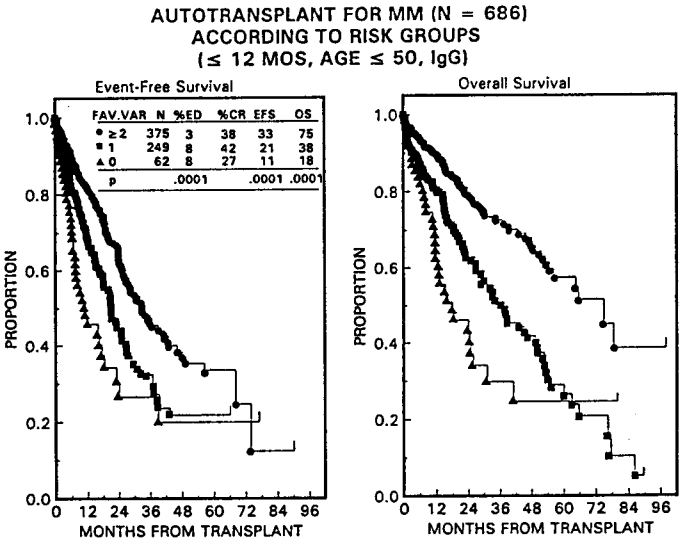

Figure 3.

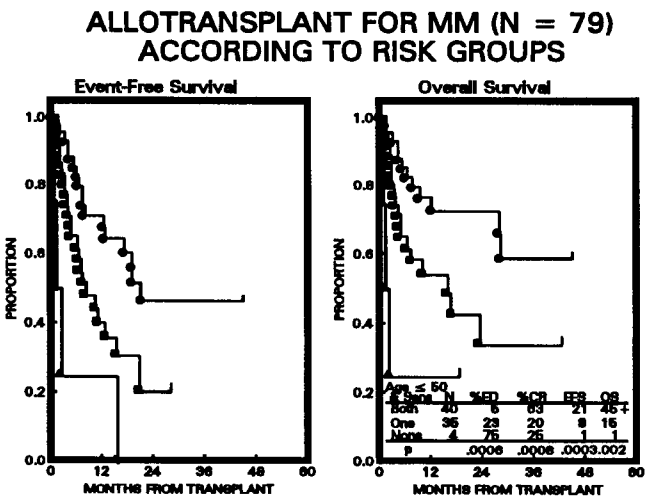

Figure 4.

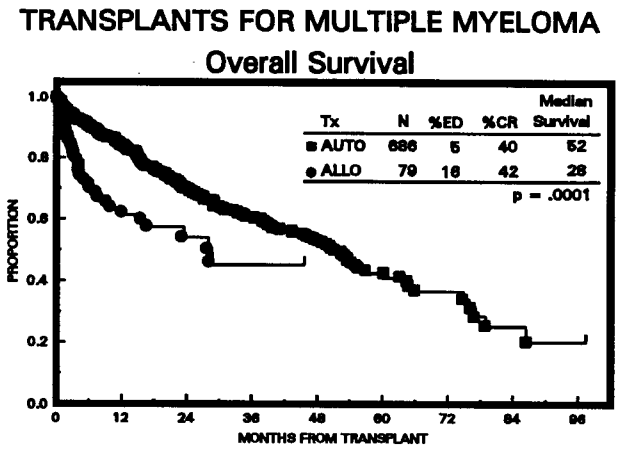

Figure 5.

mortality with autotransplants is less than 5% and about 40% with allotransplants. Plateaus are not yet apparent for either EFS or OS with autotransplants, whereas this seems to be the case after allotransplants (Figure 5). The much higher procedure-related mortality with allotransplants compared to autotransplants poses a difficult decision for both patients and physicians, since definitive data are not available as to how many patients will survive without evidence of MM beyond five years after allotransplant.

Because of the possibility of selection bias with historical comparisons, randomized trials comparing standard therapy with autotransplants are underway by the French myeloma group (revealing superior outcome for the transplant arm),[20] whereas such study has just begun in the United States under the auspices of the National Cancer Institute. Most MM investigators agree that the optimal preparatory regimen for MM transplants has yet to be defined, although we believe that the marked resistance of MM requires greater dose intensity as applied with tandem transplants (see Table 1). The issue of PBSC versus ABMT pertains to aspects both of supportive care and tumor cell reseeding. Both positive hemopoietic stem cell selection as well as tumor cell depletion ("purging") are under investigation. Post-transplant therapy has largely been limited to interferon maintenance, with a superior outcome reported by the Royal Marsden group.[12] The use of idiotype vaccines offers a highly tumor-specific approach that has been investigated in lymphomas.[21]

The use of molecular genetics has made possible the detection of minimal residual disease utilizing PCR for Ig hypervariable region genes (CDR III)[22]. Likewise, insertion of marker genes into autografts should help in the understanding of both normal hemopoietic reconstitution from PBSC versus ABMT and define the contribution to relapse from tumor cell reseeding with autografts, analogous to pilot trials in acute leukemia.[23-25] Gene therapy approaches have not yet been initiated in MM, although our group is focusing on transduction of CD16 into CD34 hemopoietic stem cells in an attempt to achieve high levels of soluble CD16 (functioning as IgG-binding factor) which has been shown to down-regulate myc, inhibit Ig secretion and induce tumor cell apoptosis in IgG MM.[26]

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CD-34 POSITIVE PERIPHERAL BLOOD STEM CELL TRANSPLANTATION IN MULTIPLE MYELOMA.

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Multiple myeloma (MM) is characterized by the accumulation of malignant plasma cells in the bone marrow which secrete monoclonal immunoglobulin (Ig) proteins. Despite the sensitivity of these cells to alkylator chemotherapy, median patient survival remains at 30 months even with the use of standard doses of multi-agent chemotherapy.[1] In an attempt to improve upon these results, dose-intensive chemotherapy with marrow transplantation has been proposed as a means of improving progression-free survival. Although allogeneic transplantation appears to be curative in some patients,[2] most cannot receive this therapy because of their elderly age at presentation and subsequent high treatment related mortality. Autologous bone marrow transplantation has therefore been performed in a greater number of patients and has produced 3 year progression-free survival rates of 40-60%. Although complete response rates are high, most patients relapse and there does not appear to be a plateau in the survival curve which would be indicative of cure.

A major potential problem of autologous bone marrow transplantation (BMT) is autograft contamination. Since myeloma is a bone marrow based disease the autograft product is most likely heavily contaminated and therefore numerous malignant cells are reinfused into the patient after the myeloablative chemotherapy has been completed. In order to reduce this tumor cell contamination, stem cell transplants involving substitution of an autologous peripheral blood product for bone marrow (BM) have been performed. This should reduce the burden of tumor cells in the product and appears to speed hematologic recovery potentially reducing treatment related morbidity and mortality. However it is clear from studies in our own lab and by others that the peripheral blood remains contaminated by tumor cells in MM patients although to a lesser degree.[3,4] In fact, recent studies have documented contamination of the mobilized leukapheresis product prior to reinfusion.[5] It should therefore be of therapeutic benefit to eliminate these malignant cells thus providing

the patient with a tumor-free autologous product that supports hematopoietic recovery. Although autograft purging procedures have been developed, the techniques described to date have resulted in prolonged engraftment times which may increase treatment related mortality.[6,7]

Recent studies show that cells positively selected for CD34, an antigen expressed on early hematopoietic cells, can support recovery following myeloablative chemotherapy.[8] Since myeloma is thought to be a malignancy of mature B lymphoid cells (plasma cells), positive selection using an antigen expressed on early progenitors, CD34, may produce a tumor-free autologous product. To verify this, we initially selected CD34 expressing cells from the BM of MM patients with advanced disease by passage over a CEPRATE immunoadsorption column (CellPro, Bothell, WA). Utilizing a sensitive PCR-based technique with patient-specific immunoglobulin gene primers, this initial selection resulted in a 1.5 - 2.5 log reduction in tumor contamination.[9] When the CD34+ cells were further purified by FAC-sorting using the HPCA-2 antibody (Becton Dickinson, Mountain View CA), no malignant cells were detectable in this highly purified population. Consequently, cells selected for expression of the CD34 antigen should yield a product capable of supporting hematopoietic reconstitution following myeloablative chemotherapy with a reduction in myeloma tumor cell load.

As a result, we conducted a multi-institutional pilot trial using CD34-selected peripheral blood progenitor cells to support hematopoietic recovery following high-dose chemotherapy in the treatment of advanced multiple myeloma. Forty-two patients with myeloma age 34-65 years (median 51 years) were entered with a median time from diagnosis to treatment of 9 months (range 4-47 months). Thirty-five patients have completed the transplantation portion of the trial. In order to be enrolled on the study, patients were required to have evidence of a response or lack of progression to conventional chemotherapy and/or high dose steroids. Three patients had nonsecretory disease but had evidence of treatment response by bone marrow analysis. After enrollment, progenitor cells were harvested 14 days after intermediate dose cyclophosphamide (2.5 g/m^2 IV), prednisone (2 mg/kg PO QD x 4), and G-CSF (10 mg/kg SQ QD x 14). Leukaphereses were performed for 2 - 5 days and CD34+ progenitor cells were purified using a cellular immunoadsorption method (CellPro, Bothell, WA) by passage over a column of avidin-coated beads after labeling with a biotin-conjugated 12-8 antibody.

The adsorbed fraction represented 0.3% - 1.4% of the starting leukapheresis cell population and the CD34 purity of the collection ranged

from 27% to 91% (median 77%). A median of 6.4 x 10^6 adsorbed cells/kg were reinfused 1 day after preparative conditioning with busulfan (0.875 mg/kg Q6h x 16 doses) and cyclophosphamide (60 mg/kg QD x 2). Following stem cell infusion, GM-CSF (500 mg IVPB) was given daily until hematopoietic recovery. Patients who achieved a complete or partial remission following the autologous transplant received alpha-interferon-2b (3 x 10^6 IU/m^2) TIW and decadron (20 mg/m^2 QD x 4 every four weeks) beginning 100 days after transplantation and continued for one year or until there was evidence of progressive disease. Engraftment data showed the median time to ANC > 500/mm^3 and untransfused platelets > 20,000/mm^3 were 13 d (range 11-16 d) and 12 d (range 9 - >52 d), respectively. The median number of units of PRBCs and platelets transfused were 7 (range 2-27) and 3 (range 0-71), respectively. The threshold dose to achieve rapid hematopoietic recovery was 2 x 10^6 CD34 cells/kg, below which platelet engraftment was prolonged and incomplete.

There have been two treatment related deaths. One patient experienced disease relapse and died from progressive disease. All remaining patients except one have shown either a complete or partial response to the transplant procedure. Because of the short median follow up time, many of the patients achieving less than complete responses continue to show ongoing reduction in paraprotein levels. Two patients have died of late pulmonary toxicity (days +177 and +217).

We are in the process of determining the efficacy of the CEPRATE immunoadsorption column in tumor reduction of the autografts used for transplantation. Since our previous studies show that the Ig heavy chain produced by the MM cells shows a high degree of somatic mutation but does not exhibit any clonal diversity,[10] the Ig heavy chain variable region (V_H) sequence can be used as a specific marker capable of identifying all malignant cells. The V_H sequence was determined by the production of cDNA from BM RNA using M-MLV (Gibco, Gaithersburg, MD) and an oligonucleotide complementary to either Cg or Ca depending upon the type of Ig secreted by the tumor cells (IgG or IgA respectively). The cDNA product was then aliquoted into six different tubes and the PCR was performed after the addition of one of six V_H family specific primers.[11] The products were then run on an agarose gel and only one band of appropriate size was noted after ethidium bromide staining, which identified the germline V_H gene family used by the myeloma clone. The PCR product was then extracted and sequenced using the Sequenase II kit from US Biochemicals (Cleveland, OH). At least three clones were sequenced and these needed to be identical to prove that the sequence

obtained was from the myeloma cell. The myeloma V_H sequence was then compared to all known germline sequences using a DNAsis program (Hitachi, San Bruno, CA) and a high degree of somatic mutation of the gene was documented in the thirteen patients studied to date. This was similar to previous results obtained by us and others which noted a high number of somatic mutations in the V_H sequences in multiple myeloma.[10,12] Oligonucleotide primers which were complementary to the V_H complementarity determining region (CDR) sequences were then designed for use in the patient specific tumor detection assay. These primers should be unique for the myeloma V_H sequence because of the additional nucleotide mutations that were present in the CDRs.

In order to improve assay sensitivity and more accurately quantitate tumor contamination we have developed a new quantitative PCR technique based on Poisson distribution analysis of positive PCR results.[13] First, DNA was extracted from leukapheresis specimens before and after CD34-CEPRATE selection, and serially diluted in placental DNA to maintain a final DNA quantity of 0.6 mg. Next, five PCR reactions were performed at each serial dilution, and tumor burden was quantitated by the percentage of positive reactions at each level of serial dilution and Poisson distribution statistics. Assay specificity was confirmed by the absence of product when placental or a different patients leukapheresis DNA was substituted for sample DNA. Six tenths of a microgram of DNA was used in each PCR which is the approximate quantity of DNA present in 100,000 cells. Since at least five replicate reactions were performed at each dilution, the assay sensitivity should be one tumor cell in 500,000 normal cells if the PCR could detect one target gene copy. This degree of sensitivity was confirmed by PCR of the same patient's BM DNA serially diluted with placental DNA. Using an assay sensitivity of 1:100,000 per tube, the calculated tumor contamination rate of the BM obtained by this assay matched the percentage of plasma cells noted on the original cytospin of the same BM sample.

Prior to CD34 selection, tumor cells were detectable in the leukapheresis product in seven of the thirteen transplanted patients studied to date. The degree of contamination varied from 0.23% to 0.0015% in these seven patients. Tumor contamination of the CD34-selected autologous product was detectable in only two of the thirteen patients, both of whom had detectable tumor in the original leukapheresis product. For these two patients, the percentage of contamination was reduced by over 1 log by the CD34 selection procedure. Moreover, in the remaining eleven cases, tumor cells became undetectable in the adsorbed population

indicative of a tumor contamination rate of < 0.0002%. In addition to an improvement in purity of the autograft, the number of cells reinfused to the patient after CD34 selection was further diminished by 2-2.5 logs. Consequently, the CD34 selection procedure resulted in an overall 3-4.5 log reduction in the number of tumor cells present in the selected stem cell product.

In conclusion, CD34-selected peripheral blood progenitor cells are an effective form of purified hematopoietic support for patients with MM undergoing myeloablative chemotherapy. Patients receiving this form of selected autograft support should receive a minimum of 2 x 10^6 CD34+ cells/kg in order to assure that engraftment occurs in a timely fashion. The selection of hematopoietic cells bearing this antigen leads to a marked reduction (3-4.5 logs) in the number of tumor cells in the autologous stem cell product without apparent prolongation of engraftment time when compared to historical controls. Further follow up will be required to determine the impact of this therapy on long-term progression-free survival. A multi-institutional phase III study comparing CD34 selected vs. unselected autologous peripheral blood transplantation for MM is being initiated and should begin enrolling patients by the end of 1994.

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IMMATURE MALIGNANT PLASMA CELLS IN G-CSF STIMULATED PBSC FROM MYELOMA

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ABSTRACT

It has been postulated that in haemopoietic malignancies autologous peripheral blood stem cell collections (PBSC) are less likely to be contaminated with malignant cells than autologous marrow. In myeloma however, cells containing rearranged immunoglobulin genes can be detected in the peripheral blood of up to 50% of patients. To investigate the nature of malignant cells in G-CSF stimulated PBSC collections in myeloma we have used flow cytometric techniques to study the PBSC collections of 6 myeloma patients following high-dose cyclophosphamide and G-CSF priming. Plasma cells, identified by the characteristic high intensity CD38 expression, comprised 2.3±1.4% of the PBSC collections. Based upon reactivity with anti-CD45 antibody, we found that the vast majority (86±12%) of these plasma cells expressed an immature phenotype (CD45+). By comparison 51±23% of plasma cells in the bone marrow of myeloma patients (n=31) were CD45+. Analysis of these CD38hi CD45+ cells in the PBSC collections demonstrated;

1. Expression of CD56 and/or light chain concordance with the malignant clone.
2. Higher plasma cell labelling indices compared to the CD45- plasma cells in 3 of 6 cases studied (mean 4.3% vs 0.9%).
3. Higher levels of nucleoside transporters compared to the CD45- population in 3 of 4 cases. This increase was marked in the high intensity CD45+ subset (up to 20,000 transporters/cell).
4. Minimal expression of the CD34 antigen (<4%).

In summary, PBSC collected following cyclophosphamide and G-CSF priming, contain primitive malignant plasma cells many of which have a significant proliferative capacity. These cells do however appear to have minimal expression of CD34 suggesting that further purification based upon CD34 selection may be of value in such patients.

INTRODUCTION

The failure of conventional chemotherapy to induce a significant number of long-term disease responses in patients with myeloma has lead to the evaluation of a variety of forms of high-dose chemotherapy and haemopoietic stem cell (HSC) transplantation for patients with this disease. Despite the encouraging long-term responses with a small number of syngeneic transplants, widespread application of allogeneic transplantation is currently limited by a variety of factors including recipient age and donor availability.[1] Autologous transplantation using either blood or bone marrow HSC is therefore the most practical alternative for patients with this disorder.

It has been postulated, that in haemopoietic malignancies autologous peripheral blood stem cells are less likely to be contaminated with malignant cells than autologous bone marrow and therefore may be the optimum source of HSC. In myeloma, such a choice is favoured by the frequent inability to obtain a morphological clearing of the malignant cells from the marrow and by the generally faster haemopoietic recovery of PBSC autotransplants compared with marrow. Finally, haemopoietic reconstitution is believed to be enhanced by the use of haemopoietic growth factors (HGF) to aid both PBSC harvesting and recovery following infusion.

It is well documentated that although plasma cells are rarely seen in the peripheral blood, circulating malignant cells are present in a large proportion of myeloma patients. Using Southern blots and flow cytometry, cells belonging to the malignant clone have been reported in the peripheral blood of about 50% of patients[2,3] while PCR results suggest an even higher frequency.[4]

Flow cytometric studies with CD19 and CD45 isoforms can be used to discriminate between immature and mature plasma cells.[2] In addition, CD56 expression appears to distinguish between malignant and nonmalignant plasma cells.[5] These parameters can be used to address the concern that PBSC collections in myeloma may be contaminated with malignant cells because of the possible effect of HGF on the malignant clone. There are preliminary data that G and GM-CSF may in fact be growth factors for myeloma cell lines, at least *in vitro*.[6]

The aim of this study was to evaluate the nature and kinetic behaviour of plasma cells and their precursors in PBSC collections from myeloma patients following chemotherapy and G-CSF priming. We

therefore were particularly interested in the maturity and other biological properties of these cells. In addition, we investigated whether such cells were present in CD34 enriched subpopulations.

METHODS

Patient Selection and Stem Cell Collections. Stem cells were mobilized from bone marrow to peripheral blood using high-dose cyclophosphamide (4 g/M^2) and rhG-CSF (5 mg/kg/d) priming in 6 patients. PBSC collections were commenced when the peripheral blood WCC was 1.0 x 10^9/L using a Baxter CS3000 Plus, with a small volume collection chamber. Between 7 and 10 L of blood were processed per collection.

Flow Cytometry. Cells were permeabilized by a gentle fixation in periodate-lysine-paraformaldehyde (pH 7.4) at -10°C for 15 minutes before incubation with unconjugated antibodies to bromodeoxyuridine,[7] kappa and lambda, CD56 and CD34 followed by anti-mouse IgG RED 613. This was followed by the simultaneous addition of monoclonal anti-CD38PE and anti-CD54FITC. Nucleoside transporters were determined by staining with 5'-(SAENTAx8)FITC,[8] anti CD38PE and antiCD45 RED 613. Three colour flow cytometry was performed on a Coulter EPICS Profile II Flow Cytometer. Plasma cells were identified by discrete bit map gating on the forward vs side scatter histogram and then setting a high fluorescent intensity threshold on the CD38 histogram as previously described.[9]

RESULTS

There were 6 patients in this study - 4 male and 2 female with a mean age of 52 years (range 35-66). An average 5.2 PBSC collections were performed per patient with a mean of 2.5 x 10^6/kg (range 0.03 - 6.4) CD34 cells collected. We have attempted to answer 5 basic questions about the myeloma cells in the peripheral blood of patients undergoing PBSC collections.

1. **Are there an increased number of plasma cells in PBSC from patients with meyloma?** High intensity CD 38 expression (CD38 ++) has previously been used by us and other groups to identify plasma cells.[8] We found a significantly greater number of CD38++ cells in the PBSC of

patients with myeloma (n=6) than in PBSC of nonmyeloma patients (5 NHL, 1 AML; n=6) (Figure 1).

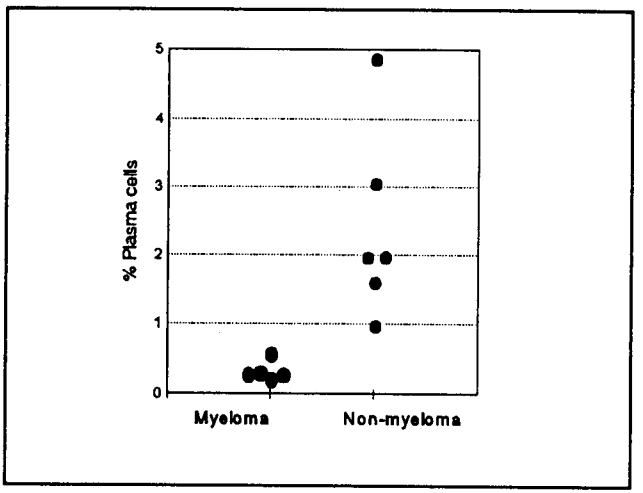

Figure 1. *CD38++ cells in PBSC collections of myeloma (n=6) and nonmyeloma patients (5NHL, 1AML).*

2. **What is the maturation stage of the circulating myeloma cells?** Coexpression of CD45 and CD19 was determined on CD38++ cells in PBSC. For each of the 6 patients studied, the vast majority of PBSCs expressed an immature plasma cell phenotype. The flow cytometry scattergram in Figure 2 illustrates the number of immature and mature plasma cells in the bone marrow and peripheral blood of a patient with myeloma. Figure 3 illustrates that the proportion of CD38++ cells expressing the immature CD45 phenotype was greater in PBSC than in bone marrow samples. In all 6 patients more than 70% of the plasma cells were immature.

3. **Are plasma cells in the peripheral blood malignant?** Three colour flow cytometry was performed to determine light chain and CD56 expression on circulating CD38++, CD45+ cells. CD56 expression has previously been shown to be present on malignant but not on normal plasma cells.[9] The results suggest that both polyclonal and monoclonal cells exist in the CD38++ population. Evidence for a monoclonal population is shown by either a significant CD56 expression

(mean=23±18%) or light chain isotype concordance present on the CD38++ cells in PBSC collections of all patients.

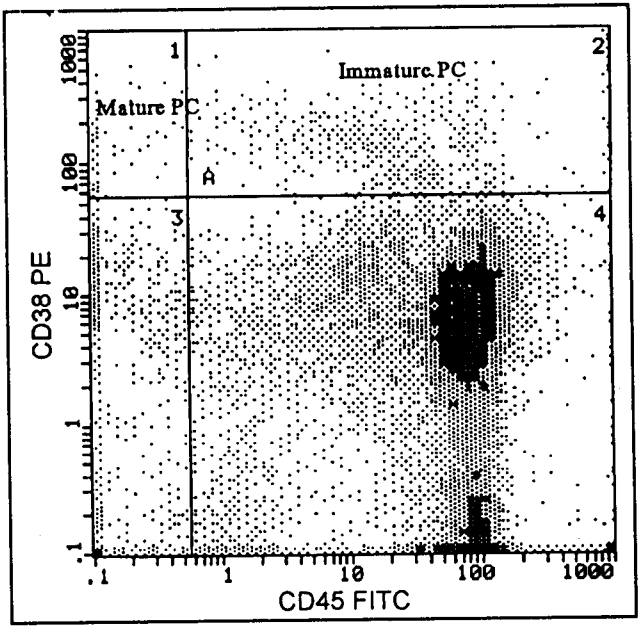

Figure 2. *Mature (CD45-) and immature plasma cells in PBSC collections.*

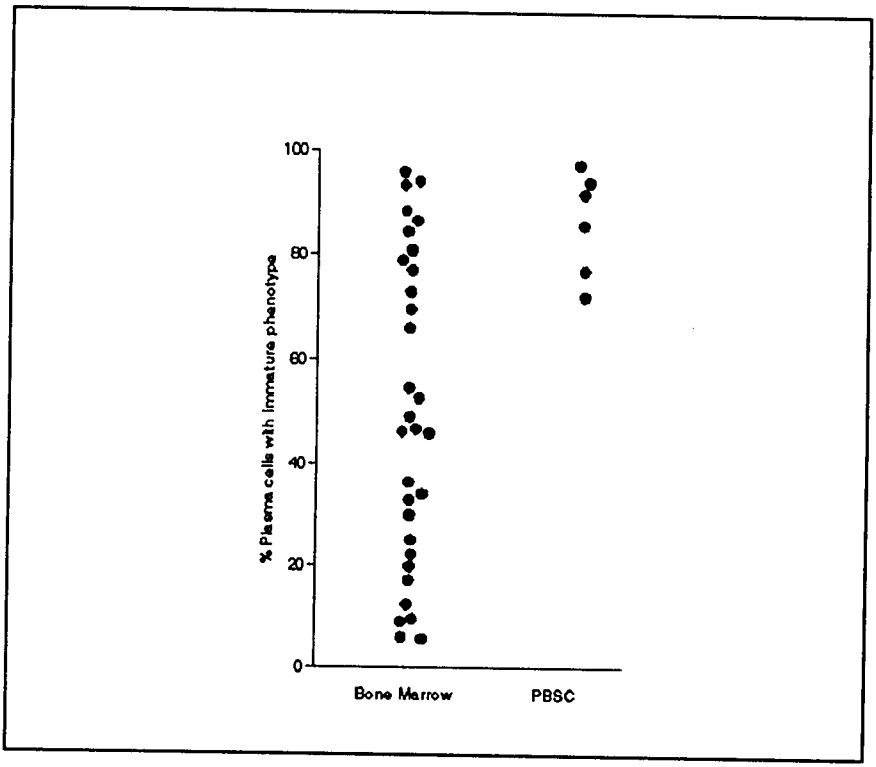

Figure 3. *Proportion of immature plasma cells in bone marrow (n=31) and PBSC.*

4. **What are the cell kinetics of the circulating myeloma cells?** The plasma cell labelling index and the number of nucleoside transporters on the surface of mature (CD45-) and immature (CD45+) CD38++ subpopulations are shown in Table 1. Both the labelling index and number of nucleoside transporters were greater in the CD45hi+ subpopulation. In a subpopulation of CD45+ cells with high intensity CD45, the nucleoside transporters were markedly increased (up to 20,000/cell).

Table 1. Proliferative Markers of Plasma Cell Subpopulations of PBSC Harvests

Patient	Labelling Index %		Nucleoside Transporters/Cell	
	Immature (CD45+) (CD45-)	Mature	Immature (CD45+) (CD45-)	Mature
1	6.8	2.6	463	610
2	0	0	8537	7683
3	1	0.9	6171	1122
4	2.9	0	2585	1073
5	0	0	7703	NP
6	3.3	0	2427	1476

NP = Not Performed

5. **Does CD34 purification of PBSC remove myeloma cells?** With 2-colour flow cytometry, it was demonstrated that CD34 expression on CD38++ cells in PBSC (n=6) was minimal (<4%) on both immature and mature plasma cells.

DISCUSSION

In this study we have answered a number of clinically relevant questions regarding circulating myeloma cells. We have recently demonstrated that in 44% of patients with myeloma there are a significant number of circulating malignant cells (>1% detected by Southern blot) and that there is no significant difference between the number of circulating myeloma cells and the stage of the disease (Joshua et al, unpublished observations). The CD38++ cells in PBSC collections are predominantly immature plasma cells. Studies of light chain and CD56 expression suggest that cells of the malignant clone are present in a significant proportion, although polyclonal plasma cells are also present, as is found in nonmyeloma PBSC. The increased expression of nucleoside transporters and the increased labelling index suggest that there are several opportunities for specific cell cycle or antimetabolite chemotherapy directed at the malignant precursor cell population. In addition, as there was a minimal expression of CD34 on these cells, we suggest that CD34 enrichment may be of value for PBSC harvests from patients with myeloma. As we have previously demonstrated that the number of malignant cells in the peripheral blood was not significantly greater in progressive than in stable disease, the timing for PBSC harvesting may not be vital. Further studies of the differences between PBSC collections obtained with and without HGF conditioning are indicated in myeloma.

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SESSION VII: SOLID TUMORS

NEW APPLICATIONS OF INTENSE THERAPY IN GERM CELL CANCER

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Nearly all patients with early stage testicular germ cell tumors are cured with either surgery or brief chemotherapy or in the case of patients with seminoma, low-dose radiation therapy. In patients with disseminated germ cell tumors, chemotherapy is remarkably effective and 75-80% of patients are cured with initial treatment.[1] This current level of success has come through careful clinical investigations over the last two decades. Current investigations represent a risk-adapted strategy in which patients with excellent prognosis are assigned treatments of limited duration, whereas patients with poor risk features at presentation are considered for more aggressive approaches and novel new agents. Many investigators feel that the standard of treatment for good risk patients is three cycles of the three-drug combination; bleomycin, etoposide and cisplatin (BEP) which results in long-term disease-free survival in over 90% of patients.[2,3] For patients presenting with poor risk features such as very bulky metastatic disease, very high serum markers or primary mediastinal non-seminoma, standard therapy is four cycles of BEP which results in cure of over half of this group of patients.[4,5] Unique to this tumor, adjunctive surgical resection after completion of chemotherapy plays a very important role in overall management.

RISK FACTORS FOR FAILURE

At Indiana University, risk assessment has been largely based on a simple system based on anatomic extent of disease and tumor bulk. This system is easily applied to nearly every clinical setting and reproducibly identifies the bulk of patients with excellent prognosis (>90% long-term survival with modern cisplatin-based treatments), or those patients in whom cure is less reliably obtained (approximately 60% long-term survival).[6] This system does not currently employ serum marker values, histologic subtype or LDH into the classification of risk. The only recent change has been the addition of any primary mediastinal non-seminomatous germ cell tumor into the advanced disease (poor risk) category.

Certainly, within the Indiana University classification system, the group that consistently has the lowest disease-free survival is the group of patients with primary mediastinal non-seminoma. A recent review highlights the rationale for these changes.[7] Between 1978 and 1993, 88 patients with mediastinal non-seminoma were identified. In summary, 48/88 (55%) patients with mediastinal non-seminoma obtained disease-free status either with chemotherapy alone (28 or 32%) or with subsequent surgery (17 - NED-ter, 3 NED-ca; 23%). Of those obtaining disease-free status, 31 (36%) are continuously free of disease with a median follow-up in excess of 30 months (range 4+ to 144+ months). Relapse from disease-free status occurred in 17 of the 48 patients with eight patients developing recurrent germ cell tumor, seven developing an associated hematologic neoplasm and two developing sarcomatous recurrences. A total of 48 patients had persistent or recurrent non-seminomatous germ cell tumor after primary treatment. Three of these patients (6%) are long-term survivors. One patient who obtained a radiographic partial remission with normal HCG and AFP did not progress. One patient developed a rising AFP at the time of surgical resection and subsequently received very high dose cisplatin/carboplatin treatment and is disease-free at 48+ months. The third patient received high dose chemotherapy with autologous marrow rescue after developing a serologic-only recurrence of mediastinal yolk sac tumor and is disease-free at 25+ months.

The current ability to assign risk on the basis of currently available clinical parameters, I believe, has been maximized. One can argue which system is the most accurate, but most commonly employed systems can assign prognosis correctly in the vast majority of patients. Current efforts at an internationally defined prognostic system may assist in supplementing local prognostic schemes and allow for accurate comparison of results across studies. However, the next level of prognostication will likely come from measurement of currently available and some experimental biologic predictors of outcome. None of these biological parameters have been investigated thoroughly enough to recommend widespread usage, but future prognostic systems will likely incorporate cytogenetics, flow cytometry, and host mechanisms of resistance into the prognostic models.

PRIMARY CHEMOTHERAPY OF POOR RISK DISEASE

Since the mid 1980s, the focus of clinical investigation in patients with poor risk features has been to improve the cure rate by intensifying

therapy or adding new agents. Rigid testing of the impact of high dose cisplatin therapy in disseminated germ cell cancer was accomplished in the successor trial of the SEGSG in advanced germ cell cancer.[8] This trial enrolled only patients with advanced disease by the Indiana classification system. Patients were assigned at random to receive standard doses of etoposide and bleomycin and either standard dose cisplatin (20 mg/M^2 daily for 5 days), or high dose cisplatin (40 mg/M^2 daily for 5 days). Between 1984 and 1989, 159 patients with advanced disseminated germ cell cancer were enrolled. 153 were evaluable for toxicity and response. Overall, 74% of patients receiving the high dose cisplatin are alive and 63% are continuously free of disease compared to 74% alive and 61% continuously free of disease on the standard dose arm. In addition, the high dose arm was associated with significantly more ototoxicity, neurotoxicity, gastrointestinal toxicity and myelosuppression. This large randomized trial found no therapeutic benefit to dose escalation of cisplatin beyond standard doses.

Subsequently, The Eastern Cooperative Oncology Group tested the substitution of ifosfamide for bleomycin in the standard combination BEP.[5] Preliminary analysis of this study suggests that the combination of etoposide, ifosfamide and cisplatin was more toxic especially with reference to myelosuppression, but no more effective with respect to therapeutic outcome. Again, BEP remains the standard therapy for poor risk disseminated disease.

A very aggressive extension of the above principles has been the use of high dose chemotherapy as primary treatment of poor-risk germ cell tumors.[9] The largest US experience comes from Memorial Sloan Kettering, where patients with poor predicted CR rates (<0.5) and treated with VAB-6 and for those patients exhibiting marker decline consistent with a prolonged half life (>7 days AFP, >3 days HCG). Twenty-eight patients were entered and 22 patients proceeded to two cycles of high dose carboplatin, etoposide, and cyclophosphamide plus ABMT. Overall, 12 of the 22 patients (55%) receiving high dose therapy and 15 of 27 (56%) overall achieved a disease-free status; two via resection of residual carcinoma. Eleven patients remain continuously free of disease with a median follow-up of 31 months.

The randomized trial from the Institut Gustave Roussey has been reported wherein poor-risk patients were randomized to receive conventional therapy with cisplatin, vinblastine, etoposide and bleomycin versus similar therapy followed by a single high dose cycle of cisplatin, etoposide, and cyclophosphamide.[10] This trial failed to demonstrate any

advantage for the high dose arm. As with many emerging new treatments however, the high dose arm of this trial would be considered substandard by modern criteria and different prognostic factors have emerged as important predictors of outcome. Accordingly, there is currently a large scale trial being planned in very poor risk patients that will compare standard therapy (probably BEP) to brief conventional therapy followed by two cycles of very high dose carboplatin, etoposide, and cyclophosphamide. It is hoped that this trial will answer definitively the role of high dose chemotherapy as primary therapy of poor risk disease.

SALVAGE THERAPY

Despite these dramatic successes, 20-30% of patients with disseminated disease and a rare patient with early stage disease will fail to achieve complete remission with first-line therapy. These individuals, as well as those who relapse from complete remission, are candidates for salvage chemotherapy. Because of the decreased efficacy and increased toxicity of second-line chemotherapy, this represents an important decision point in the treatment of such patients, and requires the expertise of individuals well versed in the intricacies of careful assessment of germ cell tumor patients and therapeutic options for this stage of testicular cancer. Also, it is in this group of patients that investigation has recently focused on high dose therapies with progenitor cell support. Currently available mature studies in these settings as well as ongoing studies of this evolving approach to high recurrent and refractory germ cell cancer will be the focus of this review.

There are several clinical situations which may mimic progressive or recurrent disease. One such situation involves the appearance of nodular lesions on chest X-ray or chest CT scan at the end of chemotherapy or soon after completion of such therapy. These nodules usually represent bleomycin-induced pulmonary injury, and are characteristically located in a subpleural region. Another clinical situation frequently mistaken for progressive disease is the syndrome of "growing teratoma." Patients with elements of teratoma in their primary lesion, and radiographically enlarging metastatic lesions during chemotherapy concurrently with appropriately declining serologic markers (as described above), is likely to represent the presence of teratomatous elements in these growing lesions. Appropriate management of such a patient includes completion of induction chemotherapy with subsequent surgical resection

of residual radiographic abnormalities, and not the administration of salvage chemotherapy.

While the presence of tumor markers and the accurate determination of these markers serve as a luxury in the management of patients with germ cell cancer, the presence of these markers can lead to errors in clinical management if not interpreted with caution. First, HCG determination can be non-specific and there is some cross-reactivity in the radioimmunoassay with luteinizing hormone. Also, HCG can be falsely elevated in patients who use marijuana. Low-levels of HCG elevation are consequently difficult to interpret. A conservative approach to this dilemma is to repeat the HCG determination to insure that the elevation is not a laboratory error. If the level is still high, the patient should be queried regarding drug usage. Testosterone should be given to insure that a hypogonadal state with resultant high levels of luteinizing hormone is not interfering with the determination of HCG. If the level remains increased, restaging procedures and investigation of sanctuary sites are in order.

In general, our policy has been to reserve initiation of salvage therapy until there is demonstration of rising markers on serial determinations. The vagaries of interpretation of low-level marker elevation makes such a policy necessary so as to assure that patients are not treated with intense salvage chemotherapy for a false positive marker elevation.

The therapeutic results with salvage chemotherapy have not been as dramatic as those for initial therapy, due primarily to the paucity of active single agents in patients refractory to cisplatin. During the two decades since the introduction of cisplatin, only 2 agents, VP-16 and ifosfamide, have demonstrated a $\geq$25% response rate in this setting. Early testing of paclitaxol suggests that this agent may have a similar level of activity.

The current standard salvage chemotherapy that serves as a basis for comparison is the regimen reported at Indiana University of vinblastine, ifosfamide and cisplatin (VelP).[11] Patients received cisplatin 20 mg/M^2 daily x 5 days, ifosfamide 1.2 gm/M^2 daily x 5 days, vinblastine 0.11 mg/kg days 1 and 2. One hundred and twenty-four patients who had not progressed on BEP received this combination as initial salvage chemotherapy. Patient characteristics reflected this poor risk population. Advanced disease by the Indiana Classification system was present in 81 patients at initial presentation and 59 (48%) at the time of VelP. Thirty-one patients had extragonadal primary site. Toxicity of the regimen in this

pretreated population was significant with 73% developing granulocytopenic fever. Transfusions of platelets (28%) and red blood cells (48%) were common. Renal insufficiency (serum creatinine >4 mg/%) was observed in 7% of patients. Three patients died related to treatment. Despite the formidable toxicity, the therapeutic results were gratifying. Fifty-six patients (45%) achieved a disease-free status with either chemotherapy alone (34 patients), or by resection of teratoma (15 patients 12%), or viable carcinoma (7 patients 6%). Twenty-nine of these patients are continuously disease-free and 37 (30%) are currently disease-free (minimum follow-up 27 months). Among the patients with extragonadal primaries, only 6 of 31 obtained disease-free status and only one is continuously disease-free. Outcome related to response to primary therapy is as follows: Of the 77 patients who never obtained a disease-free status with primary BEP, seventeen became disease-free with VelP and 11 (14%) are continuously free of disease.

HIGH DOSE CHEMOTHERAPY AS INITIAL SALVAGE THERAPY

At Indiana University, we have recently completed a phase II trial of brief conventional dose salvage treatment followed by a single high dose course.[12] Twenty-three patients in first relapse of germ cell tumors entered the trial with the intent to receive two cycles of VIP followed by carboplatin 1500-2100 mg/M^2 and etoposide 1200-2250 mg/M^2 followed by autologous marrow rescue. Eighteen of the patients completed protocol therapy. Five of the 23 did not undergo high dose therapy due to insurance refusal (1), patient refusal (1), active infection (1), CNS metastasis (1), or death on induction (1). Response to the two cycles of conventional dose therapy was complete response - 8, partial response - 12, stable disease - 2, and toxic death - 1. Two of the five patients not going on to high dose therapy are alive and progression-free with further treatment. Of the eighteen patients completing the protocol, final response status included 9 CR, 6 PR, 1 stable disease and 2 progression. Seven of these 18 (39%) remain progression-free at a median follow-up of 26 months. From these data, we conclude that such an approach is safe in this patient population (the only death was with conventional dose VIP) but, in this small trial, it is difficult to discern obvious therapeutic benefit compared to conventional salvage therapy. Our current approach is to give a single cycle of VIP followed by tandem high dose cycles with carboplatin 2100 mg/M^2 and etoposide 2250 mg/M^2. Preliminary results in

19 patients demonstrates some possibility of improved oucome. In this group of patients with testis primaries who had not progressed on initial cisplatin therapy, fifteen patients have completed treatment. Eleven of these (73%) remain disease-free with a minimum follow-up of 6 months and median follow-up of twelve months. There have been no therapy-related deaths in this group of 19 patients who have now all completed the transplant portion of their treatment. Whether this seeming improvement in outcome represents a true therapeutic advance or reflects better patient selection is unclear and will await continued enrollment and follow-up. An additional "wrinkle" to this protocol is the use of maintenance oral etoposide for patients obtaining a complete or partial remission.

HIGH DOSE CHEMOTHERAPY IN REFRACTORY DISEASE

High dose chemotherapy in the setting of multiple relapses is considerably less successful, but does offer carefully seected patients a small prospect of cure.[13-16] Patients with overt cisplatin-resistance do very poorly even with high dose treatments and chances of cure probably are in the range of 5%. Likewise patients with recurrent extragonadal germ cell tumors probably do not benefit from high dose approaches. In the patient with cisplatin-sensitive, testicular germ cell tumor, 15-20% of patients will be cured with high dose treatments with adjunctive surgery as necessary.[17] Currently, debate centers on the best preparative regimens. There is general consensus that carboplatin and etoposide for the general template for high dose treatment of germ cell tumors. The toxicity of adding ifosfamide or cyclophosphamide has not been obviously offset by an increase in therapeutic effect of three-drug combinations.

At Indiana University, we have retrospectively analyzed 81 patients who had recurrence of germ cell tumor after two or more years disease-free.[18] Sixty percent of these patients recurred beyond five years (maximum 32 years). Serum marker elevation was seen with 56% of patients having an elevated AFP and 27% of patients with an elevated HCG. Fifteen patients (19%) had a recurrence of teratoma and eight are continuously free of disase and four additional patients are currently free of disease after further surgery (27+-102+ months). Seven patients had recurrence with sarcomatous elements (+/-teratoma); four are currently disease-free. Fifty-nine patients had germ cell carcinoma as their initial late recurrence. Ten of these patients (17%) are continuously disease-free (10+-78+ months). Nine other patients are currently disease-free. Aggressive surgery was required in almost all of these patients. Overall,

65 patients received cisplatin-based chemotherapy and 17 (26%) achieved disease-free status. Twelve of the 17 have relapsed. Only two patients treated with chemotherapy alone are continuously free of disease (neither of these patients had received previous chemotherapy). Six of these patients with late relapse received high dose chemotherapy as salvage treatment with no long-term survivors.

CONCLUSIONS

Poor risk germ cell cancer is frequently defined by extensive bulk of disease, massively elevated HCG and LDH and extragonadal site. In most staging systems, such patients have less than a 50% chance of surviving the illness. Current standard therapy remains four standard dose cycles of cisplatin, etoposide and bleomycin. Planned investigations include the early use of high dose chemotherapy with carboplatin, etoposide and cyclophosphamide.

The decisions regarding initiation of salvage treatments in recurrent germ cell tumors are complex. There are clinical scenarios that can mimic recurrence and a careful assessment for the possibility of false positive marker elevation, the "growing teratoma" syndrome and sanctuary sites must be considered prior to the initiation of these intense treatments. Certain clinical features such as extragonadal primary site, late recurrence and cisplatin-resistance predict a very poor outcome and the role of salvage chemotherapy in either standard or high dose is limited. In these settings, aggressive surgery may offer the best (only?) curative option. In patients with recurrent, but cisplatin-sensitive testicular cancer, standard therapy is VelP with 30-40% of patients anticipated to be cured. Newer options include the early institution of high dose chemotherapy coupled with aggressive surgery as appropriate. The impact of these more intense options are still being evaluated.

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HIGH-DOSE CHEMOTHERAPY AND HEMATOPOIETIC STEM CELL SUPPORT IN GERM CELL TUMORS - EUROPEAN EXPERIENCE.

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The most important protocols concerned etoposide and cisplatinum, then carboplatin alone or in combination with either ifosfamide or cyclophosphamide. Bone marrow support was performed in the majority of cases. However, peripheral blood stem cell support became more popular in the 1990s. Studies with double-dose cisplatinum were performed in France between 1984 and 1991.

The mortality of high-dose chemotherapy with autologous bone marrow transplantation does not exceed 2 to 5% of patients and the toxicity is rather tolerable. Between 1984 and 1993, 312 of 365 patients who were gonadic were treated by high-dose chemotherapy and bone marrow support and were reported in the EBMT registry as: 1) 154 were known to be in relapse or progression (48.1%); 30 in first complete remission, 99 in first partial remission, 15 in first very good remission and 14 in stable disease.

The 5 year overall survival rate is 44%; results are better for patients in consolidation than in a relapse setting.

The consequence is that high-dose treatment seems of poor value in refractory disease but needs to be studied in the consolidation of salvage chemotherapy.

Few studies have addressed the question of the role of high-dose chemotherapy in the first-line treatment of poor-risk patients. The French group published two phase II trials and one phase III trial. The latter trial failed to show any advantage of high-dose treatment when compared to a conventional chemotherapy arm.

We are now beginning a new phase III trial comparing high-dose chemotherapy and autologous bone marrow transplantation after 3 VIP versus 4 VIP courses in a salvage setting.

HIGH DOSE CHEMOTHERAPY AND BONE MARROW/PERIPHERAL BLOOD STEM CELL RESCUE. EXPERIENCE IN PEDIATRIC SARCOMAS

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INTRODUCTION

Primary CNS malignancies and solid tumors constitute approximately 60% of all pediatric malignancies. The use of combination chemotherapy has improved the response rates for pediatric solid tumors resulting in improved survival. Treatment advances in the past decade for pediatric solid tumors have been largely made by using more dose intensive combination therapies.[1-4] Despite advances in modern cancer therapy, 50-75% of pediatric brain tumor patients and 30-60% of pediatric solid tumor patients will fail primary therapy.[5-6] The prognosis for refractory or metastatic pediatric solid tumors is very poor with truly resistant patients having <10% 1-year progression-free survival (PFS). Phase II studies of Ifos(I), VP-16(E),+/-CBDCA(C) have demonstrated promise in these patients with response rates of 32% (IE) and 39% (ICE) respectively.[7-8] For the ICE(Ifos/VP-16/CBDCA) regimen the maximally tolerated doses in a recent Pediatric Oncology Group trial were Ifos 6 gm/M^2, VP-16 300 mg/M^2 and CBDCA 635 mg/M^2.[8] Despite these response rates, most patients eventually recurred. In addition, complete responses were uncommon and myelotoxicity with this regimen was considerable. In particular, prolonged thrombocytopenia was dose-limiting. The limiting factor for most dose intensive salvage chemotherapy is bone marrow toxicity leading to significant myelosuppression. Preclinical and clinical studies have demonstrated significant dose response relationships for many chemotherapeutic agents suggesting that if higher doses of these drugs were given, better response rates could be achieved leading to improved survival.

Due to the excellent response rates in refractory pediatric solid tumors with ICE we designed a phase I-II chemotherapy combination with a backbone of CY and VP-16 and escalated CBDCA dosing with autologous bone marrow rescue (ABMR) in children with refractory CNS

and solid tumors. The results of the toxicities of this regimen and specific response rates for sarcomas are presented here. We analyzed several factors to determine features which predicted for response and duration of response. Although response rates were better with this approach than have been previously reported in pediatric solid tumors, most responses were not durable. This has been a significant deficiency in all high-dose regimens, especially since the models for curative chemotherapy in diseases such as acute leukemia, Hodgkin's disease, testicular cancer, and non-Hodgkin's lymphoma, all require multiple courses of therapy. Therefore, it is not surprising that a single course of high dose therapy, while providing high response rates for a number of refractory solid tumors, often results in responses of relatively short duration and complete remissions that only rarely lead to a long-term disease -free intervals. Based on data obtained by Shea et al,[9] in a dose escalation trial of CBDCA and peripheral blood stem cells collected after growth factor stimulation and infused as "rescue" after myelotoxic chemotherapy, we designed a subsequent study utilizing the combination of Taxol and escalating dose CBDCA with PBSC infusions in relapsed pediatric solid tumors. The rationale for this approach and preliminary data are also presented here.

METHODS

Protocol #1 CY/VP-16/CBDCA plus BMT: Beginning 1989, 58 children with refractory CNS or solid tumors were treated with increasing doses of CY/VP-16/CBDCA with ABMR according to an institutional protocol - Johns Hopkins Hospital (JHH) #8831. All patients (ages 3-25 yrs) had a pathologically proven solid tumor and had either relapsed (n=15), progressed (n=36), or had persistent measurable disease (biopsy proven n=7) after frontline or salvage therapy. All patients had satisfactory performance status (creatinine <2.0 mg/dl, bilirubin <2 mg/dl, platelet >100,000/mm^3, cardiac ejection fraction >45%, pulmonary function >50% of predicted) and had complete tumor workup prior to BMT.

Patients who were eligible went on to have autologous bone marrow harvested in the operating room by standard methods. Bone marrow was buffy coated, suspended in media with human plasma and 10% DMSO prior to crypopreservation by standard methods. No *ex vivo* treatment of the marrow was performed. Patients were treated in single bed isolation rooms with HEPA filtered air and all had double lumen Hickman catheters. Patients received irradiated, matched blood products

as indicated. All patients who were HSV IgG+ received acyclovir at 125 mg/M^2 every 6 hours beginning day -2 until discharge and most (n=55) received fluconazole orally at 5mg/Kg/day over the same time period. Fevers and infection were treated with broad spectrum antibiotics as indicated. Patients who demonstrated a sustained rise in absolute neutrophil counts (ANC) of greater than 500/mm^3 and had resolved all toxicities were discharged and followed as outpatients. Engraftment was defined as a sustained (≥3 consecutive days) ANC of greater than 500/mm^3 and platelets greater than 20,000/mm^3 without transfusion and confirmed by bone marrow examination. Tumor status was followed using standard imaging techniques and responses defined as below.

Protocol #2. Taxol/CBDCA + PBSC: Beginning in 1993, a second protocol, Lineberger Cancer Center #9305 (LCCC, University of North Carolina at Chapel Hill), was designed for treatment of refractory solid tumors using repetitive cycles of taxol plus escalating dose CBDCA with PBSC infusion. Eligible patients included relapsed or refractory solid tumors or primary CNS tumors with absence of bone marrow involvement and identical organ function as listed for protocol #1. Patients then received 6 days of granulocyte colony stimulating factor (G-CSF) at 10 μg/Kg/d and had collection of peripheral blood mononuclear cells by standard apheresis using a Cobe 3000 on days 5 and 6, as previously published.[10] Patients then received a 24-hour continuous infusion of taxol at 350 mg/M^2 followed by a 1-hour infusion of CBDCA.[11] Approximately 36 hours after CBDCA patients received their previously collected PBSCs. CBDCA dosing was determined based on area under the curve (AUC) target dosing using the modified Calvert equation.[12] Doses were escalated in cohorts of 3 by standard phase I methodology beginning at target AUC of 6 μg/ml/min with increments of 2 μg/ml/min. After each cycle of treatment, patients were treated with G-CSF (10 μg/Kg/d after cycle 1, 5 μg/Kg/d after cycles 2-4). Three to four collections of PBSCs were obtained during the blood count recovery after cycle 1, split into several individual bags and used for rescue after cycles 2-4.

Responses were measured with serial radiographic studies and scored by standard methods. Complete response (CR) = was defined as disappearance of all sites of disease, partial response (PR) as a greater than 50% decrease in all sites of disease. Progression (PD) was defined as growth of >25% of any lesion or appearance of new lesions. All protocols were approved by our local institutional review board.

Results Protocol #1 CY/VP-16/CBDCA plus BMT: In this phase I trial of cyclophosphamide/VP-16 with escalating dose CBDCA with

autologous bone marrow rescue, pediatric patients aged 1-25, with proven relapsed or refractory solid tumors, were treated. After collection and storage of unmanipulated autologous bone marrow, patients were treated in cohorts of 3-10 patients at 8 different dose levels followed by infusion of their autologous bone marrow. The schema for the protocol is given below:

Schema

Days	-6	-5	-4	-3	-2	-1-	0
Carboplatin	x	x	x				
Cyclophosphamide				x	x		
Etoposide	x----------						BM

CBDCA = 200-725 mg/M^2/d, Cyclophosphamide = 60 mg/Kg/d, Etoposide = 600-800 mg/M^2/d

Patient demographics are listed in Table 1. Children and young adults with a wide variety of recurrent or refractory solid tumors and primary CNS malignancies were treated. This group includes 33 patients with sarcomas, virtually all of whom had received extensive prior treatment. Overall, most (57/58) had received chemotherapy and failed a median of 2 (range 1-4) regimens and most had received radiotherapy (49/58). Patients had received prior local radiotherapy to extremity sites (n=17) or to axial sites (craniospinal, chest, whole abdomen, or pelvis; n=32).

Toxicities were significant, but reversible (see Table 2 below). All patients had grade 4 hematopoietic toxicity that was temporary. The major toxicities were transient elevations of transaminases and ulcerative stomatitis. Below doses of CBDCA of 1600 mg/M^2 stomatitis and transaminases were mild to moderate and rapidly reversible. Dose-limiting toxicity was due to elevated transaminases and mucositis at the highest dose level. The degree of mucositis and transaminase elevation at the dose of CBDCA of 2000 mg/M^2 and VP-16 2400 mg/M^2 were not limiting. One patient had a transient elevation of bilirubin with weight gain consistent with venoocclusive disease. There were 3 treatment-related deaths. Two patients died from fungal sepsis, one with acute renal failure at the highest dose of CBDCA and VP-16 (2400 mg/M^2 each). A third patient with germ cell malignancy who had prior lung irradiation developed pneumonitis and died at 30 days after BMT with pulmonary hemorrhage.

Table 1. Pediatric Solid Tumor ABMT Demographic Data

Diagnosis:	
Ewings	12
CNS tumors	12
Rhabdomyosarcoma	11
Neuroblastoma	7
Other sarcomas	6
Osteogenic sarcoma	4
Nephroblastoma (Wilms')	3
Other	3
Prior Chemotherapy (# regimens):	
1	12
2	30
3	14
4	1
Prior Radiotherapy	
Axial sites	32
Non-axial sites	17

Table 2. CBDCA/VP-16/CY ABMT Toxicity

Dose Level	CBDCA Dose (mg/M^2)	VP-16 Dose (mg/M^2)	#DLT[1]/#Patients (dose-limiting)
1	600	1800	0/3
2	900	1800	2/6
3	1200	1800	2/6
4	1600	1800	4/12
5	2000	1800	2/6
6	2000	2400	3/11
7	2400	2400	3/4
8	2175	2400	4/10

[1] *DLT = Dose-Limiting Toxicity (NCI clinical criteria)*

Responses in refractory solid tumors demonstrated ~70 PR + CR (see Table 3). Responses were similar in patients with sarcoma, with 19/28 patients with measurable disease achieving a response (13 CR, 6 PR). Of note, three patients with osteogenic sarcoma and measurable disease had significant responses. With higher doses of CBDCA, the percentage of CRs as well as duration of CR increased significantly and was correlated to the total dose of CBDCA (Table 4). Durable CRs have been demonstrated in 5 patients (median progression-free survival = 21 mos; range 15-39 mos). The use of autologous bone marrow to obviate the myelosuppressive dose-limiting toxicity of CBDCA allowed for

significant dose escalation and higher response rates. Despite the use of autologous BM, significant delay in count recovery was experienced, with neutrophil recovery to greater than 500/mm^3 and platelets to greater than 50,000/mm^3 occurring at a median of 27 and 29 days, respectively. All patients who achieved a partial response only, had early progression of their disease (median 4-6 months after BMT) while only patients achieving a CR had durable responses.

Table 3. CBDCA/VP-16/CY Response Data(Pts. with Measurable Disease)

CBDCA Dose (mg/M^2)	VP-16 Dose (mg/M^2)	# CR	# PR	%
600	1800	0	2	67%
900	1800	2	2	67%
1200	1800	0	3	60%
1600	1800	2	2	50%
2000	1800	3	1	67%
2000	2400	5	4	82%
2400	2400	2	1	75%
2175	2400	6	1	88%
Total (N=50)		20	16	72%

Table 4. CBDCA/VP-16/CY Dose Response

Total CBDCA Dose (mg/M^2)	# Pts	CR	PR	%
600-1600	27	4	9	60
2000-2400	31	16	7	82
Overall	58	20	16	72

Protocol #2. Taxol/CBDCA + PBSC. Seven patients (including 5 sarcomas) have been treated at three dose levels. Two patients had repeated the 4-cycle treatment for a total of eight cycles and a third patient had received 6 cycles. A total of 35 cycles of treatment have been given to these 7 patients with CBDCA doses at 450-850 mg/M^2 (AUC 6, 8, 10 μg/ml/min). There have only been 4 RBC (2 required for apheresis) and 3 platelet transfusions, with one patient being admitted for fever and neutropenia. All of the infusions were given as outpatients and the majority of the time, patients remained out of the hospital. There have been 5 responses with 4/5 PR in rhabdomyosarcoma.

Samples for pharmacokinetic assays have been collected. There have been no toxicities from the taxol infusions and the chemotherapy

administration has been well tolerated. Progenitor cell assays have been done and results for the cohort are listed in Table 5. There were no significant differences in mobilization of PBPCs before and after cycle 1 of treatment. Patients received a mean of ~5 x 10^5 CD34+ cells/Kg after each treatment. Of the total cycles administered, 31/35 had blood count recovery within 4 weeks of the first dose of the cycle. Only one patient has had delay of therapy beyond 4 weeks. The majority of the PBSC infusions were done as 30-60 minute procedures in the outpatient area and there were no significant toxicities from the stem cell infusions.

Table 5. Infusion Data

	MNC[1]/Kg x 10^8	CFU-GM/Kg x 10^6	CD34+/Kg x 10^5
Cycle 1	4.5	5.0	5.5
Cycles 2-4	1.9	1.9	4.5

[1] *MNC = Mononuclear Cells*

There have only been 3 platelet transfusions and 2 episodes of fever and neutropenia in 35 cycles administered. There has been minimal toxicity. Samples have been saved for pharmacokinetic analyses.

DISCUSSION

The use of high dose, combination chemotherapy with autologous bone marrow rescue has met with considerable success in terms of response rates for children with leukemias, lymphomas, neuroblastoma, and other solid tumors.[13-18] These results have been obtained even in patients with relapsed and resistant disease, supporting the concept that dose intensity may overcome some cancer cell resistance. However, toxicity and cost are high, and despite high response rates, relapses are frequent and response durations are typically short.[17-18] It is likely that a single course of high dose chemotherapy will be insufficient to completely eradicate significant body tumor burdens in most of these patients. There are many theoretical reasons for failure of this approach, including tumor cell chemoresistance, inadequate tissue delivery of drug, inadequate dosing and tumor cell kinetics. Although higher dose intensity may be able to overcome some of these mechanisms, cells that are in resting state at the time of therapy may fail to be eradicated, resulting in treatment failures.

There were several observations in our study, CY/VP-16/CBDCA with BMT, that are important in planning further drug therapy development studies in pediatric solid tumors. The data strongly suggest

that increased dose intensity appears to correlate with increased response rate and complete response rate. The likelihood of a durable response (response duration greater than 6 months) was present only in patients who achieve a complete or near complete response with the high dose chemotherapy. Finally, patients who received standard dose salvage chemotherapy at time of relapse and who had at least a minor response (responsive disease), were much more likely to have a complete response with BMT (15/21) than patients who had no response or progressive disease at time of BMT (3/15).

From these observations and the data from the study, it appears that dose intensification of combination therapy leads to greater response rates. Patients who have responsive disease with combination salvage regimens appear to have a better chance of achieving a durable complete response with high dose consolidation therapy and BMT. Patients with no response or progressive disease appear to be poor candidates for BMT and would be better off either enrolling on alternative phase I-II studies (if performance status were adequate), or receiving palliative care only.

The infusion of autologous bone marrow to obviate the myelosuppressive dose-limiting toxicity of CBDCA allowed for significant dose escalation and higher response rates. Recently, the use of peripheral blood stem cells collected after stimulation with colony stimulating factors, with or without chemotherapy, has demonstrated that: 1) the use of PBSC can result in hematopoietic recovery after high dose therapy, and 2) platelet recovery with the use of stimulated PBSCs is markedly enhanced.[19-21] In addition, the collection of PBSCs has been safely done in children as small as 7-10 Kg.[21] Therefore, it is likely that the use of PBSCs in conjunction with CBDCA may lead to marked dose escalation over doses achievable without PBSCs (with or without G-CSF).[20] The above data suggest that higher doses of CBDCA may be correlated with higher response rates and provide a chance for a better outcome.

Taxol is a novel chemotherapeutic agent which causes cell death by disruption of the microtubule system.[22,23] In a recent phase I trial in pediatric solid tumors, the MTD was 350 mg/M^2 with neuropathies occurring at 420 mg/M^2.[23] Of thirty-one patients entered, 4 had responses despite heavy prior therapy and resistant disease. In adult ovarian malignancies, taxol is a very effective agent for platinum-resistant disease in adult solid tumors.[24,25] Therefore, taxol would seem an ideal candidate to study in combination with high dose CBDCA in a phase I-II combination trial. The use of PBSCs as hematopoietic rescue may reduce platelet and white blood cell nadir duration and improve the ability to give

maximal dose intensity. This approach is much more likely to result in a chemotherapy combination regimen that can be given at maximal dose intensity to be evaluated in a subsequent phase II trial.

Our early results with the combination of taxol/CBDCA demonstrate that myelosuppressive doses of these drugs can be given in combination without major non-hematopoietic toxicity. In combination, taxol at the maximum phase I dose can be given with doses of CBDCA that are greater than the phase II single agent dose of 560 mg/M^2. This combination can be given safely with short duration neutropenia if PBSCs are given after treatment. This treatment requires minimal hospitalization and allows for complete and rapid platelet recovery despite high doses (>700-850 mg/M^2) of CBDCA. Therefore, this approach may allow significant dose escalation of CBDCA and lead to studies evaluating dose intensive, repetitive cycle treatment in these patients.

The feasibility of multiple course treatments with hematopoietic rescue will also need to be assessed as this approach has already demonstrated promise in certain situations such as testicular carcinoma.[26] Careful studies of the biology of PBPCs collected and infused in these patients, as well as correlative pharmacokinetics, are a key feature of these studies. This technique of giving multiple cycles of dose intensive chemotherapy is unique and is the only proven method for shortening platelet recovery in patients receiving CBDCA-containing regimens. Further studies are warranted to validate this approach. Finally, well planned studies comparing these therapies as intensification in first remission need to be done. The future role of high dose chemotherapy, BMT, and the use of PBSCs with repetitive cycle treatments in solid tumors, will continue to expand and, hopefully, result in higher cure rates in these patients.

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HIGH-DOSE CHEMOTHERAPY AND HEMATOPOIETIC STEM CELL SUPPORT IN BRAIN TUMORS

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Since 1986, several trials have been conducted in the Centre Léon Bérard in this field. BCNU experience: between March, 1986, and June, 1989, 103 patients with grade III and IV astrocytomas have been treated by surgery, BCNU 800 mg/m^2 followed 72 hours later by ABMT and 4 weeks later by 45 Gy total encephalic radiotherapy (60 Gy equivalent). The total treatment period planned was 2 months. Median age was 47 years, 58 were males and 45 were females. Twenty-one were grade III and 82 were grade IV astrocytomas or glioblastomas. All were treated in the initial phase of their disease. Toxicity was acceptable with 9% grade IV aplasia, 4% lung fibrosis with 2 (regressive) biologic hepatic changes, grade I and II in 20%, and 7% venous thrombosis. Finally, the toxic death rate is 7% (7 patients). Most of the patients were treated as outpatients after BCNU. Median survival is 12 months after surgery and 11 months for grade IV astrocytomas. The 3-year survival was 11% better for grade III, 36% compared with 4% for grade IV. Semi-ambulatory patients (PG $\leq$2) and less than 50 year olds have the longer survival: 17 months in 45 patients. Quality of life evaluated with the PS evolution is quite correct during the first year for semi-ambulatory patients.

Fotemustine experience: we have conducted two trials using Fotemustine, a new French nitrosourea derivative, first in phase I and II in a similar model: surgery, HD Fotemustine, radiotherapy. All the patients presented with high grade astrocytomas and a residual evaluable mass after surgery. Fotemustine was administrated in 2 days. The first level was determined as double the conventional dosage, 600 mg/m^2. Marrow reinfusion was performed after 72 hours, 33 patients were included from 600 to 1100 mg/m^2 and 12 were included at the level of 900 mg/m^2, determined as the maximum tolerated doses for further trials. Median age was 43 years. Preponderant toxicity is hematologic with a grade III and IV neutropenia and thrombopenia in 93% and 96%, respectively. We observed moderate biologic hepatic changes in all the patients, a febrile episode in 6 patients and most particularly a severe gastric pain in 9/13 patients a few hours after Fotemustine administration in the 800 mg level and higher. Sedation is easily obtained after antispasmodic drugs. No

organic lesion was noted. The toxic death rate is 14%. Results: objective response rate - 23%, stable disease - 69% and progressive disease - 8%. These results seem to correlate the conventional dosage results of Fotemustine. In our next program, we plan to integrate high dose Fotemustine in a sequential polychemotherapy regimen.

HIGH DOSE COMBINATION CHEMOTHERAPY AND BONE MARROW RESCUE FOR OVARIAN CARCINOMA: CURRENT STATUS IN THE UNITED STATES

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INTRODUCTION

While advanced epithelial ovarian carcinoma is initially chemosensitive, only 20-30% of patients enter a surgically documented complete remission (CR).[1-3] The remainder and, in fact, 50% of those entering a CR progress and eventually die of drug-resistant disease. While induction chemotherapy with taxol containing regimens may improve the survival of patients with advanced ovarian carcinoma, these regimens may not significantly improve their curability, as the pathologic CR rates are not different from non-taxol containing regimens.[3] Taxol-containing regimens may, however in combination with dose intensive consolidation therapy as described below, ultimately be a critical component to increasing the cure rates for this patient population.

While significant progress has been made in the design and performance of randomized phase III transplant trials in both high risk Stage II and Stage IV breast carcinoma, very little new data on the value of transplants for ovarian carcinoma has been published since the Sixth International Symposium almost two years ago.[4] This is primarily due to the smaller numbers of patients who develop ovarian carcinoma, and their higher median age at onset. While there are several small pilot studies published to verify that high dose chemotherapy with marrow rescue improve response rates as compared to other salvage therapies for patients with relapsed or refractory disease, up to this point, the numbers are too small to warrant a randomized phase III trial. We believe however that there are at least two regimens that deserve consideration, and they are currently being tested in a randomized phase II trial through the Southwest Oncology Group.

Mitoxantrone, Carboplatin and Cyclophosphamide

The phase I study of this regimen has been reported recently.[5] The regimen was developed by the Loyola BMT group and was designed to take advantage of the steep dose-response curve of mitoxantrone for platinum resistant ovarian cells *in vitro*,[6] the clinical data demonstrating a high response rate for carboplatin in patients with refractory ovarian carcinoma,[7] and *in vitro* synergism of multiple alkylating agents *in vitro*.[8,9] The phase II doses of this regimen are demonstrated in Table 1. The dose limiting toxicity for the regimen has been gastrointestinal; however, this is well tolerated at the phase II doses.

Table 1. Transplant Regimens

A. Mitoxantrone, Carboplatin, Cyclophosphamide

	Day -8	Day -7	Day -6	Day -5	Day -4	Day -3	Day -2	Day -1	Day -0
Mitoxantrone	x		x		x				
Cyclophosphamide	x		x		x				
Carboplatin	|					|		
Transplant									x

Mitoxantrone (25 mg/M^2/day)
Cyclophosphamide (40 mg/kg/day)
Carboplatin (300 mg/M^2/day)

B. Thiotepa, Cisplatin, Cyclophosphamide

	Day -7	Day -6	Day -5	Day -4	Day -3	Day -2	Day -1	Day -0
Thiotepa	x							
Cisplatin		|			|			
Cyclophosphamide		x	x	x				
Transplant								x

Thiotepa (600 mg/M^2)
Cyclophosphamide (1,875 mg/M^2/day)
Cisplatin (55 mg/M^2/day)

To date, 30 patients have been treated at the phase II doses of this regimen. Their clinical features are similar to that for other patients entering clinical trials for relapsed/refractory ovarian carcinoma: 67% had platinum resistant disease, and 83% had disease >1 cm at the time of transplantation. The median age was 47 and the median number of prior regimens was 2.

The toxicities associated with this regimen are similar to those currently being used to treat breast cancer. There has been only one death

in the 30 patients treated (3.3%). This patient died at day 16 post-transplant of Aspergillus pneumonia, despite full dose Amphotericin B therapy. Reversible mild to moderate diarrhea was universally seen, and transient mild ototoxicity and mucositis were seen in a minority of patients.

The clinical outcome is described in Table 2. The overall clinical CR rate was 59%. For those with platinum sensitive disease there was a borderline significant increase in CR rates as compared to those with platinum resistant disease. The overall progression-free survival for the 18 patients in CR following the transplant was 8.3 months. This increased to 10.1 months for those with sensitive disease. There appears to be a plateau on the disease-free survival curve as is seen for hematologic malignancies and Stage IV breast carcinoma, and if patients who received post-transplant radiotherapy for localized disease are included this plateau is approximately 20% at 3 years. The median overall survival for the entire group was 29 months which compares very favorably with conventional salvage therapies.

Table 2. Clinical Outcomes:
Mitoxantrone, Carboplatin, Cyclophosphamide (N=30)

Toxic Deaths		3.3%
Overall Response Rate[1]	89%	
Overall CR Rate[1]		59%
Platinum Sensitive[2]	88%	
Platinum Resistant		47%
PFS-CRs		8.3 months
Platinum Sensitive		10.1 months
Platinum Resistant		5.1 months
Overall Survival		29 months

PFS (progression-free survival)
[1] *Measurable disease*
[2] *p=0.06*
[3] *p=0.033*

Thiotepa, Cisplatin and Cyclophosphamide

This combination has been developed by the BMT group at the University of Colorado. It is based on the synergism of alkylating agents in combination, and the fact that alkylkating agent drug resistance can be

overcome by a 10-fold increase in dosage.[8-9] An initial report of this regimen has been published, in which the cisplatin was administered by the intraperitoneal route, initially by the bolus route and then as a continuous infusion.[10] They reported a high response rate of 75%, and while there were no CRs, the responses were documented by a surgical procedure and thus cannot be compared to data from other groups. Due to the technical problems associated with intraperitoneal administration of chemotherapy, the high rate of retroperitoneal involvement and the size of disease seen in this patient group, the cisplatin has been changed to the intravenous route. The current regimen is detailed in Table 1.

The regimen is well tolerated, and there have been no toxic deaths in the first 13 patients who have received the current regimen. Again these are patients with recurrent disease who have received a mean of two prior regimens. The non-hematopoietic toxicity is predominantly mucosal, with moderate reversible mucositis and mild to moderate diarrhea. No significant hepatic, or nephrotoxicity has been seen with appropriate hydration during chemotherapy.

To date, two patient groups have been treated with recurrent disease, those with measurable disease and those with minimal residual disease, as shown in Table 3. For those with measurable disease, the overall response rate is 75%, with a median progression-free survival of 6 months. For those with non-measurable disease, the PFS is 9 months. Like the mitoxantrone-based regimen described above, there does appear to be a plateau on the PFS curve at approximately 15% at 3 years from transplantation.

Table 3. Clinical Outcome: Thiotepa, Cis-platinum, Cyclophosphamide (N=13)

Toxic Deaths	0%
Overall Response Rate[1]	75%
PFS - Measurable disease	6 months
Non-measurable	7 months
2-year PFS	14%

PFS (progression-free survival)
[1] *Measurable disease*

DISCUSSION

The clinical features and outcome of these patients is similar to that of patients undergoing autologous transplantation for

relapsed/refractory hematologic malignancies, lymphomas, neuroblastoma and carcinoma of the breast.[11] While few patients appear to be cured, the results are still superior to conventional therapy. In addition we can now begin to select the patients most likely to benefit, i.e. low bulk, and platinum sensitive. However, if an impact on the disease is expected like the other tumors described above, patients must be transplanted before they develop large bulk, chemoresistant disease.

Based on the favorable results for these two preparative regimens, and that of several pilot non-randomized trials in Europe, the Southwest Oncology Group has initiated a randomized phase II trial for patients with chemosensitive disease at the time of second look laparotomy for disease-bulk that ranges from microscopic to a maximum of 3 cm. The primary endpoint of this pilot study is to determine if these regimens are exportable to other transplant centers with similar minimal toxicity. Progression-free and overall survival will also be evaluated in a phase II fashion for each of the regimens and compared to other conventional salvage therapies available to this patient group. It is not the intent of the trial however to perform a direct comparison of the two regimens, but rather to verify that it is safe and appropriate to perform a national randomized phase III trial.

A national phase III trial would be done in a similar fashion to the high risk Stage II breast carcinoma trial. Representatives from the major Cooperative Groups and the NCI recently met and agreed on the following schema: patients with stage III or IV ovarian carcinoma would, after initial debulking surgery, receive the best currently available conventional therapy: taxol and cisplatin. For those in a clinical remission after 4 cycles of this regimen, a second look surgery would be performed. Patients with chemoresponsive disease <1 cm after the second look, including those without detectable disease, would be randomized between ongoing platinum/taxol therapy versus a single transplant procedure using peripheral blood stem cells. The primary endpoint will be to compare progression-free and overall survival between the two groups.

CONCLUSIONS

As currently utilized, high dose chemotherapy with hematopoietic stem cell rescue offers a small but significant long term disease-free survival for patients with relapsed/refractory ovarian carcinoma. The patients most likely to benefit are those with platinum sensitive and low tumor burden disease. Now that several regimens are available that have little life-threatening toxicities, the goal of new trials should be to use such

therapy earlier in the course of the disease. However, the true value of such therapy will only be verified by a single national randomized phase III trial that is supported by the NCI as a high priority study and by all of the Cooperative Groups.

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HIGH-DOSE CHEMOTHERAPY WITH AUTOLOGOUS BONE MARROW TRANSPLANTATION IN OVARIAN CANCER

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ABSTRACT

In 18 patients with relapsed or persisting ovarian cancer after first-line cisplatinum-based chemotherapy, high-dose chemotherapy followed by reinfusion of autologous bone marrow was attempted. Pathologically confirmed or clinical complete remissions were seen in 10 patients. There was one toxic death. Four complete remissions persist after 6-12 years of observation. All long-term survivors started the high-dose chemotherapy with residual lesions ≤ 2 cm. It is concluded that this form of treatment could have value in patients with low-volume relapsed or persisting ovarian cancer.

INTRODUCTION

Advanced ovarian cancer is sensitive to chemotherapy, especially combination chemotherapy containing cisplatinum or one of its analogues, but the majority of patients so treated will relapse or have signs of incomplete remission after first line chemotherapy. Presently, no standard therapy, surgery, radio-, chemo- or immune therapy or combination of these modalities, offers a perspective for cure in this situation.

However, many of the chemotherapeutic agents active in ovarian cancer, exhibit a steep dose response curve and clinical information suggests a relationship between dose intensity and treatment result.[1] This has led to attempts at increasing the dose of chemotherapy, while safeguarding the recovery of bone marrow function with bone marrow autotransplantation.[2]

Most of the reports on this modality of treatment in ovarian cancer deal with short-term observations and clinically less relevant endpoints such as remission rates instead of remission duration.

We have entered patients with ovarian cancer considered to be incurable with standard second line treatment options into a number of phase I and II studies on autologous bone marrow transplantation.[2,3] In a follow-up of these studies, we report here the results of this form of intensive chemotherapy in 18 patients after a median observation time in the four long-term survivors of more than 6 years.

PATIENTS AND METHODS

Patients with histologically documented ovarian cancer were entered into the study after at least one previous line of platinum-based chemotherapy without durable (>1 year) complete clinical or, if a second look laparotomy after induction therapy had been scheduled, pathological remission. Inclusion criteria also included a WHO performance score of 2 or below and serum creatinine levels below 120 µmol/L as well as bilirubin below 30 µmol/L. Informed consent was obtained from all patients and the studies were approved by the local medical ethics committee.

Regimens

Cyclophosphamide/etoposide regimen, n=11. Cyclophosphamide was given IV in a total dose of 7 g/M^2 divided over three days, Mesna was given in a dose of 4 g/M^2. Etoposide was also given IV over the same three consecutive days in a total dose of 1 g/M^2.

Mitoxantrone based regimens, n=7. Mitoxantrone was given IV in a dose of 30-60 mg/M^2 divided over 3 days, and either melphalan 180 mg/M^2 IV, or cyclophosphamide 7 g/M^2 IV were given on the same days. Cyclophosphamide was combined with Mesna.

Supportive Care

Patients were treated in single person bedrooms without isolation. Prophylactic thrombocyte transfusions, if possible autologous frozen platelets, were given if the thrombocyte count was below 15 x 10^9/L.

Bone marrow cells, acquired and processed as described previously,[2,3] were reinfused on day seven after the start of chemotherapy, for all regimens. Growth factors were not yet available during the time of these studies.

Evaluation of Response

To assess therapeutic responses to high-dose chemotherapy, a peritoneal inspection with biopsies and peritoneal washing was performed prior to and in those patients with a clinically complete remission, after the ABMT program. Patients were considered to have residual microscopic disease when at prior laparotomy no macroscopic tumor was found, but when biopsies or peritoneal washings revealed tumor cells. Minimal residual disease was defined as the largest lesion of residual disease after surgery being ≤ 2 cm. In case of bulky disease, the largest lesion was >2 cm after surgery.

Response and toxicity of the high-dose chemotherapy were graded according to WHO criteria.[4] The duration of response in months was measured from the onset of high-dose chemotherapy until signs of recurrent disease on physical and gynecological examination or in case of increasing serum levels of CA 125.[5] Follow-up examination was performed every 4 weeks and after 1 year at 3 monthly intervals. The survival was also determined from the onset of high-dose chemotherapy. The cut off date for analysis was August 1, 1994. The survival has been analyzed by the life-table method.[6]

RESULTS

Eighteen patients were entered in this study, median age was 46, range 29-57 years. One patient received previous monochemotherapy with melphalan, all others had combination chemotherapy, three carboplatin-based, the others cisplatinum-based.

Before receiving ablative chemotherapy, 14 patients had lesions less than 2 cm and of these, three had microscopic evidence of rest tumor only. The other four had bulky residual disease.

After ablative chemotherapy there were 10 complete remissions, 9 of these were confirmed with biopsies at relaparotomy or laparoscopy. Four patients had progressive disease after recovery from the regimen, three had stable disease.

From the 10 responding patients, five relapsed within one year, the sixth relapsed after 19 months, the four remaining patients are symptom-free after more than 6 years. The relation between tumor bulk, response and survival is given in Table 1. Survival of these 18 patients is given in Figure 1.

Table 1.

Volume of residual disease	Number of patients	pCR	cCR	Median survival months	>5-yr survival
>2 cm	4	-	0	3	0
≤2 cm	14	9	10	24	4

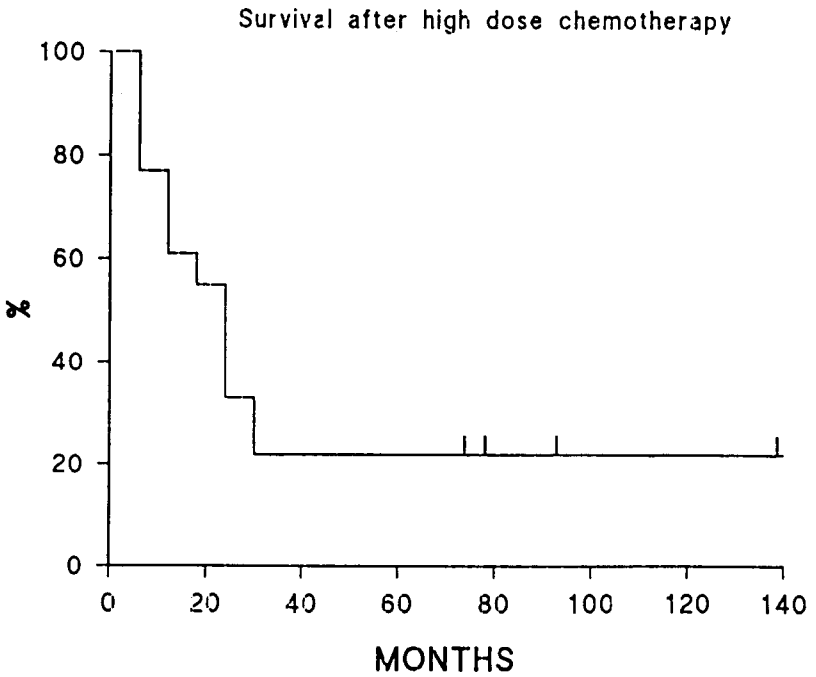

Figure 1. *Survival after high-dose chemotherapy for ovarian carcinoma (n=18). The four surviving patients have no evidence of disease.*

In this group of 18 patients, there was one toxic death due to pulmonary aspergillosis. One patient required cardiopulmonary resuscitation during bone marrow reinfusion, but made an uneventful recovery. All patients had periods of fever requiring IV antibiotic treatment. Three patients had mucositis grade 3.

The four long-term disease-free surviving patients lead a normal life, that does not seem to be compromised by the high-dose chemotherapy regimen.

DISCUSSION

Although the number of patients in this study is limited, the results permit some conclusions. The application of a high-dose regimen in patients with bulky, more than 2 cm residual lesions, does not seem to be of value. This conclusion is in agreement with results in a much larger group of patients treated by French investigators reviewed recently[7] that included 65 patients with macroscopic residual lesions after various forms of first line therapy who subsequently relapsed after high-dose chemotherapy. Better results were obtained in a more favorable subset of 52 patients with microscopic disease who demonstrated a 5-year progression-free survival after high-dose chemotherapy of 30%. In our group of 14 patients with low volume residual disease, the 6-12 year survival is 30%.

The perspective of this form of aggressive chemotherapy is therefore limited to patients who have only microscopic disease or at least disease confined to small nodules ($\leq$2 cm) before ablative chemotherapy.

In an update of results from one of the early platinum containing regimens,[8,9] the 5-year disease-free survival rate for patients with a complete pathologically documented response was 37%, comparable to 31% for the patients who reached no better than microscopic disease at second laparotomy. However, all patients in the latter group eventually relapsed within a period of 10 years.

The long-term observations in the French and our studies therefore leave the possibility that ablative treatment could have value in this group of patients with small volume residual disease, at the price of considerable toxicity as witnessed by the toxic death rates of approximately 5% in both studies. However, the recent application of peripheral stem cell harvesting with human hematopoietic growth factors has improved hematologic recovery and may lead to fewer complications.[10]

The recognition of a group of ovarian cancer patients that has a potential for cure with this form of treatment revives the discussion on second-look laparotomies, as this form of staging is probably the only method to identify these patients. Although ablative chemotherapy may cure some of these patients, other possibilities should also be considered. Among these are intraperitoneal treatment and especially the application of new drugs such as taxol.[11,12]

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SESSION VIII: CML

AN IDARUBICIN-CONTAINING REGIMEN AND G-CSF ARE ABLE TO RECRUIT A HIGH RATE OF NORMAL PROGENITOR CELLS DURING EARLY HEMOPOIETIC RECOVERY IN PATIENTS AT DIAGNOSIS OF CML

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INTRODUCTION

Our previous experience in the collection of "normal" blood progenitor cells (BPCs) in different phases of chronic myelogenous leukemia (CML) patients suggested that between 30% and 50% of patients could achieve a Philadelphia chromosome-negative (Ph-negative) BPCs if these cells were collected in the early phase of recovery after aplasia induced by an idarubicin-containing regimen.[1-4] In this report we will discuss the results achieved with this procedure even in patients at diagnosis of CML or not pretreated with α-interferon.

METHODS AND RESULTS

Between May 1993 and June 1994, nine patients were enrolled in this study. Six patients were not previously treated with chemotherapy and/or α-interferon, while three other patients had previously received hydroxyurea for a median time of 25 months (range, 12-30 months). All patients received a regimen of idarubicin at 8 mg/M^2/d on days 1-5, arabinosylcytosine at 800 mg/M^2 in 2-hr infusions daily on days 1-5, and etoposide at 150 mg/M^2/d on days 1-3. Recombinant G-CSF was given at 5μg/kg from day+8 until the total neutrophils were over 1x10^9/L for three consecutive days. Cephalosporins of third generation and aminoglycosides combined with i.v. fluconazole, were given from d+1. Peripheral blood counts were monitored daily and leukapheresis was begun on the day that the WBC exceeded 0.5-0.8x10^9/L using a CS 3000 PLUS Baxter machine. The next leukapheresis was done when the WBC reached 0.8-1.0x10^9/L and leukaphereses were subsequently performed daily until the WBC exceeded 3.0x10^9/L. The cells collected were counted and studied for chromosome analysis, bcr-abl m-RNA transcripts, *in vitro* cultures, phenotypic characteristics and LTC-IC. The total product

was frozen using Planer R-203. When the peripheral blood count had fully recovered, a sample of bone marrow was taken for cytogenetic analysis.

In untreated patients, leukopheresis was begun 13 to 21 days (median, 16) after the end of chemotherapy. The median time from the last day of chemotherapy and the first leukapheresis was different according to the two categories of patients: untreated patients: 13-16 days (median 14 days); pretreated patients with hydroxyurea: 16-21 days (median 20 days). Cytogenetic analysis of peripheral blood cells showed the absence of Ph-positive metaphases in 6/9 (66%) patients, a decrease of less than 50% of Ph-positive metaphases in other two patients and a decrease of less than 20% in the last patient.

Interestingly, the best results in terms of 100% Ph-negative metaphases, were achieved on the blood cells of the untreated group of patients (5/6 patients) versus pretreated patients (1/3).

Cytogenetic analysis was done on bone marrow cells during recovery. A decrease of Ph-positive metaphases of less than 50% was found in 4 patients (untreated group: 3 patients; pretreated group: 1 patient).

To date, the cryopreserved Ph-negative peripheral blood cells were used as an autotransplant in one patient; 4 other patients are now undergoing transplantation. Engraftment was successful in this patient with neutrophils $>1x10^9$/L at 15 days and platelets $>50x10^9$/L at 24 days. At present the patient is alive and well, with Ph-negative bone marrow 8 months after Ph-negative peripheral blood cells reinfusion. Alpha-interferon therapy at 3M/U three times weekly was given to the patients 4 months after autografting.

DISCUSSION

In a recent update of our previous experience, we demonstrated in 46 patients with CML (24 patients in chronic phase and 22 patients in accelerated phase), ineligible for Allo BMT and cytogenetically resistant to α-interferon, that BPCs were found to be Ph-ve in the collections of 17/46 patients (37%) [chronic phase: 12/24 (50%); accelerated phase 5/22 (23%)] and a decrease of less than 50% Ph-positive metaphases was seen in an additional six patients (chronic phase: 3 patients; accelerated phase: 3 patients).[5]

So far, the Ph-ve collections have been used in 16 patients (CP: 11 patients; AP: 5 patients) as autograft after high-dose radiochemotherapy. Thirteen of 16 patients engrafted and are alive; 5/11

patients are alive and well and Ph-ve at 5+, 13+, 17+, 18+ and 29+ months. The last patient is also PCR-negative while the other 4 patients are PCR-positive to varying degrees.

Interestingly, the chronic phase group had better results than the accelerated phase group which in turn fared significantly better than the blastic phase group.

According to the excellent results in terms of Ph-ve BPCs collections and tolerability in the earlier phase of the disease, we decided to evaluate the impact of the same procedure in untreated patients. To date, six patients have been given the mobilizing procedure. All patients achieved an excellent overshoot of Ph-ve BPCs. In particular, 5 patients achieved only diploid cells on a median of 3 leukaphereses and one patient had a decrease of less than 50% Ph-positive metaphases. Interestingly, with respect to our previous experience in CP-CML, CFU-GM, CD34+/DR- cells, CD34+/Lin-/Thyl+ cells and LTC-ICs were highly increased in this group of untreated patients. Moreover, the time to discharge from hospital was shorter and no patient experienced infections and/or fever. The results achieved in this group of patients demonstrate that it is possible to collect a high number of Ph-ve BPCs, which are able to express higher numbers of CFU-GM, LTC-ICs and CD34+/Lin-/Thyl+ cells.

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AUTOLOGOUS TRANSPLANTATION IN CHRONIC MYELOGENOUS LEUKEMIA: EUROPEAN RESULTS

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ABSTRACT

Autologous stem cell transplantation (ASCT) was performed in 95 patients with chronic myelogenous leukemia (CML) in chronic phase and their data were reported to the European Bone Marrow Transplant Registry. Most patients presented bad prognostic factors. The results were analyzed by September 1, 1993. The actuarial proportion of patients who achieved a complete hematological response at one year was 87.3±7.5% (95% CI). The 3-year percentage of major cytogenetic response was 38.6±13% (95% CI). The actuarial risk of transformation for the evaluable patients was 31.5±14% (95% CI) and the actuarial survival at four years was 83.3±10% (95% CI). These encouraging results suggest that ASCT by itself could play a role to prolong survival in CML patients.

INTRODUCTION

Autologous stem cell transplantation (ASCT) was first used to treat patients with Philadelphia chromosome (Ph) positive chronic myeloid leukemia (CML) in transformation, but clinical results were disappointing as the patients, although they achieved a second chronic phase in most cases, had recurrent transformation within one year after transplantation.[1,2] However, some of these latter patients exhibited a cytogenetic conversion after transplantation and they seemed to survive longer than the other patients. This clinical observation corresponds to the findings of Coulombel et al[3] who demonstrated, using long-term culture techniques, that normal (Ph negative) hematopoietic precursors are present during the chronic phase of the disease and have a proliferative advantage (when transplanted) over leukemic Ph positive progenitor cells. Thus, in an attempt to obtain cytogenetic conversion, leading to a possible prolongation of survival, ASCT was proposed to patients in chronic phase. During the last few years, an increasing number of patients have been transplanted both in the US[4] and in Europe.[5] The preliminary results indicate that the transplant related mortality was acceptable and a prolongation of survival could be obtained, at least in some cases.[4] We

have previously reported the experience of the EBMT Group.[5] This is an update of these latter results which concern a higher number of patients.

MATERIALS AND METHODS

Patients

Ninety-five CML patients who were transplanted during chronic phase were reported to the EBMT registry. For these patients (median age 42 years, [11-64], the median interval between diagnosis and ASCT was 15 months [1-96]).[6] The indications for transplantation were the presence of bad prognostic factors at diagnosis according to Sokal's classification or the absence of clinical or cytogenetic response following IFN treatment.[6] However, 26 patients who responded to IFN, or chemotherapy subsequently underwent ASCT in complete hematological (n=10) or even cytogenetic response (major = 8; minimal = 8).

The median interval between diagnosis and stem cell collection was 7 months (0-56). The patients were transplanted using either peripheral blood stem cells (PBSC) (n=55) collected by leukaphereses at diagnosis in most cases, or bone marrow harvested at diagnosis or during chronic phase. In 82 cases, there was no attempt for *in vitro* purging. However, in 13 patients, bone marrow cells were cultured using the technique described by the Vancouver group (n=9) or treated with mafosfamide (n=4) before being reinfused.

Before ASCT, 59 patients were conditioned with a combination of busulfan (4 mg/kg/day, 4 days) and melphalan (60 to 140 mg/M^2). The other 36 patients were treated with busulfan combined with cyclophosphamide (n=16) or other regimens (n=20) including total body irradiation in only five cases. Depending on the policy used in each center, some patients received recombinant alpha interferon (IFN) as soon as the hematopoietic recovery was achieved following ASCT.

Hematological and cytogenetic responses following ASCT were defined according to the Houston Criteria.[7]

The survival of the patients from ASCT to death or time of last follow-up (September 1, 1993), and the actuarial risk of transformation, were calculated using the Kaplan-Meier method. The log-rank test was used for comparison of the survival curves. Similar statistical methods were used to evaluate the actuarial proportion of patients who achieved either a complete hematological response (CHR) or a major cytogenetic response (MCR) (≥65% Ph^1 negative metaphases).

RESULTS

I. Hematological and Cytogenetic Responses. The actuarial proportion of patients who achieved a complete hematological response at 12 months is 87.3±7.5% (95% CI) (Figure 1). We did not find any patient or disease-related variables that significantly influenced the CR rate. The actuarial proportion of patients achieving a major cytogenetic response was 37.2±10% at 12 months and 40.7±11% at 36 months (Figure 2). The

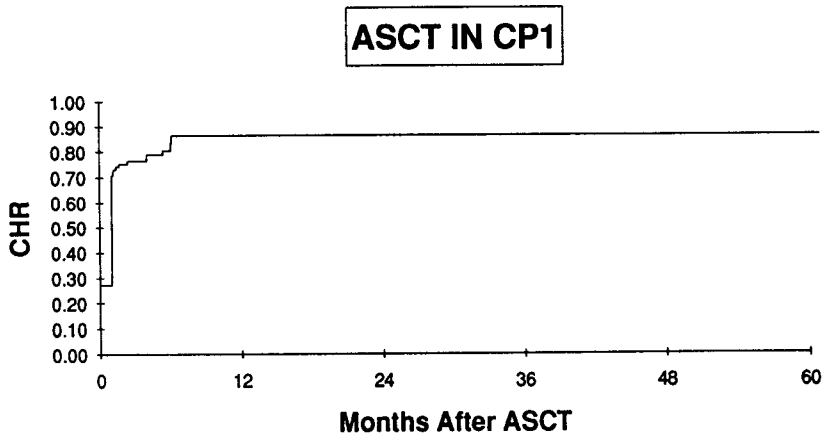

Figure 1. Actuarial proportion of patients who achieved a complete hematological response (CHR). Day 0 represents the day of ASCT (n=95).

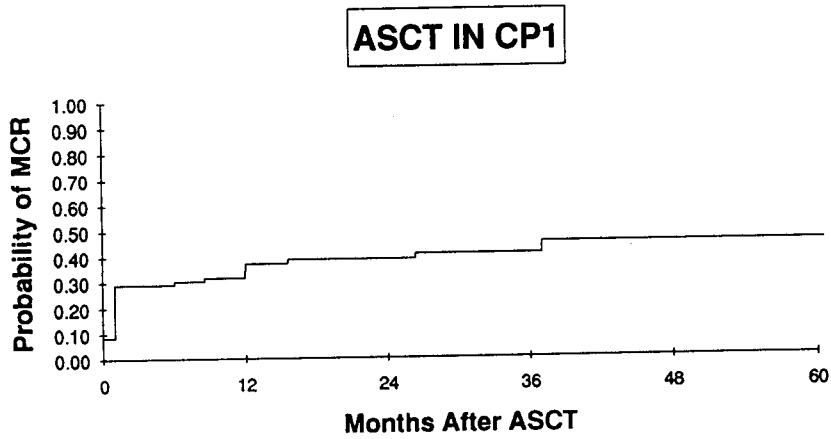

Figure 2. Actuarial proportion of patients who achieved a major cytogenetic response (MCR) after ASCT (≥65% Ph-negative marrow metaphases) (n=95)

MCR rate was influenced by the interval between diagnosis and ASCT but the difference did not reach the statistical level (p=0.06). The patients who received IFN before ASCT had a higher CHR rate than the patients treated with hydroxyurea or busulfan or those who received no treatment (p <0.003). Other variables had no significant effect on the MCR rate.

II. Survival. The 4-year probability of transformation (accelerated phase or blast crisis) was 31.5±14% (95% CI). The risk of transformation was not different for patients transplanted in chronic phase as compared to patients transplanted while in CHR or cytogenetic response. The use of IFN (either before or after IFN) did not influence the risk of transformation. There was a trend for a lower risk of transformation for patients who achieved either CHR or MCR after ASCT than for the others (p=NS).

The 4-year transformation free survival (TFS) was 57±13% (95% CI) (Figure 3) and was not influenced by the disease status at transplant (CHR versus cytogenetic response versus Ph-positive chronic phase). However, the patients who achieved CHR (p <0.005) or MCR (p=0.06) after ASCT had a longer TFS than other patients. Among the other variables, only the age of patients was found to influence significantly the TFS (p=0.04) (longer TFS for younger patients). Marrow purging was not associated with a better TFS (data not shown).

The 4-year survival was 83.3±10% (95% CI) (Figure 3). As for TFS, the overall survival was influenced by the age of patients (p=0.06), the presence of MCR (p=0.06) or CHR (p=0.004) after ASCT. Other factors did not significantly modify the survival of patients.

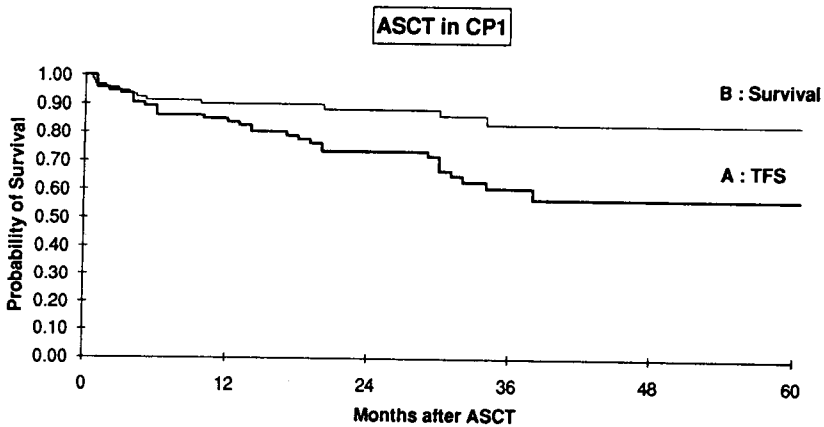

Figure 3. Transformation free survival (A) and survival (B) from ASCT for the 95 patients

DISCUSSION

Autologous bone marrow or blood stem cell transplantation has recently been proposed as treatment for CML patients in chronic phase.[4] Long lasting cytogenetic conversions have been reported using bone marrow cells purged *in vitro* using long-term culture techniques, gamma or alpha IFN or hydroperoxy cyclophosphamide but similar observations have also been made when peripheral blood stem cells collected at diagnosis, were used to restore hematopoiesis.[8] Thus, it cannot be concluded that "*in vitro*" purging is more efficient than the transplantation of unmodified (blood or bone marrow) stem cells. The retrospective analysis of the EBMT registry cannot provide sufficient data to analyze the effect of *in vivo* or *in vitro* purging. This question has to be addressed prospectively.

Another question concerns the possibility of prolonging survival by ASCT. This question also needs to be addressed prospectively. In a recent analysis from U.K., the overall survival of transplanted patients was better than the survival of historical cohorts of patients treated with hydroxyurea or busulfan.[9] In the retrospective analysis of 200 patients undergoing ASCT in Europe or in the US, the 5-year survival of patients transplanted in chronic phase was close to 60% and compared favorably with that achieved with standard chemotherapy. Thus, for patients who did not achieve a major cytogenetic response after IFN, ASCT could be an option when allogeneic BMT is not feasible.

Contributing teams: Besancon (Hôpital Jean-Minjoz; Prof J.Y. Cahn); Bolzano (Ospedale Generale Regionale, Prof P. Coser); Bordeaux (Hôpital Haut-Levêque; Prof J. Reiffers); Caen (Centre Hospitalier; Dr. X. Troussard); Dijon (Hôpital du Bocage, Dr. D. Caillot); Huddinge (Karolinska Institute; Dr. B. Bjorkstrand), London (Hammersmith Hospital; Prof J. Goldman); Lyon (Hôpital Edouard Herriot and Hôpital Debrousse; Dr. J. Troncy, Dr. G. Souillet); Paris (Hôpital Pitié Salpétrière, Dr. V. Leblond); Roma (University degli Studi, La Sapienza Dr. G. Meloni).

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AUTOGRAFTING IN CHRONIC MYELOID LEUKEMIA WITH CULTURED MARROW: UPDATE OF THE VANCOUVER PILOT STUDY

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INTRODUCTION

In 1987 we began a study to evaluate the feasibility of using cultured marrow cells as autografts to allow intensive treatment of patients with chronic myeloid leukemia (CML) who were ineligible for allogeneic bone marrow transplantation (BMT).[1] The laboratory studies upon which this trial was based have been reported,[2-4] as have the results.[5] This report will serve to provide an update.

PATIENTS AND METHODS

Between April 1987 and December 1992, 100 patients (aged ≤60 years, in morphological chronic phase of Philadelphia chromosome [Ph]-positive CML and ineligible for allogeneic BMT) had their marrows assessed after 10 days of long-term culture (LTC) for normal and leukemic LTC-initiating cell (LTC-IC) numbers using previously described procedures.[1,5] In 40 patients (40%), normal LTC-IC in these cultured samples were present at >2% of normal marrow values and leukemic LTC-IC were not detectable. Only patients meeting these criteria were considered eligible for autografting.

Twenty-six patients (aged 22 to 59 years, median 42), 20 in first chronic phase (Group 1) and 6 in accelerated phase or beyond first chronic phase (Group 2), were autografted with 10-day cultured marrow after either a busulfan-based (19 patients) or total body irradiation-based (7 patients) regimen. The methods employed for manipulating the autograft were as previously described.[1,5] In brief, ~ 2 x 10^{10} nucleated cells were

set up in culture, and 10 days later all cells present (range 0.7 to 4.4 x 10^8/kg, median 1.6) were collected and infused.

The experience with the first 22 patients has been reported[5] and this has been combined with that of the subsequent 4 patients (treated on a modified protocol which incorporates elective splenectomy pre-autograft and the administration of low-dose interferon-α [IFN-α] early post-autograft) to produce the following summary of results.

RESULTS

Hematological recovery occurred in 20 of the 26 patients with a median time to 0.5 x 10^9 neutrophils/L and 20 x 10^9 platelets/L of 26 and 46 days post-autograft, respectively. Neutrophil ($p < .0001$) and platelets ($p = .032$) recovery was significantly faster in patients who had previously undergone splenectomy than in those who had not. There was no correlation between the speed of hematological recovery (either neutrophil or platelet) and the number of nucleated cells, clonogenic cells or LTC-IC infused.

One patient died on day 35 in spite of early neutrophil recovery. In the other 5 patients, satisfactory neutrophil recovery was not achieved during the first 6 weeks after autografting. All 5 of these were then infused with unmanipulated reserve cells. In each case this was followed by a recovery of the neutrophil count, although 2 of these patients died, nevertheless, of therapy-related toxicity. There was no correlation between graft failure and the size of the spleen, marrow fibrosis, or the number of nucleated cells, clonogenic cells or LTC-IC infused.

Marrow samples from 14 of the 20 patients who had satisfactory hematological recovery after infusion of cultured autologous marrow showed a complete cytogenetic remission (100% Ph-negative marrow cells) and in 5 of the other 6, a partial cytogenetic remission (66-99% Ph-negative marrow cells) was obtained.

In all but 1 (who died in remission) of the 14 patients in whom a complete cytogenetic remission was achieved, Ph-positive cells became detectable between 4 and 36 months (median 12) post-autograft. Nine of these 14 patients were subsequently treated with low dose IFN-α (typically 3 x 10^6 units, 3 days/week). Six of them returned to a complete cytogenetic remission, but in the other 3 the proportion of Ph-positive cells in the marrow continued to increase.

Sixteen of the 20 patients in Group 1 are alive 1.1 to 6.6 years (median 3.7) after autografting (3 died of therapy-related toxicity and 1 of

blast phase disease). Four of these patients are in complete cytogenetic remission (3 continue on low dose IFN-α), 2 are in partial cytogenetic remission (both continue on low dose IFN-α), 6 are in chronic phase, 1 has accelerated phase disease and 3 have developed blast phase disease.

Three (all treated in accelerated phase) of the 6 patients in Group 2 are alive 3.7, 4.8 and 5.1 years after autografting (2 died of blast phase disease and 1 of therapy-related toxicity). One of the patients is in complete cytogenetic remission (continuing on low-dose IFN-α), 1 is in chronic phase and 1 has accelerated phase disease.

DISCUSSION

These results are encouraging from a number of standpoints. First, the demonstration of a consistent and sustained restoration of normal hematopoiesis in most patients establishes the feasibility of using cultured CML marrow to protect against the "myeloablative" effects of intensive therapy regimens. Second, it appears that elective splenectomy may be a useful maneuver to hasten hematological recovery and might, therefore, reduce therapy-related mortality to <10%. Third, low dose IFN-α was well tolerated and effective in reducing the recurrent leukemic population in two-thirds of the patients, 6 of whom continue with predominantly Ph-negative hematopoiesis now >3 years post-autograft. Finally, only 4 of 23 patients autografted for either first chronic phase or accelerated phase disease have developed blast phase disease, suggesting that some patients may have derived benefit from the therapy received.

These observations notwithstanding, the method of patient selection (on the basis of marrow content of primitive Ph-negative cells) has confounded independent analysis of the effectiveness (in terms of hematological recovery, achievement of cytogenetic remission and survival) of this approach. A study to evaluate the modified protocol (i.e., elective splenectomy pre-autograft and low dose IFN-α early post-autograft) in an unselected population of patients is now underway.

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CHRONIC MYELOGENOUS LEUKEMIA TREATED WITH BONE MARROW TRANSPLANTATION FOLLOWED BY IMMUNOTHERAPY

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ABSTRACT

Patients with chronic myelogenous leukemia (CML) can be cured with allogeneic bone marrow transplantation. It is the establishment of this therapeutic modality that has led to unequivocal evidence for the efficacy of immunotherapy in the treatment of myeloid leukemias in general, and especially in CML. Immunological mechanisms, in the form of graft-versus-leukemia, are integral to the success of allogeneic bone marrow transplantation in CML. Because of the toxicity of allogeneic transplantation as well as limitations imposed by the lack of suitable donors and patient age, autologous bone marrow transplantation (ABMT) has been employed for the therapy of CML using a variety of pre- and post-transplantation manipulations. This report describes the rationale for a current clinical trial using the immunomodulator roquinimex following autologous bone marrow transplantation, in an attempt to stimulate immunological responses thought to be critical for successful therapy in CML.

INTRODUCTION

The use of autografting in chronic myelogenous leukemia (CML) remains controversial. Over the past two decades there have been many reports of autologous bone marrow transplantation (ABMT) all stages of CML, but it has not yet been accepted as standard therapy. Many reports suggest that intensive therapy for CML, with or without autologous transplantation, can resotre Ph-negativity and prolong life.[1-7] However,

most of these reports are small and, to date, no large clinical trials have demonstrated that ABMT in CML extends the duration of chronic phase or prolongs survival.

ABMT for CML has been performed using a variety of methods.[8,9] Most of the early studies attempted to treat patients with CML in accelerated phase or frank blast crisis using cryopreserved cells obtained during the chronic phase.[6,10-13] In these reports either unmanipulated bone marrow or peripheral blood stem cells, or both, were used. Unmanipulated ABMT has also been used, with some success, for patients in first chronic phase.[14,15] More recently, several centers have reported their experience of ABMT in CML using *in vivo* or *in vitro* manipulations of marrow designed to increase the overall response rate. Some have exploited high dose therapy in order to mobilize Ph-negative stem cells and use this as the source of the autograft.[16,17] Together with this *in vivo* approach, which appears to be very promising, several *in vitro* manipulations have been attempted in an effort to reduce posttransplant relapses from "contaminated" autografted leukemia cells. These include attempts to purge the marrow by long-term culture techniques,[18] or direct purging with cytotoxic agents,[19] antibodies directed against the *bcr-abl* fusion protein[20] and the use of IFN-γ.[21]

It is now recognized that the efficacy of allogeneic BMT for acute or chronic myelogenous leukemia is only partially related to the conditioning regimen. Evidence for a graft-versus-leukemia (GVL) effect is primarily based on the increased rate of relapse in recipients of syngeneic marrow;[22] allogeneic marrow transplants without graft-versus-host disease (GVHD),[23] and in most reports of T-cell depleted donor transplants.[24] It was initially thought that T-lymphocytes mediate both GVHD and GVL, with the two processes being interdependent. However, a large survey from the International Bone Marrow Transplant Registry (IBMTR),[25] reported that the *relative risk* for relapse for patients receiving T-lymphocyte depleted marrow transplants was greater than that observed for patients undergoing syngeneic transplants (*relative risk* 5.14 versus 2.95) and both were significantly greater than the risk of relapse in patients undergoing undepleted allogeneic transplantation in whom GVHD did not develop. This finding suggests a T-lymphocyte mediated antileukemic effect that may be independent of GVHD or, at least, clinically apparent GVHD.

Due to the close association of GVHD and GVL, it was originally thought that any beneficial effect of GVL could only be demonstrated in the allogeneic BMT setting. This idea posed a theoretical limitation to the

efficacy of autografting in CML due to the lack of immunological effects that appear to be so critical in allogeneic BMT. In an attemp to overcome this limitation, efforts have recently been made to identify the effector cell, or cells, associated with GVL and develop methods to induce their proliferation or reactivity in circumstances devoid of classic GvHD, such as ABMT. Several immunomodulating agents have been studied are now undergoing clinical trials, mostly in acute myelogenous leukemia (AML). These include interleukin-2,[26,27] cyclosporine[28-30] and roquinimex (Linomide) Table 1.[31,32]

Table 1. The Most Important Immunotherapeutic Mediators in the Myeloid Leukemias

Immunocompetent cells	Immunomodulating agents
T-lymphocytes	IL-2
NK cells	Roquinimex
Macrophages	Interferons
	Cyclosporine A

There is increasing evidence that of all hematopoietic neoplasms, CML may be most susceptible to immune regulation.[33] In allogeneic BMT, the effect of T-cell depleted grafts, or absence of GVHD, on relapse rate is greatest in CML patients.[25,34] Additionally, patients who relapse after allogeneic BMT for CML have recently been shown to respond to infusions of donor T-lymphocytes with complete reversal of all cytogenetic abnormalities.[35,36] In a recent summary of the published literature regarding the therapeutic use of buffy coat infusions for CML patients who relapse after allogeneic BMT,[37] it was reported that in 46 patients, the overall clinical response was 83% with a cytogenetic remission in 20 of 25 evaluable patients (80%). Eighty percent of patients were also reported to have suffered from grade I-IV GVHD. These data suggest that CML may be a particular candidate for immunomodulation following autologous bone marrow transplantation.

Roquinimex (Linomide), a quinoline derivative (Figure 1), is an orally active novel immunomodulator that enhances T cell, NK cell and monocyte/macrophase activity.[38-42] In animal models, roquinimex has been associated with therapeutic effects in both primary tumors and metastases as well as parasitic and viral infections.[43,44] A pilot study in 5 patients with AML following ABMT[45] demonstrated a significant increase in the classic NK immunophenotypes, CD16+/CD3- and CD56+/CD3-, concurrent with roquinimex administration. Functional studies also

indicated enhanced cytotoxic activity of patient cells against the NK-sensitive K562 cell line. Other immunophenotypes reportedly increased during roquinimex administration include CD14+ monocytic cells as well as T lymphocytes, mostly CD4+. Serum IL-6 levels are also increased.[46,47] At the present time, major randomized phase III placebo-controlled-studies are underway in the USA, Europe and Australia evaluating roquinimex in AML post-ABMT.[31,46] Owing to the immunological mechanisms inherent in CML, a phase II study was begun in 1992 at the University of Rochester, Rochester, NY, USA, to evaluate the role of roquinimex when given post-ABMT to patients with CML.

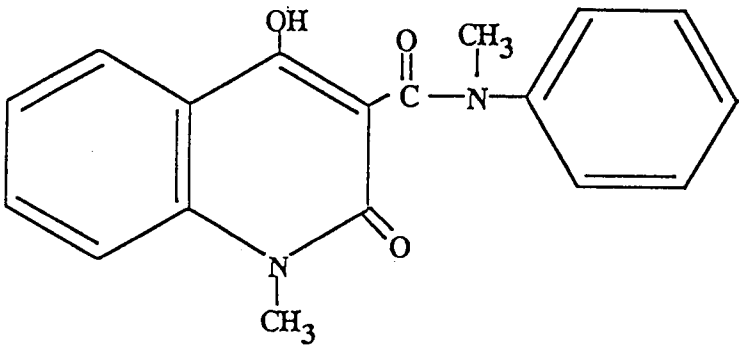

Figure 1. *The structure of roquinimex.*

STUDY DESIGN

Patients eligible for this study were those aged 18-65 with Ph-positive CML at any stage of the disease, excluding blast crisis. Patients in the second chronic phase were eligible as were those who had previously been treated with interferon. Patients who had become cytogenetically negative following therapy with interferon were eligible for this study, provided an abnormal clone could be detected by molecular analysis using a panel of restriction enzymes and/or Southern Blot analysis. Patients less than 45 years of age who have a histocompatible sibling, as well as those with a poor performance status or with major organ toxicity were excluded from the study. The study was approved by the Human Investigations Committee by the University of Rochester, Rochester, NY and all patients signed informed consent. The study design is shown schematically in Figure 2. The conditioning regimen consists of

busulfan 1 mg/kg p.o. q.6h for 4 days followed by 2 days of cyclophosphamide 60 mg/kg IV. Following a single "rest day," unmanipulated bone marrow (usually $3x10^8$ NBC/kg body weight, but not less than $1x10^8$ NBC/kg body weight) or peripheral blood (at least $>5x10^8$ NBC/kg or $1x10^4$ colony forming units-granulocyte colony macrophage [CFU-GM]/kg body weight) is infused as a source of stem cells. Following engraftment (ANC >100/μL), patients are treated with roquinimex for 2 years as outlined in Figure 2. The primary objective of the study is to determine the efficacy of the treatment regimen as measured by: 1) the rate and duration of cytogenetic conversion; 2) the time from BMT to clinical progression; and 3) overall survival from bone marrow transplantation. The secondary objectives are to determine the toxicity of roquinimex as administered in this study and concurrently to determine immunological parameters in an attempt to correlate these factors with clinical response. Between March 1992 and August 1994, 14 patients entered the study. All patients were cytogenetically positive for the Ph chromosome pretransplant and all required significant doses of myelosuppressive therapy prior to transplantation to maintain stable blood counts. Nine patients were in first chronic phase (CP), two in accelerated phase (AP) and two patients were transplanted in the second chronic phase (CP2). The latter two patients presented in blast crisis, were successfully reinduced with high dose chemotherapy consisting of daunorubicin 70 mg/M^2 x 3 days and cytosine arabinoside 200 mg/M^2 x 9 days, and were then reinfused with marrow obtained in the second chronic phase as their source of stem cells. The two patients in accelerated phase were reinfused with marrow obtained during the accelerated phase. The median age of the patients was 48 years (range 12-56) and the median time from diagnosis to ABMT was 33 months.[8-37]

There has been no procedure-related mortality, but two patients died from progressive disease at 4 months (CP2) and 20 months (CP1).

The median follow-up of the patients was 17 months (2-29 months) and the median time to engraftment (neutrophils >500/μL) was 16 days (12-35 days).

Although the study allowed the use of peripheral blood as a source of stem cells, this was required for only one patient, in accelerated phase, in whom an adequate number of stem cells could not be obtained from the bone marrow. It is important to emphasize that unmanipulated stem cells were used throughout with no attempt at *in vitro* manipulation of the reinfused cells, which in every patient were 100% Ph-positive. To avoid significant heterogeneity in the patient population, patients were treated

pretransplantation with hydroxyurea to achieve a WBC <20,000/μL and reduce the spleen size to ≤8 cm below the left costal margin.

This study is ongoing and some significant clinical as well as cytogenetic responses have been observed in this population (data not shown).

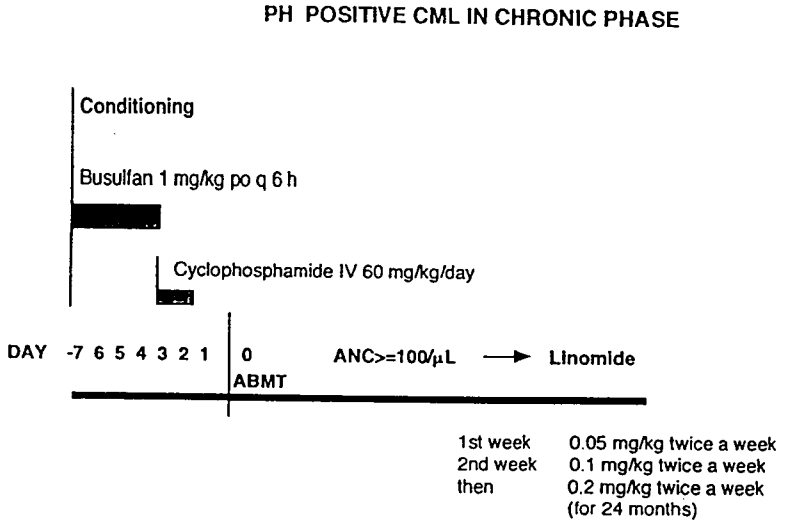

Figure 2. *Schematic representation of the study design.*

The primary toxicity on this regimen consisted of edema, usually periorbital or ankle, or both. This was observed in half the patients receiving roquinimex and typically occurred within 4-6 weeks of starting this medication. Four of the patients had skin rashes that were histologically indistinguishable from grade II graft-versus-host disease while five of the patients developed a more unusual skin rash consistent with eccrine sweat gland necrosis.[48] One of the patients developed severe gastrointestinal toxicity during hospitalization for pancytopenia about 6 months posttransplant. The pattern of the GI toxicity, as assessed radiologically, strongly suggested a "graft-versus-host" effect (Figure 3). The gastrointestinal toxicity resolved promptly after administering a short course of corticosteroids.

Serial immunophenotypic analyses have been performed on all of the patients. However, since patients with CML may have a unique immunological response,[33] it is difficult to define an adequate control group, as this is not a randomized study. The ideal control for this study would be CML patients autografted, but without receiving subsequent roquinimex therapy. Furthermore, it is difficult to know how to interpret the number of NK cell counts post ABMT for CML. Since there appears

to be a deficiency of NK cells with patients with CML,[49,50] it may be that a normalization of NK cell number and function following Linomide therapy is clinically significant. Finally, functional studies on NK cell subsets will prove more relevant than the cell counts.

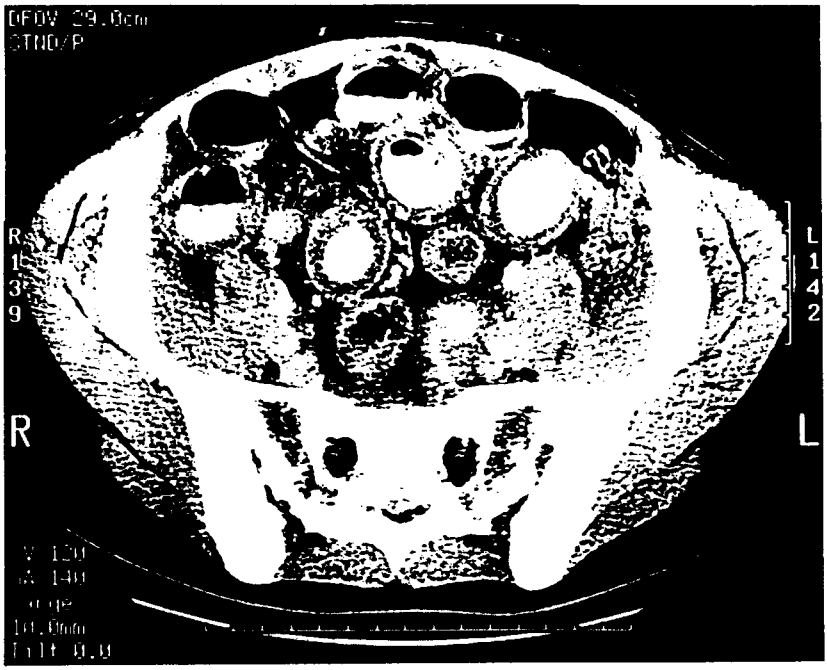

Figure 3. *CAT scan appearance of thickened small bowel in patient with acute gastrointestinal toxicity following roquinimex administration.*

This trial is clearly in its early phases and although the data are encouraging, further accrual and follow-up is needed before any definitive conclusions can be made. Whether or not a true graft-versus-leukemia effect can be induced in CML patients receiving roquinimex remains to be seen.

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INTENSIVE TREATMENT IN ORDER TO MINIMIZE THE PH-POSITIVE CLONE IN CHRONIC MYELOGENIC LEUKEMIA (CML)

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INTRODUCTION

The poor prognosis of patients with CML on conventional symptomatic therapy is well known. Allogeneic bone marrow transplantation (BMT) can result in prolonged disease-free survival and may also be curative. There are now also reports indicating that autologous BMT (ABMT) may be an effective treatment in CML.[1-3]

A significant reduction of the malignant clone in CML might prolong time to metamorphosis. In addition to ABMT, interferon (IFN) and intensive chemotherapy[4,5] are also reported to reduce or eliminate the Ph-positive clone in CML. In the present study, in patients ≤55 years, we have therefore combined these three treatment modalities to see whether a significant reduction of the malignant clone could be maintained for a longer period of time and also to see whether survival could be improved compared with historical controls.

PATIENTS AND METHODS

From September 1, 1989 to June 1, 1994, 125 patients ≤55 years with Ph-positive (or Ph-negative, BCR-ABL-positive) CML were registered in the study. Eighty were males, 45 females and the median age was 41 (range 19-55).

The study design is shown in Figure 1. Patients having a donor were allotransplanted, while all other patients, after peripheral stem cell

harvest at diagnosis, were offered treatment with hydroxyurea (HU) and IFN. If the patients were not cytogenetically normal after IFN+HU treatment for six months, they received 1-3 courses of intensive chemotherapy, interfoliated in most cases by IFN+HU. When Ph-negativity occurred, bone marrow was harvested and the patient was autotransplanted. Patients with less than 50% Ph-positive metaphases after the third chemotherapy course were also offered ABMT.

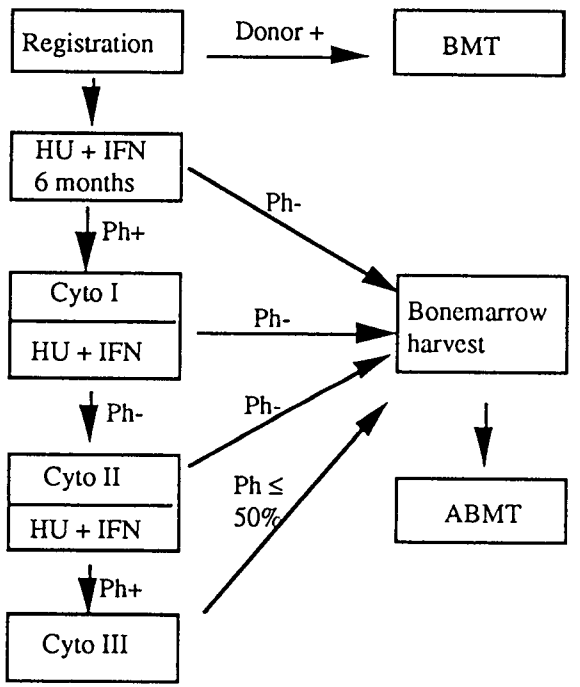

Figure 1. *Design of treatment. HU + IFN = Hydroxyurea 1-3 g daily and Interferon (Introna,[R] Schering-Plough, Sweden) 5-10 x 10^6 IU/M^2 sc. daily in order to keep WBC <2-4 x 10^9/L and/or platelets 100-150 x 10^9/L. Cyto I = Daunorubicin 50 mg/M^2 1h x III, Ara-C 200 mg/M^2 24h x VII. Cyto II = 1 g/M^2 2h x 2 x IV. Cyto III = Amsacrine 75 mg/M^2 x 1 x IV and Ara-C 1 gm/M^2 x 2, 2h x IV.*

RESULTS

As shown in Table 1, IFN+HU therapy reduced the percentage of Ph-positive metaphases in 46/84 patients (55%) and 4/84 (5%) became Ph-negative. The corresponding figures after two intensive chemotherapy

courses were 67/92 (70%) and 25/92 = 30%, respectively. The third chemotherapy course had only a minor effect.

Table 1. Outcome of Different Therapies on Number of Ph-positive Metaphases in Bone Marrow

	IFN + HU	Cyto I	Cyto II	Cyto III
Ph-negative	4	15	10	1
Ph-reduction ≥10%	42	31	11	4
No Ph-effect	38	11	14	8
n	84	57	35	13

After the first two intensive courses, median time for ANC <0.5 x 10^9/L was 13 days (range 0-60) and for platelet count <20 x 10^9/L (range 0-70+) days.

In thirty patients all analyzed bone marrow cells became cytogenetically normal. Twenty of these patients were autotransplanted, two had bone marrow harvested and are awaiting ABMT, one was allotransplanted, while in six there was progression to Ph-positivity.

In all, twenty-six patients were autotransplanted. Three have not yet been analyzed for Ph after ABMT, eleven remain Ph-negative 1-48+ months while twelve have relapsed with Ph-positivity 3-22 months after ABMT.

Twenty-three patients are alive and well after ABMT, while three have died (1 interstitial pneumonia, 2 blastic transformation) after 1, 7 and 18 months, respectively. Of the remaining 99 patients in the study, 38 are in continuous chronic phase, 46 are allotransplanted and 15 are dead (five during chemotherapy, one refusing transfusions, nine in blastic transformation). With thirty months median follow-up, the 6-year actuarial survival from diagnosis is 68%.

DISCUSSION

There are several reports showing that IFN-treatment in newly diagnosed CML reduces the frequency of Ph-positive bone marrow cells in approximately 2/3 of the patients. We have achieved the same result after a relatively short treatment period with HU added to IFN. A further reduction was then achieved after the intensive chemotherapy. It can, however, not be excluded that the same result could have been reached by chemotherapy alone.

The side effects of intensive chemotherapy were the same as observed in patients treated for acute leukemia.

Our study also shows that ABMT is possible with bone marrow harvested close to IFN-treatment. The hematological recovery was slow, but did not differ from that seen after ABMT for acute leukemia. However, the number of patients autotransplanted is small and we therefore recommend a peripheral stem cell harvest at diagnosis as backup.

Around 50% of the patients are in cytogenetic (in 6 cases also analyzed with PCR) remission 3 to 48+ months after ABMT.

At present we can make no firm conclusions but it seems reasonable to assume that our treatment might prolong survival in CML. The actuarial six-year survival of 68% and a median survival not yet reached suggests a better outcome than is reported for historical controls.

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AUTOLOGOUS TRANSPLANT FOR CHRONIC MYELOGENOUS LEUKEMIA WITH MAFOSFAMIDE PURGED MARROW

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INTRODUCTION

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder due to an acquired abnormality in a pluripotential hematopoietic stem cell.[1,2] Busulfan or hydroxyurea may control clinical symptoms but are not able to delay blastic transformation, although hydroxyurea has been reported to prolong survival.[3] Recently, it has been shown that recombinant interferon-alpha may delay disease progression and prolong survival,[4] but a complete cytogenetic remission with polyclonal hematopoiesis has been reported in 10 to 20% of patients.[4,5] Allogeneic bone marrow transplantation remains the only curative treatment for CML,[6,7] but more than 70% of patients will be ineligible for this procedure.[8]

Several experimental and clinical evidences suggest that functionally competent polyclonal Ph-negative hematopoietic stem cells are present in CML,[9-16] thus supporting the concept that autologous bone marrow transplantation (ABMT) may represent a valuable alternative for CML patients in chronic phase.[17] Current clinical results show that ABMT in CML patients leads to 5% long-term disease-free survival, with leukemic relapse being the main cause of treatment failure.[18,19] To become an effective treatment modality, it is likely that either purging of leukemic stem cells or selecting for nonleukemic stem cells will be required.[20,21] *Ex vivo* marrow purging with the cyclophosphamide derivative mafosfamide has been reported to be able to exert a selective killing effect on Ph-positive cells either cultured in suspension[22] or in semisolid media.[23] Recently, it has been demonstrated that the primitive progenitors generated by CML marrow cells adherent to allogeneic marrow stroma are significantly enriched in Ph-negative clonogenic cells,[24] due to a defective capacity of CML progenitors to adhere to allogeneic marrow stroma.[25] In addition, mafosfamide significantly reduces the frequency of Ph-positive

progenitors within the stroma-adherent fraction, supporting an antileukemic effect of this drug at a primitive clongenic cell level.[24,26]

In the present report, the results of a pilot study testing the efficacy of *ex vivo* marrow treatment with mafosfamide followed by autografting are summarized.

PATIENTS AND METHODS

Patient Population. Ten adult patients, six males and four females with a median age of 44 years (range, 36-52 years) and a diagnosis of Ph-positive CML were treated with high-dose chemotherapy followed by autografting with mafosfamide-treated marrow. The clinical characteristics of the patients are reported in Table 1. All patients fulfilled the following criteria set for entry into the study: 1) Ph-positive CML; 2) ≤60 years old; 3) ineligible for allogeneic or unrelated marrow transplant. At the time of transplant, 7 patients were in chronic phase (5 in first, 2 in second) and 3 in accelerated phase. The median interval between diagnosis and ABMT was 37 months (range, 8-67 months).

Pre-Harvest Test. Patients to be harvested were selected on the basis of a laboratory assessment of the percentage of Ph-negative stroma-adherent progenitor cells within mafosfamide-treated marrow.[24] Briefly, mafosfamide-treated (100 μg/ml), plastic non-adherent mononuclear cells (5×10^5/ml) were plated onto confluent allogeneic stroma for 2 hours (37°C, 5% CO_2) to allow attachment of stroma-adherent cells. The non-adherent cells were removed by extensive washing, and the stroma-adherent cells were cultured in suspension for 3 days over the allogeneic stroma. Then, stroma-adherent cells were harvested by repeatedly washing each dish and incorporated in methylcellulose to assay the growth of clonogenic cells to be analyzed at the cytogenetic level. Only patients showing ≥50% Ph-negative stroma-adherent mafosfamide-treated progenitors were considered eligible for marrow harvest and autografting. Between January 1991 and November 1993, 25 patients fulfilling the criteria set for autografting had their marrow assessed for normal and leukemic stroma-adherent mafosfamide-treated progenitors. Sixteen patients (64%) showing ≥50% Ph-negative stroma-adherent mafosfamide-treated progenitors were considered eligible for marrow harvest and autografting.

Marrow Treatment. Marrow was harvested after a median interval from diagnosis of 23 months (range, 5 to 57). Patients were taken off all antileukemic therapy at least 7 days prior to bone marrow harvest.

Table 1. Clinical Characteristics of the Patients

Case	Age/Sex	Disease Stage	Previous Therapy	Conditioning Regimen	Cell Dose x10e^8/Kg	CFU-GM x10e^4/Kg	Days to ANC ≥500/μL	Day to Plt ≥20,000/μL	Survival from Diagnosis (months)	Survival from Post-BMT (months)
1	45/M	AP	HU IFN	BU CY	1.7	0.7	28	28	41	16
2	42/M	CP	HU IFN	BU CY MEL	2.1	0.4	33	37	39	20
3	49/F	CP	HU IFN	BU CY MEL	0.5	0.1	17	24	45+	4+
4	50/F	CP	HU IFN	BU CY MEL	1.2	1.4	NE	NE	61	NE
5	48/F	2nd CP	HU IFN CT	BU CY MEL	0.7	0	70	151	75+	8+
6	39/M	AP	HU IFN	BU CY MEL	0.8	0.3	48	57	62	12
7	52/M	CP	HU	BU CY MEL	1.1	1.6	25	27	31+	22+
8	37/F	AP	HU IFN	BU CY VP-16	0.9	0.2	32	43	47	17
9	36/M	2nd CP	BU IFN CY	BU CY	1.5	0.2	32	41	66	31
10	43/M	CP	HU IFN	BU CY VP-16	1.3	2.1	72	79	39	7

AP = accelerated phase; CP = chronic phase; HU = hydroxyurea; IFN = interferon alpha; CT = chemotherapy; BU = busulfan; CY = cyclophosphamide; MEL = melphalan; VP-16 = etoposide; ANC = absolute neutrophil count; + = indicates that the patient continues to survive

The method of marrow purging has been described in detail elsewhere.[27] Briefly, mafosfamide (ASTA Medica, Bielefeld, FRG) was provided as lypophilized compound and reconstituted with saline at 10 mg/ml. Marrow was incubated with the drug (100 μg/ml, 30 min, 37°C) with gentle agitation every 5 min, then the reaction was stopped by immersion in ice-cold water (4°C). After treatment, marrow was cryopreserved in 10% dimethyl sulfoxide, 55% autologous plasma, and stored in liquid nitrogen.

Conditioning of the Patients. Three preparative regimens were used. Six patients received: busulfan 4 mg/kg/d x 4d, cyclophosphamide 60 mg/kg/d x 2d and melphalan 90 mg/sqm/d x 1d; two patients received busulfan 4 mg/kg/d x 4d and cyclophosphamide 60 mg/kg/d x 2d; and two received busulfan 4 mg/kg/d x 4d, cyclophosphamide 60 mg/kg/d x 2d and VP-16-213 30 mg/kg x 1d.

Cytogenetic and Molecular Analysis. Cytogenetic analysis and standard GTG- or QFQ-banding techniques were performed in each case according to standard methods.[28] Single colony karyotyping was performed according to the technique of Dubè et al.[29] Karyotyping of individual progenitors was extensively used to cytogenetically characterize the stroma-adherent progenitors both prior to harvest and within marrow graft. The percentage of Ph-negative stroma-adherent progenitors within marrow graft was evaluated in each patient and correlated with the posttransplant occurrence of nonleukemic engraftment. DNA analysis was performed according to standard techniques.[30] The filters were hybridized with a 3' probe for the M-bcr (Oncogene Sciences, Manhasset, USA) previously oligolabelled with ^{32}P and exposed to photographic film for 3-5 days.

RESULTS

The mean (±SD) number of infused nucleated marrow cells was $1.18 \pm 0.49 \times 10^8$/kg (range, 0.5 to 2.1 x 10^8/kg). The mean number of CFU-GM transplanted per kg body weight was $0.70 \pm 0.73 \times 10^4$/kg (range, 0 to 2.1 x 10^4/kg). The median time to achieve 500 neutrophils/μL was 32 days (range, 17 to 72). A platelet count of 20,000/μL was achieved at a median of 40 days (range, 24 to 151). Patients required a median of 10 (range, 2 to 27) units of red cells and 206 (range, 50 to 578) units of platelets.

Standard cytogenetic analysis of marrow harvest cells revealed 100% Ph-positive metaphases in 8 of 10 patients, whereas at autografting

all patients were 100% Ph-positive. During the initial phase of hematopoietic regeneration, direct cytogenetic analysis revealed 100% Ph-negative marrow metaphases in 6 out of 9 analyzable patients. In these patients, the median duration of Ph-negative hematopoiesis was 6.5 months (range, 4 to 30). In all patients who achieved complete disappearance of Ph-positive marrow metaphases, Southern blot analysis confirmed the disappearance of the *bcr/abl* rearrangement. Of the remaining 3 patients, two (nos. 6, 8) revealed only Ph-positive metaphases from the first cytogenetic analysis performed posttransplant, whereas in case no. 5 a substantial proportion (75%) of Ph-negative marrow metaphases could be detected but gradually were replaced by Ph-positive cells so that only Ph-positive metaphases were detected at +3 months.

At the time of marrow infusion, a thawed aliquot of mafosfamide-treated cells was used to determine the percentage of Ph-negative clonogenic cells generated by the stroma-adherent fraction. As shown in Table 2, three samples (cases nos. 5, 6, 8) revealed 75%, 56% and 69% Ph-negative stroma-adherent progenitors, respectively. In contrast, the remaining seven patients had 100% Ph-negative stroma-adherent progenitors. Following autograft, patients with ≤75% Ph-negative progenitors within the stroma-adherent fraction engrafted Ph-positive, whereas patients with >75% Ph-negative progenitors within the stroma-adherent fraction engrafted Ph-negative.

Nine patients were evaluable and have been followed for a median of 16 months (range, 4 to 31). One patient (no. 4) died of stroke by day 19. None of the patients received therapy following transplant until hematological relapse occurred. Five patients grafted in accelerated phase (n=3) or in 2nd chronic phase (n=2) evolved into blast crisis at +5, +10, +11, +13 and +31 months, respectively. Three cases (nos. 1, 6, 9) relapsed in blast crisis following a period of hematological and cytogenetic remission, whereas in two cases (nos. 5, 8) evolution into blast crisis was preceded by a period of leukocytosis and thrombocytosis which was successfully controlled by interferon-alpha or hydroxyurea. Of the remaining patients, two (nos. 2, 10) died of non-hematological causes, one (no. 3) is in a stable, Ph-negative, chronic phase not requiring therapy at +4, and one (no. 7) remained in a stable, Ph-negative, chronic phase for 6 months. At that time, administration of interferon-alpha was successfully started to control leukocytosis. The patient is still in chronic phase, but Ph-positive, at +22 months following autografting.

Table 2. Results of Cytogenetic Analysis Before and After Autografting

	Ph-negative metaphases (%)																
				After Autograft (months)													
Case	Harvest*	Stroma-Adherent Progenitors**	Autograft*	1	2	3	4	5	6	7	8	9	10	11	12	30	31
1	0	100	0	100	100	100	100	100	100	100	100	100	80	0			
2	0	100	0	100	100	100	100	100	100	83	79	62	14	0			
3	40	100	0	100	100	100	100										
4	0	100	0	NE***													
5	0	75	0	75	40	0											
6	0	56	0	0													
7	0	100	0	100	100	100	100	100	100	0							
8	0	69	0	0													
9	10	100	0	100	100	100	100	100	100	100	100	100	100	100	100	100	0
10	0	100	0	100	100	100	100	100	100	100							

* *Percentage of Ph-negative bone marrow metaphases obtained after routine cytogenetic analysis of freshly aspirated bone marrow.*

** *Percentage of Ph-negative bone marrow stroma-adherent mafosfamide-treated progenitors obtained by single colony karyotyping (see Materials & Methods).*

*** *Not evaluable. The patient died of stroke by day 19.*

DISCUSSION

The use of autologous unmanipulated cells for autografting CML patients either after transformation or even in chronic phase has induced a durable benefit in a few cases.[31-33] Elimination of malignant stem cells or isolation of Ph-negative stem cells from the graft is the prerequisite for autografting CML patients.[20] Several approaches have been proposed to purge autologous marrow.[19] These approaches include purging *in vivo* with chemotherapy[34,35] or *in vitro* with interferon-gamma,[36] *in vitro* selection by growth in long-term culture,[37] *in vivo* therapy with combinations of antileukemic agents or with interferon-alpha followed by single or double autotransplant.[32,38]

In this paper we report the results of a pilot study including 10 patients with CML treated with high dose chemotherapy and transplantation with *ex vivo* mafosfamide purged marrow. Our patients were harvested in chronic phase and grafted either in accelerated phase or chronic phase. Despite the clinical and biological heterogeneity of our patients as well as their long therapeutic history, six out of nine evaluable patients engrafted Ph-negative with an overall duration of Ph-negative hematopoiesis of 6.5 months. *In vitro* purging with mafosfamide may effectively reset the balance between normal and neoplastic clones and may influence stem cell dynamics by transiently depleting Ph-positive stem cells and thus favoring the repopulating activity by normal stem cells. Attempts to further enrich normal marrow cells *in vitro* or to deplete Ph-positive stem cells prior to autotransplant might take advantage not only of the greater mafosfamide toxicity for Ph-positive versus Ph-negative progenitors, but also of the differences in stromal adherence between normal and leukemic cells, provided that an adequate quantity of these cells will be available by *ex vivo* expansion procedures.[24,26]

A transient re-establishment of Ph-negative hematopoiesis has also been reported following autograft using unpurged hematopoietic stem cells.[31-33,38] Since our study was designed as a pilot trial, neither a control group nor historical controls are available and, currently no definitive conclusion concerning the real impact of mafosfamide marrow purging on the natural history of CML can be drawn. Prospective controlled trials are required to investigate the relative efficacy of autografting with both purged and unpurged marrow. Prior to ABMT, all but one patient had been treated with interferon-alpha. Since interferon-alpha is known to exert a cytotoxic effect on progenitor cells and restore their adhesive properties,[39] the question then arises as to whether pretreatment with

interferon-alpha may eventually influence mafosfamide efficacy. Due to the small number of patients reported herein, the possibility that the combined cytoreductive and stroma-adhesion restorative properties of interferon-alpha could somewhat affect mafosfamide action cannot be ruled out.

We have extensively characterized the cytogenetic status of the stroma-adherent progenitors to evaluate the percentage of Ph-negative progenitors infused with the graft. The correlation between the percentage of infused Ph-negative stroma-adherent progenitors and the *in vivo* occurrence of either normal or leukemic hematopoiesis resulted in an interesting observation. In fact, patients who had >75% Ph-negative stroma-adherent progenitors engrafted Ph-negative, whereas those patients with ≤75% Ph-negative stroma-adherent progenitors engrafted Ph-positive. The predictive clinical value of this parameter requires confirmation in a larger group of patients.

Increasing evidences suggest that long-term remission of CML by marrow transplant is not only dependent on the eradicating effect of chemoradiotherapy but also on the even more important antileukemic role of the graft-versus-leukemia (GVL) effect.[40] Our patients did not receive any posttransplant therapy until this was required due to the clinical evolution of each individual patient. The possibility to therapeutically induce a GVL effect following autograft with either interferon-alpha, interleukin-2 or other cytokines must be considered an important issue.[19]

In conclusion, the results of the present pilot study are encouraging in that they demonstrate that engraftment can occur from Ph-negative stem cells selected by mafosfamide purging. In selected CML patients, mafosfamide seems to be effective in reducing the size of the malignant clone and inducing a transient period of Ph-negative hematopoiesis. However, prospective controlled studies on a larger group of patients with disease in first chronic phase will be required to evaluate the potential of mafosfamide marrow purging. In addition, further information about how to assess normal and leukemic hematopoietic stem cells is needed to optimize any *in vitro* manipulation. Finally, modifications of the purging procedure as well as posttransplant manipulation of the immune-hematopoietic system will be required to prolong either survival or cytogenetic remission or cure CML patients ineligible for allogeneic or unrelated BMT.

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SESSION IX: PERIPHERAL STEM CELLS

PROGENITOR THRESHOLD EFFECTS IN HAEMOPOIETIC RECONSTITUTION

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Increasing experience with haemopoietic transplantation and its demonstrated safety have widened the age limits and the disease type indications for high dose therapy. Both the very young and the over sixties have been transplanted, so are patients with chemosensitive solid tumors. Haemopoietic growth factors such as granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage (GM)-CSF have improved neutrophil recovery following bone marrow rescue, yet the most significant advance in rapid haemopoietic reconstitution (HR) and reduced resource utilization has been the use of mobilized peripheral blood instead of bone marrow for haemopoietic rescue.[1,2] This benefit of rapid HR is seen in some, though not all, patients receiving mobilized autografts so it is important to understand the contributing variables.

Non-Progenitor Dose-Related Variables Influencing HR

Patient factors have long been recognized as influencing HR. Pediatric patients often have higher yields from mobilization than adults and therefore tend to recover more rapidly. It has been suggested that the haemopoietic system is more vigorous early in life although no quantitative measures are yet available. Moreover, cord blood has a much higher level of progenitors than adult blood. Among adult patients, age has not been found to influence HR although progenitor yield from mobilization may vary up to 10-fold in comparable patients undergoing a standard G-CSF mobilization protocol so intrinsic factors, as yet to be defined, are obviously important.[3,4]

It has been reported that the amount of previous chemotherapy significantly influences HR.[5] The amount of previous chemotherapy also affects progenitor yields from mobilization[6] so is probably a reflection of bone marrow damage. Bone marrow damage is most commonly regarded as damage to the haemopoietic stem/progenitor cells although a recent observation suggest that stromal damage may also affect HR.[4] A cohort of

patients who have poor prognosis carcinoma of breast had peripheral blood mobilized by G-CSF prior to any chemotherapy. The mobilized blood was used for haemopoietic recovery following 3 cycles of high dose cyclophosphamide and epirubicin. Surprisingly, there is progressive slowing of HR with subsequent cycles in spite of identical haemopoietic rescue, thus suggesting that stromal damage plays a significant role in HR. These patient factors are undeniably important although most are difficult to quantitate and not often controllable. Use of G-CSF/GM-CSF post-transplant hastens neutrophil reconstitution [7,8] and is therefore another nonprogenitor dose-related factor influencing HR.

Types of Progenitor Threshold

The correlation of progenitor dose (the number of progenitors infused) with HR has been described in several studies [1,9] and 2 recent reviews highlight the complex relationship between them.[10,11] Besides the significant correlation between the rate of HR and the progenitor dose as measured by the myeloid progenitor (CFU-GM) or $CD34^+$ assays, a number of progenitor thresholds can also be recognized:

1. A minimum progenitor threshold below which rapid HR is unlikely.
2. An optimum progenitor threshold above which rapid HR is assured.
3. A progenitor threshold for sustained HR.
4. A threshold of rapid HR that could not be overcome even with high numbers of progenitors and the use of haemopoietic growth factors post transplant. 3. and 4. has been dealt with [10,11] and so will not be further discussed here.

In practice, progenitors can also be measured by erythroid, megakaryocytic, multilineage clonogenic assays, subsets of $CD34^+$, or in research laboratories, as pre-progenitors[12], long-term culture-initiating cells (LTCIC)[13], cobblestone area forming cells (CAFC), SCID-hu [14] or neonatal sheep [15] models. Nonetheless, most reported data were based on CFU-GM and/or $CD34^+$ cell measurements so this article will discuss progenitor thresholds using these two. It is important to appreciate that HR is, in all likelihood, a polyphasic process with the primitive haemopoietic progenitors responsible for long term HR (long term marrow repopulating cells, LTMRC) and the more mature progenitors responsible for the early phase of HR but probably unable to sustain haemopoiesis lifelong (short-term marrow repopulating cells, STMRC). CD34 measurement includes both LTMRC and STMRC and increasing

evidences suggest that these cells can be differentiated based on co-expression of maturation and lineage markers. Most oligopotent clonogenic assays probably identify STMRC while the specialized research laboratory assays probably identify cells much closer to LTMRC.

The standardization of progenitor assays to make inter-institutional comparisons valid is a major task facing transplant centres. Clonogenic assays have a large number of biological components such as the colony stimulating activity used and different batches of fetal calf serum that are difficult to standardize. Different laboratories employ different culture conditions and scoring criteria and scoring validation between different observers are usually not done. The establishment of a serum-deprived culture system and an accepted combination of recombinant haemopoietic growth factors will be critical developments although the increased costs and the consensus required are formidable hurdles. Hence the current trend in many clinical laboratories is to move to $CD34^+$ cell assay by flow cytometry. Standardization of $CD34^+$ assays is just as critical because the measurement of rare events by flow cytometry has it own stringency.[16] It is also important to bear in mind that flow cytometric analysis does not measure proliferative capacity so the two approaches are complimentary to a degree.

Progenitor threshold effects are clinically relevant. Firstly, the minimum threshold provides us with a measure of the minimum progenitor dose that we should try to reinfuse to avoid delayed HR, important when assessing marginal progenitor yields from patients with compromised bone marrow reserve. Secondly, the optimum threshold provides us with a target for designing high dose therapy protocols when rapid HR and low procedure-related morbidity and mortality are important, especially in adjuvant studies. Thirdly, the trend towards multiple high dose therapy imposes a demand for sufficient progenitors for multiple rescues so a threshold dose for rapid HR is required. Lastly, the increasing complexity of *ex vivo* stem cell processing, such as $CD34^+$ selection, tumor cell purging, maturation/expansion and gene therapy makes it imperative that measures of graft adequacy are defined.

Threshold Dose Based on CFU-GM

The N500 (number of days post transplant to reach $0.5x10^9$ neutrophils/L): CFU-GM dose correlation shown in Fig. 1 suggests that N500 is unlikely to be <11 days if the CFU-GM dose is less than $10\text{-}20x10^4$/kg BW, so this would be the minimum threshold. The same figure

suggests that N500 is unlikely to be >15 days if the CFU-GM dose is above 40-50x10^4/kg BW, so this would be the optimum threshold.

The P50 (number of days post transplant to reach 50x10^9 platelets/L): CFU-GM dose correlation shown in Fig. 2 does not give a clear indication of a minimum threshold but suggests that P50 is unlikely to be >15 days if the CFU-GM dose is above 50x10^4/kg BW. Hence 50x10^4/kg BW appears to be the optimum CFU-GM threshold. In general, CFU-GM dose predicts platelet reconstitution less well than neutrophil reconstitution. In particular it fails to predict the secondary fall seen in patients with acute myeloid leukemia transplanted with mobilized peripheral blood [17] and fails to predict delayed and incomplete platelet reconstitutions sometimes seen.[18] We also found that there is poor correlation between CFU-GM and megakaryocyte progenitors in mobilized blood (Dyson et al, manuscript in preparation). Hence megakaryocyte progenitor dose may be a better predictor for platelet reconstitution.

Threshold Dose Based on $CD34^+$

Our HR: CD34 data are less extensive but 8x10^6 $CD34^+$ cell/kg BW appears to be the optimum threshold (data not shown). This threshold is similar to that proposed by Siena et al.[19] The minimum threshold is probably at 1.5-2x10^6 $CD34^+$ cells. The $CD34^+$ and CFU-GM thresholds for reconstitution suggested here are quite consistent with the generally accepted $CD34^+$: CFU-GM ratio of 4-8 : 1.

Subsets of $CD34^+$ cells that co-express lineage markers may predict the rate of reconstitution of their respective lineages although even fewer data are available. We have shown that the number of $CD34^+$ $CD33^+$ cells (a candidate STMRC) in mobilized peripheral blood autotransplants is much higher than that in bone marrow transplants while the number of $CD34^+$ $CD38^-$ cells (a candidate LTMRC) is at least comparable.[20] Table 1 shows the estimated numbers of subsets of $CD34^+$ infused in bone marrow transplants, G-CSF-mobilized peripheral blood and chemotherapy-mobilized peripheral blood in authors' institution.

Data on subsets of $CD34^+$ cells expressing platelet markers are not yet available, largely due to the methodological difficulty of differentiating false positive events due to platelets adherent to cells.

Table 1. A comparison of $CD34^+$ cells and their subsets in haemopoietic transplantation.

	$CD34^+$ Cells ($x10^6$/kg BW)	$CD34^+CD33^+$Cells ($x10^6$/kg BW)	CD34+CD38- Cells ($x10^6$/kg BW)
Bone Marrow Transplants (n=34)	0.97 ± 0.12	0.37	0.04
G-CSF mobilized peripheral blood (n=10)	6.3 ± 1.4	1.77	0.76
Chemotherapy mobilized peripheral blood (n=10)	3.62 ± 2.48	3.35	0.04

CONCLUDING REMARKS

Recognizing and defining progenitor threshold has major clinical relevance in performing safe transplants but are especially important when multiple high dose therapy and graft engineering are considered. Standardization of progenitor assay is crucial and improving the sophistication of ST- and LT-MRC measurements will enhance our assessment of graft adequacy.

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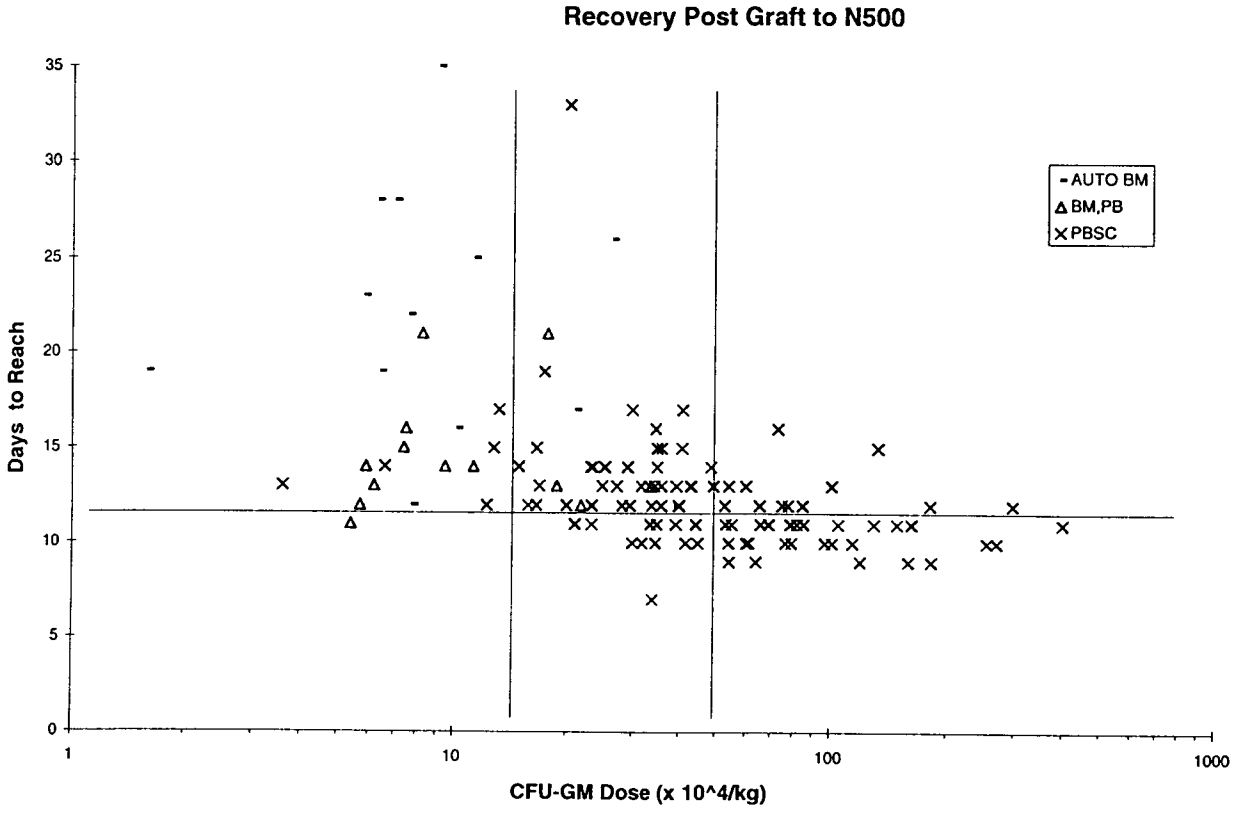

Figure 1: The relationship between CFU-GM dose and the rate of neutrophil reconstitution as measured by the number of days post transplant to reach $0.5x10^9$ neutrophils/L.

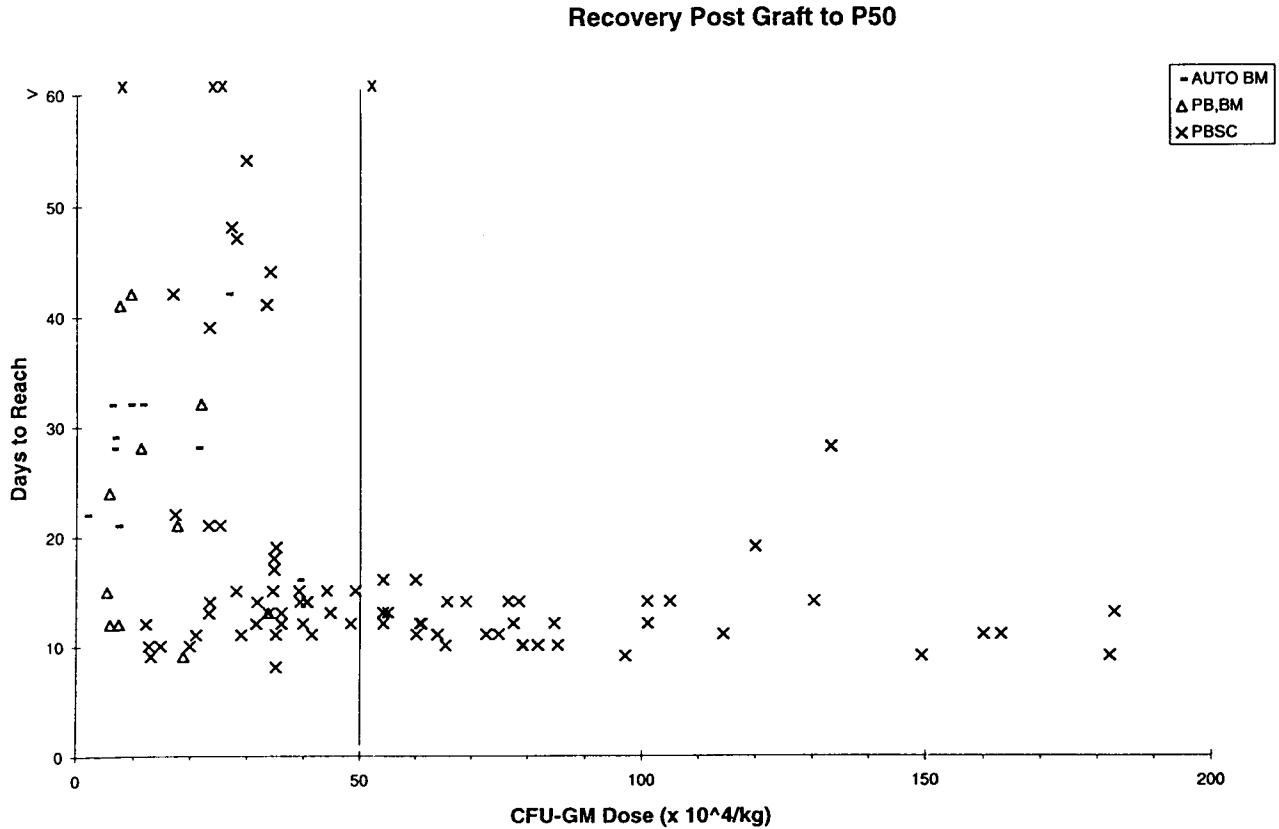

Figure 2: The relationship between CFU-GM dose and the rate of platelet reconstitution as measured by the number of days post transplant to reach $50x10^9$ platelets/L.

MOBILIZING ACTIVITY OF RECOMBINANT CYTOKINES AFTER HIGH-DOSE CYCLOPHOSPHAMIDE THERAPY IN CANCER PATIENTS

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INTRODUCTION

Hematopoietic progenitor cells harvested from peripheral blood are capable to restore the hematopoiesis irreversibly demaged after myeloablative cancer radiotherapy and/or chemotherapy.[1] The utilization of peripheral hematopoietic progenitor cells has revealed an effective means for the substantial amelioration of the therapeutic index of high-dose cancer therapy. This has offered clinicians the tool for addressing the issue of whether or not some malignancies may be curable with high doses of drugs and radiation.[2]

In our initial experience, hematopoietic progenitor cells were collected on large scale from peripheral blood after high-dose cyclophosphamide (HD-CTX);[3] in particular, blood leukocytes were harvested by leukapheresis during the period of rapid leukocyte recovery occurring after pancytopenia induced by HD-CTX, i.e., when hematopoietic progenitor cells circulate and peak in peripheral blood.[4] In more recent years, the administration of recombinant hematopoietic growth factors after HD-CTX resulted not only in shorter duration of iatrogenic neutropenia (with GM-CSF) [5,6] and trombocytopenia (with IL-3) [7], but also in larger expansion of the circulating progenitor cell pool, and thus in the possibility of harvesting larger quantities of circulating progenitor cells (CPCs). The extremely rapid reconstitution of all hematopoietic lineages achievable by autografting with mobilized CPCs, but not with the sole bone marrow or by utilizing any of the presently available growth factors, is likely to be due to the enormous number of relatively mature hematopoietic progenitors comprised among mobilized

CPCs that are capable of proliferating and differentiating in approximately one week to mature blood cells. Pilot studies have indicated that similar favorable results can be achieved by autografting with mobilized CPCs also without bone marrow.[8,9]

Clinical studies have indicated that hematopoietic growth factors, alone or in combination, are capable per se of expanding CPCs pool without preceding chemotherapy.[10,11] The combined use of chemotherapy and hematopoietic growth factors, however, accomplishes the dual aim of treating cancer and further expanding CPCs compartment: as a consequence, the yield of the collection is more predictable and an optimal number of circulating progenitors can be harvested through a limited number of leukaphereses.

The aim of this analysis was the comparison of the mobilizing activity of G-CSF, GM-CSF and IL-3 administered after HD-CTX chemotherapy in cancer patients.

PATIENTS AND METHODS

Patients treated with HD-CTX and cytokine were: stage II-IV diffuse large-cell non-Hodgkin's lymphoma patients (Working Formulation G and H), that were randomized to receive first-line treatment with high-dose sequential chemotherapy program [12], or women with resected breast cancer and >9 axillary lymph nodes involved, receiving high-dose a sequential chemotherapy program as adjuvant treatment.[13] Twenty-two patients received G-CSF, 27 received GM-CSF, and 22 patients received IL-3 after HD-CTX therapy. Fifteen patients did not receive hematopoietic growth factors after HD-CTX. Most patients receiving cytokine were chemotherapy-naive, except for 6, 5, and 1 patient in the G-CSF, GM-CSF, and IL-3 group, respectively. Eligible patients were required to be less than 56 (breast cancer) or 61 (lymphoma) years old, to have a Karnofsky performance status $\geq$70, as well as adequate cardiac, renal, hepatic and hematologic functions. All patients gave written informed consent to the treatment program. Cyclophosphamide was administered at the dose of 7 grams/m^2 in five divided 1-hour infusions every 3 hours over a 13-hour period.[6] For urothelial protection, all patients were given sodium-2-mercapto-ethan sulfonate intravenously at the dose of 1.5 g every 3 hours for 5 doses, followed by 1 g for 7 additional doses, starting from the end of the first cyclophosphamide infusion. Intravenous hydration (3 L/m^2/day), urine alkalinization, and

acetazolamide were administered over 48 hours starting 8 hours before the first cyclophosphamide dose.

Cytokines were administered according to doses and schedules specified below:

G-CSF: Four patients received G-CSF (Amgen Inc., Thousand Oaks, CA) at the dose of 30 μg/kg/day, twelve patients received 10 μg/kg/day, and six patients received 5 μg/kg/day for 14 days by continuous IV infusion, or until completion of the last leukapheresis procedure.

GM-CSF: Thirteen patients received glycosylated GM-CSF (Sandoz/Schering-Plough, Basel, CH, and Kenilworth, NJ), 5.5 μg/kg/day as protein in continuous intravenous (IV) infusion, starting 24 hours after the first dose of cyclophosphamide and continuing for 14 days, or until completion of the last leukapheresis procedure. Some of these patients were already described in a previous report.[3] Fourteen patients received non-glycosylated GM-CSF (Sandoz/Schering-Plough), 5.0 μg/kg/day in continuous IV infusion with the same schedule of administration.

IL-3: Non-glycosylated IL-3 was provided by Sandoz (Basel, CH) as a lyophilized powder, and administered in continuous IV infusion. Groups of three patients each received 1.0, 2.5, 5.0, and 10 μg IL-3 per kg body weight per day, starting 24 hours after the first cyclophosphamide dose and continuing for 14 consecutive days, or until leukocyte counts reached a level greater than 10,000/μL. After evaluation of hematologic effects in these 12 patients, 7 additional patients received 5 μg per kg per day, and 3 patients 10 μg per kg per day of IL-3, respectively.

CPCs were evaluated by both flow cytometry and colony-forming assay. Cells expressing the surface membrane CD34 and/or CD33 antigen(s) were identified by flow cytometry using either indirect CD34/direct CD33 immunofluorescence or dual-color direct immunofluorescence analysis.[4, 14] For colony-forming (CFU-GM) assay, a leukocyte-enriched fraction from whole blood or from leukapheresis cell suspension was cultured in triplicate in semisolid medium.[15]

RESULTS

Expansion of the circulating progenitor cell pool was a transient phenomenon occurring during the second and third week after HD-CTX. Since magnitude of the expansion did not significantly differ among various G-CSF and IL-3 dose levels, data for each cytokine were pooled and analyzed together. Table 1 shows the peak values of both CD34-

positive cells and CFU-GM in peripheral blood following either HD-CTX alone, or HD-CTX plus either G-CSF, GM-CSF, or IL-3.

Table 1. Median peak values of CPCs after HD-CTX cancer therapy, without or with cytokine.

	Peak of	Day after	
	CD34+/uL	CFU-GM/mL	HD-CTX
Steady-state	0	149	-
HD-CTX plus:			
no cytokine	68	3,620	18
G-CSF	539	35, 933	14
GM-CSF	376	13,335	14
IL-3	81	2,536	14

The administration of either G-CSF or GM-CSF after HD-CTX induced a dramatic increase of CPCs (from 4- to 10-fold)in comparison to HD-CTX alone. By contrast, after IL-3 infusion the peak of CPCs was superimposable to that of patients treated with HD-CTX alone. Addition of either cytokine to HD-CTX resulted in the anticipation of the day of peak from day 18 (HD-CTX alone) to day 14 (HD-CTX plus either G-CSF, GM-CSF, or IL-3).

DISCUSSION

One of the most promising aspects of the clinical application of cytokines in oncology is their ability to ameliorate and shorten chemotherapy-induced myelosuppression, thus allowing the safe administration of myelotoxic drugs at higher doses and/or after shorter intervals. A similarly important effect of hematopoietic growth factors is their ability to induce release from the bone marrow of large amounts of CPCs, particularly when cytokine infusion is preceded by administration of selected chemotherapeutic agent(s). HD-CTX exerts a potent anticancer effect on chemosensitive tumors such as lymphoma, breast cancer, and myeloma, while increasing circulation of CPCs at the time of recovery from chemotherapy-induced myelosuppression.[4, 6] Our data confirm that administration of GM-CSF and of G-CSF after HD-CTX is a potent tool to further expand and anticipate the circulation of hematopoietic progenitors. IL-3 given after HD-CTX did not cause expansion of CPCs, but anticipated their peak in comparison to historical controls given HD-CTX alone. While ineffective per se in expanding circulating progenitor cell

pool, IL-3 expands the compartment of bone marrow progenitor cells [16], and may thus favor optimal mobilization when given prior to cytokines with documented effect on circulating progenitor cell counts like GM-CSF [17, 18] or G-CSF. Our preliminary results indicate that administration of a short course of IL-3 followed by G-CSF or GM-CSF after HD-CTX further anticipates the peak and increases the number of CPCs, thus allowing collection of optimal quantities of CPCs though a single leukapheresis (AM Gianni et al, unpublished results).

In conclusion, the possibility of harvesting large amounts of committed as well as undifferentiated progenitors from peripheral blood though utilization of chemotherapy plus cytokine(s) renders application of myeloablative therapies an attractive and safe tool for treatment of chemosensitive tumors.

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COMPARISON OF CELL COLLECTIONS AND RATES OF POSTTRANSPLANT GRANULOCYTE RECOVERY WHEN G-CSF AND GM-CSF ARE USED AS MOBILIZERS OF PERIPHERAL BLOOD STEM CELLS FOR AUTOTRANSPLANTATION

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ABSTRACT

We have employed both granulocyte colony stimulating factor (G-CSF, filgrastim) and granulocyte-macrophage colony-stimulating factor (GM-CSF), sargramostim) for the expansion of blood-borne hematopoietic progenitors, i.e., peripheral blood stem cells (PBSC), in preparation for collection for autotransplant. Patients received either GM-CSF (10μg/kg) or G-CSF (16 μg/kg) for five days preceding collection of their circulating leukocytes. Leukocyte collection was carried out for four to six days, until a minimum of 5 x 10^7 CD34+ cells were obtained. Growth factor administration was continued through the penultimate day of collection. Collected cells were stored in liquid nitrogen pending autotransplant. Following collection, there was a slightly greater number of nucleated cells recovered from patients who had received G-CSF, although the number of CD34-positive cells collected was identical for both sets of patients. The number of colony forming unit-granulocyte-macrophage cells (CFU-GM) collected was remarkably greater from patients who had received G-CSF. Patients were conditioned for autotransplant with combination drug therapy comprising either ifosfamide, carboplatin, and etoposide (ICE) or mitoxantrone and thiotepa (MITT), following which their cells were thawed and returned. In the posttransplant course, patients who had had their PBSC collected with G-CSF experienced a significantly more rapid recovery of circulating neutrophils than did those who had their PBSC collected with GM-CSF.

INTRODUCTION

It has long been known that hematopoietic progenitors are present in circulating blood, albeit in lower numbers relative to the bone marrow in adult animals.[1-3] It has also been observed that the circulating stem cells are capable of reconstituting hematopoiesis in myeloablated individuals.[4] For peripheral blood hematopoietic progenitors to be a suitable source of stem cells for routine transplant, however, the efficiency of their recovery, and thus their numbers, must be increased. In fact, while there have been reports of the use of unmobilized PBSC for transplantation,[5] the majority of reported PBSC transplantations have used mobilized circulating progenitors.

Increase in circulating stem cells is associated with hematopoietic recovery following myelosuppressive chemotherapy[6-8] and with administration of hematopoietic growth factors.[9-11] Accordingly, the regimens used to mobilize PBSC fall into two basic categories: 1) the use of myelosuppressive chemotherapy, with PBSC collection during recovery from the resultant leukopenia. The mobilizing effect may be augmented by the administration of a growth factor following chemotherapy; 2) the administration of hematopoietic growth factors without preceding chemotherapy. While mobilization of circulating hematopoietic progenitors with chemotherapy may have some advantage of concomitant cytoreduction, it poses serious risks to a patient who might otherwise not require cytotoxic therapy. Moreover, scheduling of PBSC collections is difficult, as collection cannot commence until there are sufficient circulating leukocytes to support pheresis, and it cannot accurately be predicted how many days following chemotherapy that white blood cell (WBC) recovery will begin. Conversely, the use of defined growth factor regimens for PBSC mobilization is considerably safer and allows for precise scheduling of the collection process.

Peters et al[12] have reported comparative data on PBSC collected following mobilization with G-CSF and GM-CSF. However, in that study, the patients were reinfused with both conventionally collected autologous bone marrow and with the PBSC, resulting in rapid recoveries with both G-CSF and GM-CSF mobilized PBSC. We have a group of patients for whom bone marrow harvesting is not an option, due to fibrosis, hypocellularity or infiltrating disease. We have mobilized PBSC in these individuals with either G-CSF or GM-CSF and, following high-dose chemotherapy, have reinfused the cells collected. We have also collected PBSC under both of the aforementioned mobilization stimuli from patients

who had already undergone bone marrow harvesting, and have reinfused these cells alone (i.e., not with the bone marrow product). We have found substantial differences in the makeup of the collected cells and in the hematopoietic recovery which they generate.

MATERIALS AND METHODS

Patients

The individuals who were enrolled in this study were patients of the Bone Marrow Transplant program at the H. Lee Moffitt Cancer Center in Tampa, Florida. These studies were reviewed by the Scientific Review Committee of the Moffitt Cancer Center and the Institutional Review Board of the University of South Florida. Patients gave written informed consent to participate in these and other studies as part of their transplant course. All patients enrolled were adults, and their age distribution by decade is indicated in Table 1. The majority of the patients in these studies had breast cancer, although other solid tumor types were represented as well. These demographic data is provided in Table 1. It should be noted that by Chi-square analysis, there is no demonstrable difference between the two groups with respect to these parameters.

Table 1. Age Group and Disease of Patients Studied. Breakdown by Patients Whose PBSC Were Mobilized with GM-CSF and Those Whose PBSC Were Mobilized with G-CSF. For Both Age Groupings (by decade) and Diseases, Pearson's Chi-Square Statistic is Presented, Indicating no Statistical Difference Between Groups.

	Mobilization Regimen		
Age Grouping	G-CSF	GM-CSF	Total
At least 20, below 30	1	0	1
At least 30, below 40	2	8	10
At least 40, below 50	13	15	28
At least 50, below 60	5	8	13
At least 60, below 70	0	2	2
Column Totals	21	33	54

Pearson Chi-square = 5.016212 df=4 p=.28566

	Mobilization Regimen		
Disease	G-CSF	GM-CSF	Total
Breast cancer	19	29	48
Lymphoma	1	2	3
Adenocarcinoma	0	1	1
Non-Hodgkin's lymphoma	1	0	1
Hodgkin's disease	0	1	1
Totals	21	33	54

Pearson Chi-square = 2.892857 df=4 p=.57592

Mobilization regimens and PBSC harvesting. Patients whose PBSC were mobilized with GM-CSF were treated with 10 μg/kg/day of sargramostim beginning on the fifth day preceding PBSC collection. Sargramostim administration was maintained through the penultimate day of PBSC collections. Patients whose PBSC were mobilized with G-CSF received growth factor and had their PBSC collected on the same schedule, but they were treated with 16 μg/kg/day of filgrastim.

Leukaphereses were carried out using a Haemonetics V-50 (Haemonetics, Braintree, Massachusetts) fitted with a pediatric-bowl (125 mL). The standard lymphocyte apheresis protocol was used with sixteen draw and reinfuse cycles performed per procedure. Each pheresis harvest was reprocessed, to remove as many erythrocytes as possible, and sent to the laboratory for final processing and freezing, and for hematopoietic progenitor assessment.

Each day's leukapheresis product was post-processed over a Ficoll density gradient (1.077 gm/mL), resuspended in medium 199 supplemented with 10% autologous plasma and 10% DMSO, and frozen in a controlled-rate freezer. The cells were stored immersed in liquid nitrogen.

Collection was continued until at least 50 million total CD34+ cells were collected. In cases where this was not achieved by the end of six collections, the affected patient was deferred from transplant with growth-factor-only mobilized PBSC.

Evaluation of PBSC collections. Total nucleated cells, CD34+ cells and CFU-GM cells were enumerated in each collection. The total for each class of cell was computed for all collections.

CD34+ and CFU-GM were measured as reported previously.[13] Briefly, CD34+ cells were enumerated by incubating Ficoll-light density cells with fluorochrome labelled CD34 specific antibody (HPCA-1 or HPCA-2, Becton-Dickinson, San Jose, CA). Excess antibody was washed

out and the cells were dropped onto a microscope slide, covered with a coverslip, and examined with an ultra violet. illuminated mciroscope. The average total number of cells per field of view was computed, then at least 30 fields were examined for the presence of fluorochrome labelled cells. The total number of labelled cells observed was divided by the approximate total number of cells observed (average cells per field x fields examined) to yield the fraction of CD34+ cells. CFU-GM were evaluated by plating 2 x 10^5 Ficoll-light density cells in Iscove's medium supplemented with 1.2% methylcellulose, 1.5 mg/mL GM-CSF, and 20% fetal bovine serum. The plated cells were incubated for 14 days, and the resultant colonies were enumerated.

High-dose chemotherapy, stem cell transplant, and hematologic recovery. Patients were prepared for stem cell transplant with one of two regimens. ICE is comprised of ifosfamide, carboplatin and etoposide, while MITT is comprised of mitoxantrone and thiotepa. The scheduling and dosing of each regimen has been described previously.[14]

Stem cells were reinfused after thawing in a 37°C water bath. Patients' daily WBC and absolute neutrophil count (ANC) were monitored, and the days to reach an ANC of greater than 500 /μL for two or more consecutive days was recorded.

Statistical analysis. Data was maintained in MEDLOG (Information Analysis Corporation, Lake Tahoe, CA) and dBase (Borland, Scotts Valley, CA) files. Analysis was performed using the CSS Statistica programs (StatSoft, Tulsa, OK). Because of the apparent lack of normal distribution in the data, cell collections were analyzed using two non-parametric tests. In the Kruskal-Wallis ANOVA test, data are ranked and the distribution of the rankings is analyzed. In the median test, distribution of data points above and below the combined median value is tested using a Chi-square statistic. In all cases, both tests were confirming in their outcome. Analysis of the periods to reach an ANC of greater than 500 was carried out by Mantel's generalized Mantel-Haenszel method.[15] For analysis of nominal and ordinal data, Pearson's Chi-square test was employed.

RESULTS

Nucleated cells, CD34+ cells, and CFU-GM collected. PBSC were collected from 33 patients who were treated with GM-CSF for mobilization, and from 19 who were treated with G-CSF for mobilization (45 of these patients subsequently went on to transplant). Four to six

leukaphereses were performed on each of the patients. The distribution of number of phereses by mobilization regimen employed is presented in Table 2.

Table 2. Numbers of Apheresis Collections Performed as a Function of the Mobilization Regimen Employed.

	Mobilization Regimen		
Number of Collections	G-CSF	GM-CSF	Row-Total
4	20	14	34
5	0	2	2
6	1	17	18
Totals	21	33	54

Pearson Chi-square = 15.37357 df=2 p=.00046

The number of pheresis procedures employed is different, by Chi-square analysis, due to a greater number of phereses procedures required to accumulate the minimum CD34+ cell number with GM-CSF mobilization. Cumulative numbers of total nucleated cells, CD34+ cells, and CFU-GM colony forming cells were computed by summing the recoveries from all collections (Figure 1). The total number of CD34+ cells recovered (and thus the number returned to each patient) may be seen to be statistically identical (Kruskal-Wallis p=.4879, Median test p=.7734). Conversely, the total number of CFU-GM colony-forming cells recovered from each patient was much greater if G-CSF was used as the mobilizing agent (Kruskal-Wallis p=.0001, Median test p=.0021). This was also true of the total number of nucleated cells recovered and subsequently returned (Kruskal-Wallis p <.0001, Median test p <.0001).

Hematopoietic recovery following transplant. Recovery of circulating neutrophils (to ANC >500) following infusion with PBSC mobilized with G-CSF was much more rapid than when PBSC mobilized with GM-CSF was infused (Figure 2, p=0.00041). Because of data which indicate that patients who are prepared for transplant with our ICE regimen recover more quickly than those prepared with our MITT regimen,[16] we grouped our patients by preparative regimen, and reexamined the rates of hematopoietic recovery. Within each group, the results remained the same (Figure 3), that is, patients who received G-CSF mobilized cells consistently engrafted more rapidly than did those receiving GM-CSF mobilized cells (ICE patients, p=0.00222, MITT patients, p=0.00353).

DISCUSSION

It is clear from the data we have presented that the mobilization regimen employed in the harvesting of hematopoietic progenitors from circulating blood can have a significant impact not only on the number of hematopoietic progenitor cells recovered, but on the types of cells recovered and on the ultimate rate of hematopoietic recovery.

Specifically, we have employed two different hematopoietic growth factors, GM-CSF and G-CSF, for the mobilization of hematopoietic stem cells. Both of these proteins have been associated with increased numbers of circulating hematopoietic cells,[17-23] albeit when used following chemotherapy. In our application, as in that of Peters et al,[12] the growth factors have been administered to individuals who were not recovering from chemotherapy. With both factors, circulating leukocyte counts were observed to increase, and the fraction of circulating cells which was CD34 antigen positive was similarly increased. Conversely, the number of circulating CFU-GM was only increased when G-CSF was administered, and not when GM-CSF was used.

It might be speculated that GM-CSF drives all of the CFU-GM to terminally differentiate, thus depleting that pool within the hematopoietic progenitor population. If this were the case, then initiating cell collection earlier in the course of growth factor administration may result in greater CFU-GM yields. Peters et al[12] report on cell collection following six days of GM-CSF and following twelve days of GM-CSF and, in fact, they do observe reduced CFU-GM collection after twelve days of growth factor administration. Bishop et al[24] report cell collection within 24 hours of initiating GM-CSF administration. In their hands, the cells collected in the manner produce accelerated engraftment following autotransplant, relative to PBSC collected without mobilization.

Of particular note, is the fact that the number of CD34 positive cells infused into each patient was essentially the same. CD34 cell numbers have been proposed as a means of ensuring the engraftment potential of a stem cell product.[25-27] From our data, it would appear that the total number of nucleated cells or the number of CFU-GM colony forming cells would be a better indicator.

The use of hematopoietic progenitors derived from circulating blood for stem cell rescue following myeloablative dose chemotherapy has proven to be an effective therapeutic technique. The efficiency with which these cells may be collected is dependent on some form of "mobilization." The data presented here demonstrate that the mobilization regimen

employed can profoundly affect the distribution of progenitor cell types recovered and, in turn, the pace of hematopoietic reconstitution following return of those cells.

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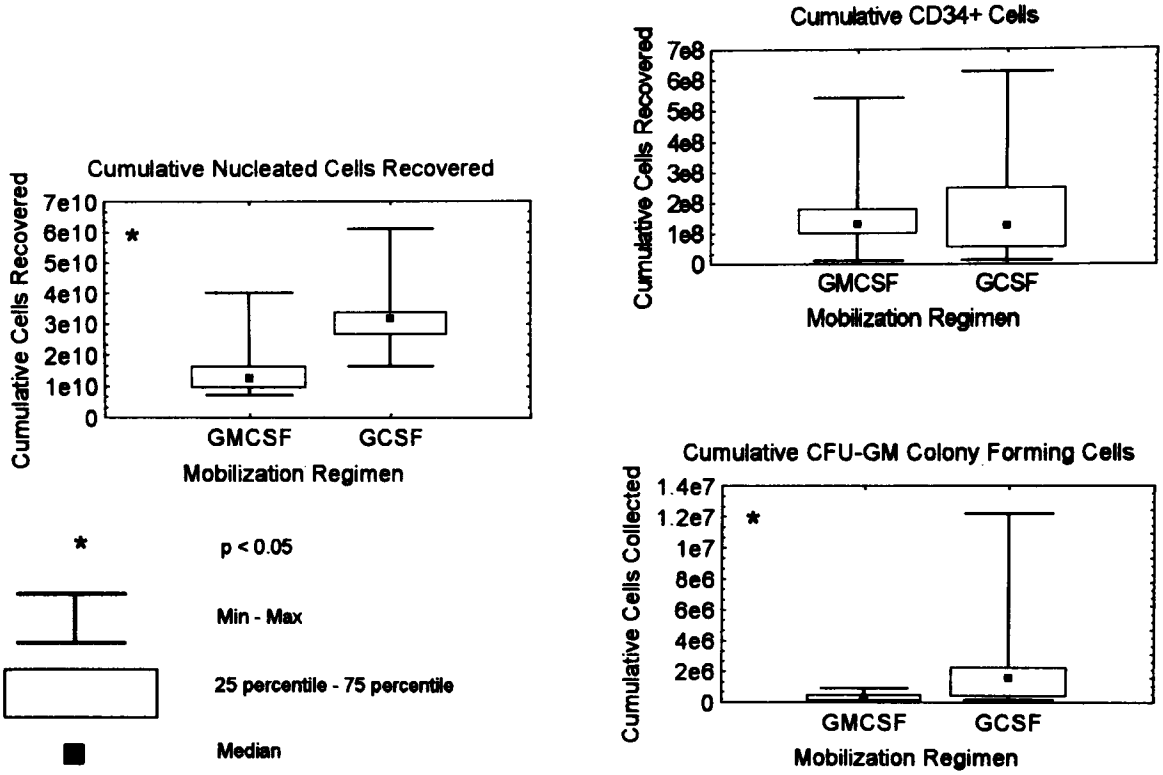

Figure 1. *Numbers of nucleated cells, CD34+ and CFU-GM colony forming cells recovered as a function of mobilization regimen: Cumulative numbers over all collections of each identified type of cell were recorded for each patient. Numbers were evaluated by both Kruskal-Wallis ANOVA ranks test and median test. Asterisk denoted $p<0.05$ with both tests (actual p values are identified in the text).*

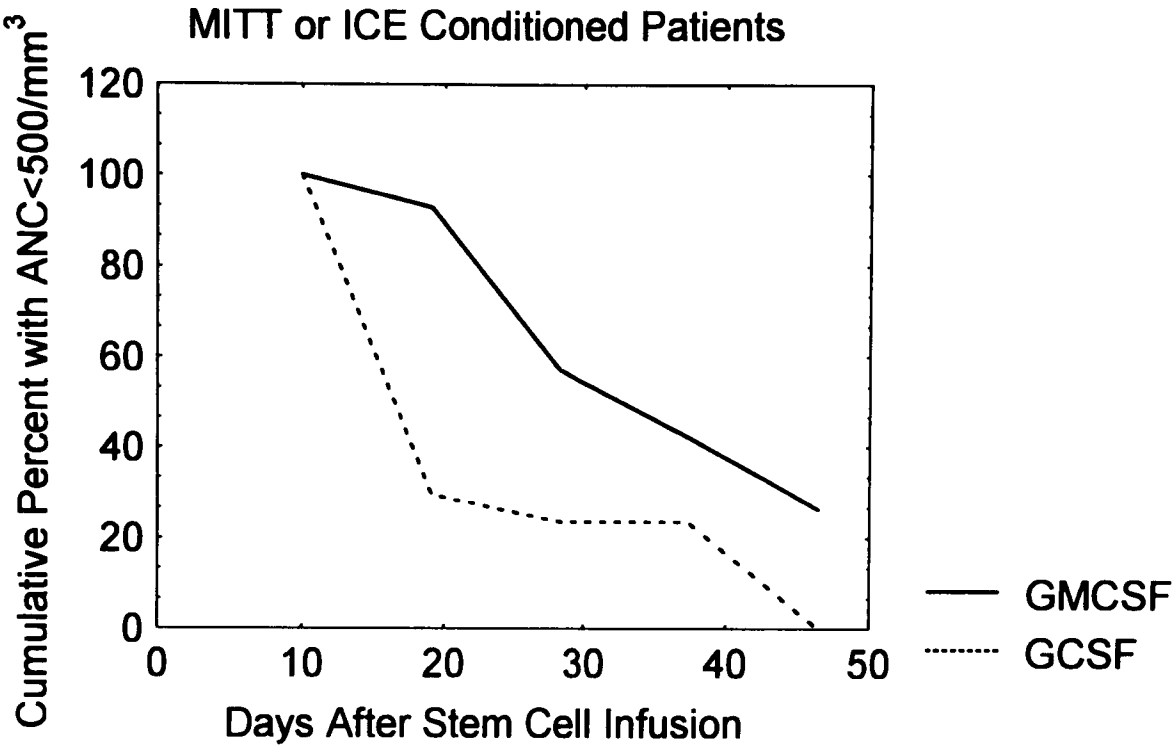

Figure 2. *Days to achieve ANC >500/mm3 in all patients studied: The number of days post stem cell infusion to achieve an absolute neutrophil count of >500 for three consecutive days was recorded for all patients. Patients were grouped according to PBSC mobilization regimen but without consideration of any other parameters, and comparative analysis was done using Mantel's generalized Mantel-Haenszel technique (p=0.00041).*

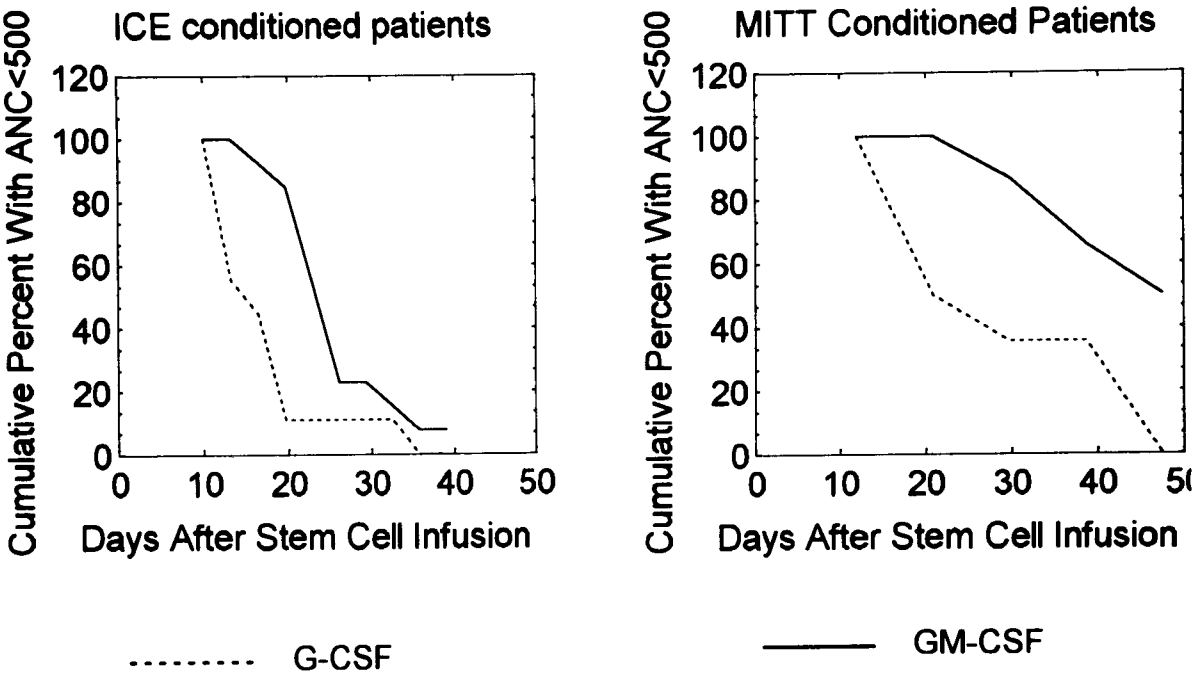

Figure 3. *Days to achieve ANC >500/mm3 broken down by ICE or MITT conditioning regimens: Enumeration and analysis were done as in Figure 2, except that patients were first grouped according to whether their transplant conditioning was with the ICE or MITT chemotherapy regimen. For patients who were conditioned with ICE (n=22), p=0.00222. For patients conditioned with MITT (n=23), p=0.00353.*

PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) COLLECTED AFTER CHEMOTHERAPY PLUS RECOMBINANT HUMAN GRANULOCYTE (rhG-CSF) AND GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR (rhGM-CSF): AN ANALYSIS OF FACTORS CORRELATING WITH MOBILIZATION AND ENGRAFTMENT

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INTRODUCTION

Today, peripheral blood hematopoietic progenitors are often collected after mobilization by chemotherapy plus growth factors. The standard growth factors are granulocyte and granulocyte macrophage colony stimulating factors. The aim of this study was to identify after priming by chemotherapy plus G-CSF or GM-CSF, parameters predictive of collection efficiency and recovery posttransplantation.

PATIENTS AND METHODS

We studied ninety-one patients, including forty-eight males, transplanted between November, 1992 and January, 1994. The mean age was thirty-five years. Thirty-six patients had non-Hodgkin's lymphoma, nineteen had breast carcinoma, sixteen had Ewing's sarcoma and twenty had other diseases. The 91 patients underwent 251 collections performed on Spectra (Cobe), CS 3000 (Fenwal) and AS104 (Fresenius) cell separators. Various mobilization protocols were used. Mobilizing chemotherapy was based on cyclophosphamide alone in 22 cases, and combined with anthracycline in 23 cases, antracycline-vindesine in 28 cases and other drugs in 26 cases. G-CSF was used, in association with chemotherapy, in 62 patients and GM-CSF in 29 patients. Patients were grafted after conditioning regimens, including TBI in 49 cases and high-dose chemotherapy in 42. Forty-five patients received G-CSF

posttransplantation, 29 received GM-CSF and 19 patients did not receive growth factors.

The following parameters likely to influence collection efficiency and engraftment were analyzed:

Patient characteristics (age, sex and diagnosis), the patient's past history, including time from diagnosis to collection, the number of prior chemotherapy courses, the number of chemotherapy courses less than 6 months before PBSC harvest, the type of drugs received in the past (Nitrosourea, melphalan, Busulfan, Cyclophosphamide and Anthracycline) and prior extensive irradiation.

We also analyzed the **influence of the mobilization regimen**, i.e. of the type of chemotherapy and growth factors, together with the influence of *white blood cell and platelet counts in peripheral blood* on the day of collection, and whether the *disease was sensitive or progressive* at the time of collection.

Finally, we analyzed other parameters potentially influencing recovery posttransplantation: *tumor status at transplantation, component characteristics* (number of white blood cells, mononuclear cells and hematopoietic progenitors infused), *conditioning* with or without TBI, and administration of growth factors posttransplantation.

We studied the influence of previously described parameters on collection and engraftment; collection efficiency was estimated by the number of hematopoietic progenitors harvested, counted using a CFU-GM assay. CD34+ cells were evaluated in 42 cases; engraftment was estimated by the median time required to recover a granulocyte count over 10^9/liter, a platelet count over 50 x 10^9/l and platelet independence of transfusion support. Data analyses included univariate analysis, correlation tests and linear regression analysis with stepwise inclusion of variables; if necessary, numeric parameters were log transformed to obtain a normal distribution of variables.

RESULTS

Parameters Influencing Collection

The total number of CFU-GM infused was $11.5 \pm 10.4 \times 10^5$/kg and the total number of CD34+ cells was 8.7×10^6/kg. In our laboratories the minimum target for grafting is 2×10^5 CFU-GM/kg and 2×10^6 CD34+ cells/kg.

The main parameters influencing progenitor collection were the patient's past history, tumor status at collection and peripheral blood counts at the time of collection. In patients who had previously received Busulfan and irradiation, the number of progenitors collected was 3 times lower than in other patients respectively 4.2 versus 12.2 x 10^5 CFU-GM/kg for Busulfan ($p=0.03$) and 4.8 versus 12.7 x 10^5 CFU-GM/kg for irradiation ($p=0.008$). There was a strong correlation between the number of prior courses of chemotherapy and the number of progenitors collected with a significant difference maximal over 5 courses ($p<0.001$). Progressive disease had a negative influence on collection, the number of progenitors collected being 5 times lower than in patients who were in remission 3.1 and 12.9 CFU-GM/kg, respectively ($p<0.001$). The peripheral WBC count was highly predictive of collection efficiency. When patients were mobilized with G-CSF, a significantly higher number of progenitors were collected if the harvest was performed when the WBC was over 20 x 10^9/l, in comparison with 10 to 20 x 10^9/l and less than 10x10^9/l. After mobilization with GM-CSF, the differences were significant when the peripheral WBC count was over 6 x 10^9/l. In the same way, when patients recovered platelet counts over 100 x 10^9/l after mobilizing chemotherapy, the number of progenitors collected was significantly increased.

No influence of the mobilizing regimen was detected; the yield was similar whatever the type of chemotherapy used for mobilization; in the same way, G-CSF and GM-CSF led to similar yields, 12.2 and 11.2 x 10^5 CFU-GM/kg, respectively. This was the case in the entire population and also in the very uniform subgroup with breast cancer.

Parameters Influencing Engraftment

Granulocyte recovery occurred at a mean of 11.7 days, with a standard deviation of 2.5 days (median 10 days). By multivariate analysis, the main factor predictive of granulocyte recovery, with a p-value less than 0.001, was the number of CFU-GM infused ($r=0.42$). The correlation was optimal at 5 x 10^5 CFU-GM/kg. The number of CD34+ cells infused also correlated strongly with granulocyte recovery. The number of WBC or MNC infused had no influence.

Clinical status was highly predictive as granulocyte recovery in patients with progressive disease occurred 2 days later than in patients who were in remission, 11.5 and 13.6 days with a p-value of 0.03. Patients mobilized with G-CSF showed significantly earlier granulocyte recovery

than those mobilized with GM-CSF, 11.4 and 12.4 days, respectively. In the same way, in patients who received G-CSF posttransplantation, the duration of neutropenia was reduced by 1 day in comparison with patients who received GM-CSF, and 2 days when no growth factors were administered, 11.2, 11.2 and 12.6 days, respectively. The last factor which emerged from the multivariate analysis of granulocyte recovery was the number of chemotherapy courses prior to collection.

A platelet count of more than $50x10^9/l$ was obtained after a mean of 23 days, with a large standard deviation of 22 days (median 13 days). Six patients did not show platelet recovery. Independence of transfusion support was achieved after a mean of 16 ± 17 days (median 10 days).

Certain parameters which influence granulocyte recovery were also predictive of platelet recovery: this was the case of the number of CFU-GM infused and status at transplantation. The number of CFU-GM infused correlated strongly with the duration of thrombocytopenia ($n=0.49$, $p<0.001$) with an optimum number of CFU-GM evaluated between 5 and 9 x 10^5/kg. The number of CD34+ cells infused also correlated strongly with platelet recovery. The number of WBC and MNC infused had no influence. In patients with progressive disease, platelet recovery occurred at a mean of 10 days later than in patients who were in remission, 32.1 and 21.7 days, respectively.

The patient's past history had a significant influence on duration of thrombocytopenia; patients who had been extensively irradiated recovered from thrombocytopenia on day 45 versus day 19 in nonirradiated patients. In the same way, thrombocytopenia lasted 52 days in patients who received Busulfan, versus 20 days in the others.

Other predictive parameters included the diagnosis (patients with breast cancer had short thrombocytopenia) and the number of chemotherapy courses prior to collection. We found no influence of the type of chemotherapy or growth factors used for mobilization; patients mobilized with G-CSF showed platelet recovery after 25 days compared to 20 days with GM-CSF. In the same way, the duration of thrombocytopenia was independent of growth factor administration posttransplantation.

In conclusion, these simple parameters should help in predicting both the feasibility of peripheral progenitor harvest and the approximate recovery time after transplantation.

Table 1. Description of the Sample

Continuous Items	median	range	(n)	Qualitative Items	n	%
Age at PBSC collection	38	2-63	(91)	Sex (female)	43	47
Past chemotherapies				Past irradiation		
total number	5	1-23	(89)	any	14	15
number in the last 6 mos.	3	1-10	(89)	pelvis	8	
total dosage (g/m^2)						
nitrosurea	--	300-400	(2)	Mobilization regimen		
melphalan	32	12-36	(3)	chemotherapy		
busulfan	3.85	1.2-8.4	(8)	ACVBP	28	31
cyclophosphamide	3.34	0.75-11.55	(68)	cyclo alone	22	24
anthracycline	0.126	0.024-0.825	(87)	cyclo + anthracycline	23	25
PBSC collection						
Time to diagnosis (weeks)	6	1-68	(91)	with G-CSF	62	68
Speed of recovery (%)	9.1	0.2-43.2	(91)	with GM-CSF	29	32
1st day blood collection						
WBC (10^9/l)	12.5	1.2-50	(91)	Disease status at collection		
MNC (10^9/l)	1.6	0.3-4.6	(91)	sensitive	78	86
Plts (10^9/l)	126	31-392	(91)	progressive	13	14
Volume (l)	19	5-33	(90)			
Total blood collection				Conditioning regimen		
WBC (10^6/kg)	6.6	0.9-53.0	(90)	TBI + chemo	48	53
MNC (10^8/kg)	3.4	0.5-12.5	(90)	high dose chemo	43	47
CFU-GM (10^5/kg)	7.3	1.3-46.8	(91)			
				Disease status at BMT		
No. RBC trans. post BMT	2	0-16	(91)	sensitive	79	87
Last plt trans. post-BMT	10	4-100	(91)*	progressive	12	13
				PostBMT growth factors		
Time to engraftment (days)				G-CSF	45	49
granulocytes >0.5x10^9/l	12	5-20	(91)	GM-CSF	29	32
plts >50x10^9/l	13	5-100	(91)	none	17	19

*4 patients had still not recovered 50x10^9 plts at day 100.

Table 2. Parameters affecting the time to granulocyte recovery (>10^9/l)

Parameters	Category	Time to recovery (days) mean ± SD	median	(n)	P*
Diagnosis	Ewing's	12.9±1.9	13	(16)	.009
	Other	11.5±2.6	11	(75)	
Prior therapies					
Total no. of cycles	<5	10.5±1.8	11	(43)	.001
	≥5	12.9±2.6	13	(46)	
No. of cycles in the last 6 months	<3	11.1±1.6	11	(41)	.015
	≥3	12.3±3.1	12	(48)	
Cyclophosphamide dosage (g/m^2)	<1.3	10.8±1.3	11	(23)	.021
	≥1.3	12.2±2.6	12	(45)	
irradiation	pelvis	15.0±3.7	14.5	(8)	.007
	no or other	11.4±2.2	12	(83)	
PBSC collection					
time to diagnosis (weeks)	<10	11.3±2.2	11	(64)	.020
	≥10	12.9±2.9	12	(27)	
	<6	10.5±1.8	10	(44)	.001
	≥6	12.9±2.6	12	(47)	
Disease status	sensitive	11.5±2.3	11	(78)	.020
	progressive	13.1±3.3	13	(13)	
Mobilization chemo	ACVBP	10.8±2.4	11	(28)	.019
	others	11.8±2.1	11	(41)	
	cyclo alone	12.9±3.1	13	(22)	
Growth factor	G	11.4±2.8	11	(62)	.010
	GM	12.4±1.6	12	(29)	

BMT					
Disease status	sensitive	11.5±2.4	11	(79)	.003
	progressive	13.6±2.6	13	(12)	
CFU-GM infused (10^5/kg)	<700	12.5±2.8	12	(43)	.011
	≥700	11.1±2.1	11	(48)	
Growth factor	yes	11.5±2.7	11	(74)	.028
	no	12.6±1.5	12	(17)	
	G	11.2±3.1	11	(45)	.047
	GM	12.0±1.8	12	(29)	

*Mann Whitney test

Table 3. Parameters affecting the time to platelet recovery (>50x10^9/l)

		Time to recovery (days)			
Parameters	Category	mean ± SD	median	(n)	P*
Diagnosis	Breast	14.4±7.5	12	(19)	.018
	Ewing's NHL	22.2±21.3	13	(52)	
	Other	33.6±28.8	21	(20)	
Prior therapies					
Total number of cycles	<5	15.0±8.8	12	(43)	.001
	≥5	31.2±27.6	18	(46)	
No. of cycles in the last 6 mos.	<4	18.6±17.0	12	(57)	.004
	≥4	31.7±27.6	20.5	(32)	
Busulfan	no	20.3±18.1	13	(83)	.002
	yes	52.5±36.3	46	(8)	
Cyclophosphamide dosage (g/m^2)	<1.3	12.9±5.1	12	(23)	.007
	≥1.3	24.6±22.7	14	(45)	
Anthracycline dosage (g/m^2)	<0.3	21.3±19.7	13	(65)	.040
	≥0.3	30.6±28.1	18	(22)	
Irradiation	no	19.1±15.9	12	(77)	.001
	yes	45.2±35.3	30.5	(14)	
	pelvis	58.3±40.2	13	(8)	.005
	no or other	19.7±16.2	61.5	(83)	
PBSC collection					
Time to diagnosis (weeks)	<10	17.6±15.1	12	(64)	.001

	≥10	36.1±29.5	23	(27)	
Disease status	sensitive	19.0±17.8	12	(78)	.001
	progressive	47.5±29.0	50	(13)	
Mobilization chemo	ACVBP or Cyclophosphamide + anthracycline	17.3±13.1	12	(51)	.023
	Others	30.5±28.8	16.5	(40)	
Speed of recovery (%)	<10	27.1±25.7	14	(49)	.052
	≥10	18.1±15.5	12	(42)	
1st day blood collection					
WBC (10^9/l)	<10	30.5±28.6	14.5	(34)	.055
	≥10	18.6±15.6	12	(57)	
Plts (10^9/l)	≤100	34.8±30.2	18	(27)	.014
	>100	18.1±15.2	12.5	(64)	
BMT					
Disease status	sensitive	21.7±21.6	12	(79)	.007
	progressive	32.1±23.6	30	(12)	
No. of CFU-GM	<700	34.8±27.2	25	(43)	.001
infused (10^5/kg)	≥700	12.6±5.2	12	(48)	

*Mann Whitney test

KIT POSITIVE PERIPHERAL BLOOD CELLS COLLECTED AFTER CHEMOTHERAPY AND G-CSF PRIMING

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ABSTRACT

In a preliminary study we have measured the numbers of cells expressing KIT, the receptor for stem cell factor, on the mononuclear cells of the leukapheresis product obtained after chemotherapy and G-CSF priming. Forty-two PBSC collections from 12 patients were evaluated and KIT+ cell numbers correlated with CD34+ cell numbers, CFU-GM and total mononuclear cells. Significant correlations were found between KIT+ cells and CD34+ cells (r=0.94), KIT+ cells and CFU-GM (r=0.76) and CD34+ cells and CFU-GM (r=0.69). These preliminary observations suggest that enumeration of KIT+ cells may be of use in the evaluation of the quality and quantity of PBSC collections. Further studies are however required.

INTRODUCTION

Despite early concerns over the reliability of long-term engraftment, it is now well established that peripheral blood haemopoietic stem or progenitor cells (PBSC) are capable of long-term haemopoietic reconstitution following high-dose chemotherapy. PBSC collected after priming chemotherapy and cytokine treatment are generally thought to provide the most enriched source of stem cells. The optimum timing of PBSC collection and the best method of assessment of the quantity and quality of the harvested progenitors have however not definitively been determined. Commonly used techniques include nucleated cell counts, *in vitro* haemopoietic colony growth, usually CFU-GM, and CD34+ cell numbers.

The CD34 surface molecule is expressed on a minor subset of normal marrow cells that has been shown to contain virtually all *in vitro* colony forming progenitor cells as well as their precursors.[1,2] This subset also probably contains those cells that can establish haematopoiesis following myeloablative chemotherapy. Assessment of progenitor cell

numbers of flow cytometric enumeration of CD34+ cells is widely applied because of assay speed and the established correlation between total CD34+ cells infused and subsequent engraftment. However, other methods of stem cell assessment may have advantages such as better selection of the timing of the PBSC collection and assessment of the quality of the stem cells in those collections with low total cell numbers and, in particular, low CD34 numbers.

KIT is a transmembrane tyrosine kinase receptor encoded by the proto-oncogene *c-kit*. The ligand for KIT is the multipotential haemopoietic growth factor, stem cell factor (SCF). Acting in synergy with a variety of other cytokines, SCF significantly promotes the growth and proliferation of several classes of haematopoietic progenitor cells including, the high-proliferative-potential-colony forming cells (HPP-CFC) and the long-term bone marrow culture-initiating cells (LTC-IC).[3,4] However, little is known about the numbers of KIT+ cells in PBSC collections and the relationship, if any, between KIT+ cell numbers and the established methods of assessing the quantity and quality of haemopoietic progenitors in PBSC collections.

The aims of this study were to investigate the expression of KIT on the mononuclear cells in peripheral blood stem cell harvests, obtained by leukopheresis following chemotherapy and G-CSF. We also correlated the number of KIT+ cells with other methods of assessing PBSC collections such as the nucleated cell number, CD34+ cell numbers and CFU-GM.

METHODS

Forty-two PBSC collections from 12 patients were evaluated. This group consisted of 7 patients with non-Hodgkin's lymphoma who received priming chemotherapy with high-dose cytosine arabinoside and etoposide, 3 patients with myeloma primed with high-dose cyclophosphamide, one patient with Burkitt's lymphoma primed with high-dose cyclophosphamide, and one patient with bi-phenotypic acute leukaemia collected following chemotherapy with cyclophosphamide, vincristine, cytosine arabinoside, prednisone and idarubicin. After chemotherapy, all patients received rhG-CSF (Filgastrim 5 mg/kg per day) until stem cell collection was completed. Apheresis procedures were commenced when the white cell count was $>1.0 \times 10^9$/L using a Baxter CS3000 plus with a small volume collection chamber. Seven to 10 liters of blood were processed per collection with an average of 3.5 collections

per patient. Five patients have subsequently been transplanted and all have engrafted with a mean of 9 days to an absolute neutrophil count of 0.5 x 10^9/L and 11 days to a platelet count of 20 x 10^9/L.

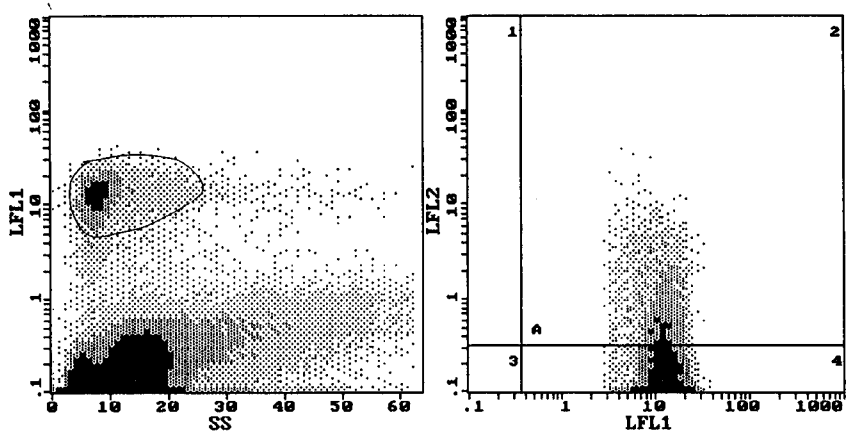

Figure 1. *CD34FITC (LFL1) and side scatter (left) is used to gate cells and then (right) CD34FITC (LFL1) and KIT+ expression (LFL2) is analyzed.*

An Epics Profile II flow cytometer was used for the CD34 and KIT analysis. Cells were labeled with anti-CD34 FITC (HPCA-2) and anti-KIT PE (clone 95C3). CD34 cells were gated from the FITC versus side scatter histogram and then analyzed for KIT expression using Elite Software (Figure 1). The total cell population was also analyzed for KIT expression. The CFU-GM were assayed in a semisolid agar culture system with colonies scored on day 14.

RESULTS

Table 1 shows the levels (/kg of body weight) of KIT+ cells, CD34+ cells and cells co-expressing both KIT and CD34, per collection and per patient. Also shown are the CFU-GM and the total nucleated cells collected and the peripheral white cell count at the time of collection.

Table 2 shows a correlation matrix between the five variables. The significant correlations were between the KIT+ cells and the CD34+ cells (r=0.94), KIT+ cells and CFU-GM (r=0.76), KIT+CD34+ cells and CFU-GM (r=0.74) and CD34+ cells and CFU-GM (r=0.69). All these correlations were statistically significant with a P value <0.001.

Table 1. Details of Patient's PBSC Collections

Parameter	Units/kg*	n	Per Collection	Per Patient
KIT+	$x10^6$	28	0.00 - 7.0	0.7 - 9.2
CD34+	$x10^6$	42	0.1 - 24.4	0.9 - 28.2
KIT+ CD34+	$x10^6$	42	0.01 - 5.9	0.2 - 8.2
CFU-GM	$x10^4$	27	0.1 - 22.9	1.5 - 41.1
Total WCC	$x10^8$	42	0.2 - 2.6	2.2 - 7.7
Peripheral WCC	$x10^9$	42	1.0 - 39.5	-

*patient body weight

Table 2. Correlations Between the Parameters Evaluated in this Study

	CD34+/kg	CFU-GM/kg	KIT+/CD34+ /kg	KIT+/kg	WCC
CD34+/kg	1	0.69 (n=27) P<.001	0.93 (n=42) P<.001	0.94 (n=28) P<.001	-0.17 (n=41) P=NS
CFU-GM/kg	0.69 (n=27) P<.001	1	0.74 (n=27) P<.001	0.76 (n=20) P<.001	0.06 (n=26) P=NS
KIT+CD34+/kg	0.93 (n=42) P<.001	0.74 (n=27) P<.001	1	0.98 (n=28) P<.001	-0.16 (n=41) P=NS
KIT+/kg	0.94 (n=28) P<.001	0.76 (n=20) P<.001	0.98 (n=28) P<.001	1	-0.16 (n=27) P=NS
WCC	-0.17 (n=41) P=NS	0.06 (n=26) P=NS	-0.16 (n=41) P=NS	-0.16 (n=27) P=NS	1

DISCUSSION

The results of these preliminary experiments demonstrate that the number of KIT+ mononuclear cells collected after chemotherapy and G-CSF priming correlate strongly with the traditional measures of the adequacy of stem cell collection namely CD34+ cells and the number of CFU-GM. It is important to note that in these experiments neither CD34+ cell numbers, CFU-GM nor KIT+ cell numbers correlated with total mononuclear cells collected. The previously reported correlation between CD34+ cells and CFU-GM has been confirmed. Although further work in this area is needed, the expression of KIT on the mononuclear cells of the leukopheresis product may potentially be another factor of value when

assessing the quality and quantity of haemopoietic precursors in PBSC obtained following chemotherapy and G-CSF.

ACKNOWLEDGEMENTS

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RANDOMIZED IN VIVO STUDY OF G-CSF (PRIMING) VS (NO PRIMING) PRIOR TO HIGH DOSE THERAPY (HDT) WITH CYCLOPHOSPHAMIDE-ETOPOSIDE-CISPLATIN (CVP)

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INTRODUCTION

The importance of dose intensification of chemotherapeutic agents in the treatment of solid tumors has been demonstrated in multiple studies over the last decade.[1-3] Hematopoietic recovery post high-dose therapy has been affected by reversible but significant absolute neutropenia and thrombocytopenia responsible for the observed morbidity and mortality of those regimens.[1-3]

Subgroups of patients had been reported in which high doses of CVP has demonstrated efficacy in prolonging survival of patients with metastatic breast cancer.[4] We have shown that high-dose CVP is highly myelotoxic but not myeloablative.[5,6] Growth factors have been extensively studied and used to reduce but not eliminate chemotherapy induced neutropenia.[7]

We studied the potential benefit of expanding the bone marrow and peripheral blood of progenitor cells with the hematopoietic growth factor, granulocyte colony stimulating factor, given prior to the administration of cytotoxic agents. The purpose of this approach is to expand hematopoietic progenitors *in vivo*, in a natural environment, in an attempt to shorten the duration of neutropenia.

PATIENTS AND METHODS

Seventeen patients with histologically confirmed solid tumors with low curability rates were entered on this treatment project. See Tables 1 and 2. All patients have adequate performance status (Karnofsky Performance of >70%).

These seventeen patients were randomized in two groups. Arm I received priming treatment with G-CSF 5 mcg/kg every 12 hours subcutaneous for seven consecutive days, this was followed by a two-day resting interval. Arm II patients did not receive any growth factors before chemotherapy. Chemotherapy regimen was identical for both arms: Cyclophosphamide 1.75 gm/M^2 (days 1-3), VP-16 400 mg/M^2 (days 1-3

and cisplatin 55 mg/M^2 (days 1-3) per course. Both arms received G-CSF 5 mcg/kg sc. every 12 hours post-chemotherapy until absolute neutrophil counts recover to 1.5 x 10^9/L and platelets to 100 x 10^9/L. Treatment design is that both arms will receive a second course of same regimen when they recover from marrow and extramedullary toxicities observed during the first treatment.

Kaplan-Meier estimates were calculated between primer and no primer groups after course one and course two of chemotherapy. The Wilcoxon test was used to determine statistical differences between neutrophil and platelet recovery for the patients stratified in the two different groups.

Table 1. Patient Characteristics

	No Primer	Primer
Malignancy		
Breast III/IV	4	3
Melanoma	1	2
Sarcoma	1	1
Lung NSC/SC*	2	1
Adenocarcinoma		2
Previous Radiation		
Pelvis/chest wall	1	
Pelvis alone	1	
Chest wall	3	1
Extremity		1
Prior Mitomycin	1	1

*NSC - non small cell; SC - small cell

Table 2. Prior Chemotherapy Cycles

Group	Median (range)
Primer	7 (3-14)
No primer	4 (3-9

RESULTS

During Course 1, eight patients were randomized to receive primer, and nine patients to the no primer arm. Fourteen patients in the study received Course II, seven from each arm. Three patients did not receive a second course. In the primer arm, one patient refused Course Ii. In the no primer arm, one patient suffered early death during Course I and one patient refused Course II.

The use of hematopoietic growth factor (G-CSF) as a primer did not show significant shortening in the duration of neutropenia or thrombocytopenia following chemotherapy in this project design. See Tables 3 and 4. During Course I, the duration of absolute neutrophil count <100, <500 p values are 0.809 and 0.382 and, during Course II, the duration of absolute neutrophil count <100, <500 p values are 0.267 and 0.608 respectively.

Table 3. Duration Absolute Neutrophil Count

# Days	Course I Primer/No Primer median (range)	Course II Primer/No Primer median (range)
ANC <100	9 (5-13) / 10 (6-13)	9 (6-12) / 8 (5-10)
ANC <500	10 (5-16) / 13 (8-19)	12 (8-15) / 12 (7-13)
ANC <1000	12 (5-16) / 13 (8-21)	16 (9-17) / 14 (8-24)

Table 4. Duration Platelet Count

# Days	Course I Primer/No Primer median (range)	Course II Primer/No Primer median (range)
PLT <20	10 (7-16) / 9 (2-22)	11 (9-13) / 12 (5-35)
PLT <50	15 (9-18) / 12 (10-27)	18 (10-34) / 17 (14-50)
PLT <100	21 (15-29) / 18 (12-33)	22 (12-51) / 25(19186)

A possible trend to less infection (pneumonia, bacteremia, fungemia) in the primer group was observed. An observation in this project is the fact that three patients who with priming reached white blood cell counts above 70,000/ul have shorter duration of neutropenia than five patients that did not prime greater than 70,000/ul. See Table 5.

Table 5. Primed >70,000

Patient	WBC day 7 ($x10^9$/L)	Days ANC <100
#1	76.8	5
#2	74.0	7
#3	76.1	6

There was no difference in days febrile, of hospitalization, or use of antibiotics between both groups. Incidence of infections during treatment are summarized in Table 6. One early mortality occurred in the no primer arm due to fungemia after the patient recovers absolute neutrophils count >1500/ul.

Table 6. Infections Rate

	Primer % Course I/II	No primer % Course I/II
Fever	60/50	60/78
Pneumonia	0/0	0/22
Bacteremia	12/0	0/14
Fungemia	0/0	11/14

Primer arm: eight patients Course I, seven patients Course II.
No primer arm: nine patients Course I, seven patients Course II. Neutropenia: absolute neutrophil count less than 100/ul.
Fever: temperature above 37.0° C.
Pneumonia: radiographic identifiable infiltrate.
Bacteremia: identifiable positive blood culture with or without clinical sepsis.
Fungemia: identifiable positive blood culture.

DISCUSSION

The optimal priming (duration, dose, or combination) is yet to be described. Even though this study did not show a different neutrophil recovery between the two arms, it should be emphasized that there was a small sample size of patients in this study. There are data reported by other investigators that priming using a shorter duration of G-CSF to treat hematologic malignancies shows significantly faster recovery of neutrophils in the primed patients compared to the no primer arm.[8]

Kinetic response of human marrow progenitor cell to growth factors could be the determinant factor in the selection of the adequate hematopoietic stimulating growth factor used in this project design. A recent publication has shown that G-CSF stimulated progenitor cells will

still be rapidly proliferating two to four days after G-CSF has been stopped.[9] This longer period of proliferation after stopping growth factor makes the hematopoietic progenitors more vulnerable for a longer period of time to cytotoxic drugs, therefore the resting interval after stopping G-CSF may be needed to be longer than four days. A different observation has been reported after GM-CSF priming. After GM-CSF priming, hematopoietic progenitors have demonstrated return to a slower cycling or non-cycling state within one to two days of stopping GM-CSF. This quicker return to a non-cycling state was observed to be maintained for at least a week.9 This one-week interval may offer a chemoprotective effect.

Future directions involving the best combination of growth factors, as well as an optimal dose, duration and schedule of administration should be considered. It may also be informative to obtain myeloid progenitor cell assays to assess the effect of growth factors on hematopoietic progenitor cells *in vivo*.

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HIGH-DOSE CHEMOTHERAPY AND PERIPHERAL BLOOD PROGENITOR CELL TRANSPLANTATION: EFFECTS OF GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR ON THE AUTOGRAFT

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ABSTRACT

Between June 1989 and September 1993, 169 patients participated in sequential clinical trials using peripheral blood progenitor cells (PBC) as their sole source of hematopoietic rescue following high-dose chemotherapy. All patients had received prior extensive combination chemotherapy and had marrow defects which precluded autologous bone marrow transplantation. The initial 86 patients (Group 1) had PBC collected without mobilization. Beginning in April 1991, PBCs were mobilized solely with recombinant granulocyte-macrophage colony stimulating factor (rGM-CSF). Thirty-four patients (Group 2) received rGM-CSF at 125 $\mu g/M^2$/day by continuous intravenous infusion, and 24 patients (Group 3) and 25 patients (Group 4) received rGM-CSF at 250 $\mu g/M^2$/day by continuous intravenous infusion. Patients underwent at least six aphereses and had a minimum of 6.5 x 10^8 mononuclear cells/kg collected. Groups 1, 2, and 3 did not receive cytokines immediately after transplantation. Group 4 received rGM-CSF at 250 $\mu g/M^2$/day by continuous intravenous infusion starting the day of PBC transplantation. Mean time to hematopoietic recovery in days (d) in evaluable patients was as follows:

Group	N	rGM-CSF	ANC>500	Hb>8 gm	Plt >20K
1	86	None	26d(11-93)	22d(4-135)	24d(9-266)
2	34	125$\mu g/M^2$	23d(12-49)	27d(2-74)	24d(7-102)
3	24	250$\mu g/M^2$	18d(10-46)	15d(6-41)	15d(8-41)
4	25	250$\mu g/M^2$+	12d(9-16)	20d(3-70)	22d(7-94)

(+ = rGM-CSF post-PBSCT)

Time to recover granulocytes after transplant was significantly shorter for the mobilized groups. Transplantation of PBC mobilized with rGM-CSF resulted in shorter time to platelet and RBC transfusion independence. Administration of rGM-CSF post-PBCT resulted in more rapid granulocyte recovery, but had no or little effect on RBC and platelet transfusion independence. Mobilization of PBC with rGM-CSF alone is a practical, effective method for patients, who have received prior chemotherapy and have bone marrow abnormalities, resulting in rapid and sustained restoration of hematopoietic recovery following high-dose chemotherapy.

INTRODUCTION

The use of autologous peripheral blood progenitor cells (PBC) for transplantation has permitted patients, whose bone marrow is unsuitable for collection, to receive high-dose therapy (HDT) for a variety of malignancies.[1] In early clinical trials, PBCs were collected while myelopoiesis was in a steady-state.[2] Transplantation of steady-state PBC following HDT resulted in sustained hematopoietic recovery at rates similar to that obtained with autologous bone marrow transplantation (ABMT), but multiple aphereses were required to collect an adequate number of cells for transplantation. The efficiency of PBC collection was improved when cells were collected following mobilization by cytotoxic chemotherapy and hematopoietic cytokines, used alone or in combination.[3-7] The optimal method of PBC mobilization is highly dependent upon such variables as the patient population being treated, the degree of prior treatment, and the practicality of the mobilization method. We have previously reported improved efficiency of PBC collection following mobilization with recombinant human granulocyte-macrophage colony stimulating factor (rGM-CSF) in a patient population with bone marrow unsuitable for ABMT.[8] These trials were able to demonstrate that cytokine-mobilization resulted in a more effective autograft, as hematopoietic recovery was improved despite the fact that cytokines were not admitted following PBC transplantation. The following trial investigates the effect of cytokines on hematopoietic recovery following transplantation of PBC mobilized with rGM-CSF.

MATERIALS AND METHODS

Patient Characteristics

The trials reported here are sequential. Patient eligibility requirements for HDT included malignancies that were refractory to conventional curative strategies, age <60, a Karnofsky performance status of at least 70, and no major organ dysfunction. Eligibility requirements for peripheral blood progenitor cell transplantation (PBCT) included the presence or history of morphological tumor contamination of the bone marrow or bone marrow hypocellularity. Patients with evidence of circulating malignant cells on peripheral smear were ineligible for these trials. Between June 1989 and September 1993, 169 patients who were candidates for HDT and PBCT at the University of Nebraska Medical Center were eligible to participate in these trials. Written informed consent for PBC collection with or without cytokine mobilization and for autologous stem cell transplantation was obtained from each patient. The characteristics of these patients and their malignancies are presented in Table 1.

Table 1. Patient Characteristics

	Non-mobilized		*Mobilized*	
	(Group 1)	(Group 2)	(Group 3)	(Group 4)
Number of patients	86	34	24	25
Male:Female	43:43	17:17	11:13	13:12
Median age (range)	38(18-62)	44(19-59)	38(17-59)	47(26-66)
Malignancies:				
Hodgkin's disease	38	7	7	4
Non-Hodgkin's disease	27	14	14	16
Breast cancer	15	11	1	4
Other	6	2	2	1

Peripheral blood progenitor cell mobilization. The initial 86 patients had PBC collected while myelopoiesis was in a steady-state (Group 1). Eighty-three patients had PBC collected following mobilization with Sargramostim (Immunex, Seattle, WA), a yeast-derived form of rGM-CSF, administered as a continuous intravenous infusion through an ambulatory infusion pump (Model CADD+, Pharmacia-Deltec, St. Paul, MN) in an outpatient setting. Beginning April 1991 through December 1991, 34 patients received rGM-CSF at 125 $\mu g/M^2$/day (Group 2). Beginning January 1992, 49 patients received rGM-CSF at 250

$\mu g/M^2$/day (Groups 3 and 4). For patients in Groups 2, 3, and 4, apheresis began when the peripheral white blood cell count (WBC) reached 10 x 10^9/L. The administration of rGM-CSF was discontinued on the day of the final apheresis procedure. The dose of rGM-CSF was reduced by 50 percent for a suspected drug toxicity or to maintain the WBC below 25 x 10^9/L. If after five days of rHuGM-CSF administration the WBC had not reached 10 x 10^9/L, human recombinant granulocyte colony-stimulating factor (rG-CSF), filgrastim (Amgen, Thousand Oaks, CA), at a dose of 5 μg/kg/day administered subcutaneously was substituted, and apheresis was immediately initiated.

Stem cell collection and processing techniques. All patients had a minimum of 6.5 x 10^8 mononuclear cells (MNC)/kg-patient weight collected with at least six apheresis procedures. The minimum number of MNC and apheresis procedures were based upon results of previous trials at the University of Nebraska Medical Center using non-mobilized PBC as the sole source of hematopoietic rescue following high-dose chemotherapy.[9] The apheresis procedures were repeated no more than five times weekly. If, at the end of six apheresis procedures, a total of 6.5 x 10^8 MNC/kg-patient weight had not been collected, the procedures were continued until the target number of MNC was obtained. All collections were cryopreserved using a previously reported technique.[10] Briefly, the cells were cryopreserved in a 10 percent concentration of dimethyl sulfoxide (DMSO) and stored in the vapor phase of liquid nitrogen using a controlled rate freezer (Cryo-Med, Mt. Clemens, MI).

The colony forming unit-granulocyte/macrophage (CFU-GM) content of the combined collections from each patient was determined both before and after cryopreservation using a modification of a previously reported culture method, where recombinant human interleukin-3 (200 U/ml), rGM-CSF (200 U/ml), and rG-CSF (200 U/ml) were also added as growth factors.[9]

High-dose chemotherapy and peripheral blood progenitor cell transplantation. Most patients subsequently received high-dose chemotherapy and PBCT. The HDT these patients received was determined by their underlying malignancy, but in no instance did the therapy include total body irradiation. Patients with Hodgkin's disease received a single regimen of carmustine 300 mg/M^2, etoposide 125 mg/M^2 administered every 12 hours for six doses, and cyclophosphamide 1.5 gm/M^2/day for four days (CBV). Patients with non-Hodgkin's lymphoma (NHL) were included in three consecutive studies of high-dose chemotherapy regimens based upon histologic diagnosis and previous

tumor responsiveness. The first regimen consisted of carmustine 300 mg/M^2, etoposide 100 mg/M^2 every 12 hours for eight doses, cyclophosphamide 35 mg/kg/day for four days, and cytarabine 100 mg/M^2 every 12 hours for eight doses (BEAC). The second regimen consisted of carmustine 300 mg/M^2, etoposide 150 mg/M^2every 12 hours for six doses, cyclophosphamide 2.5 gm/M^2/day for two consecutive days, and hydroxyurea 1.5 gm/M^2 every six hours for 12 doses (BECH). The third regimen consisted of ifosfamide 3 gm/M^2/day for four days, carboplatin 300 mg/M^2/day for four days, and etoposide 400 mg/M^2/day for four days (MICE). Patients with breast cancer received cyclophosphamide 50 mg/kg/day for four days, thiotepa 150 mg/M^2/day for four days, and hydroxyurea 1.5 gm/M^2 every six hours for 12 doses (CTH). All other patients with solid tumors received high-dose chemotherapy regimens consisting of various alkylating agents.

Cytokines were not routinely administered at the time of the transplantation to Groups 1, 2 and 3. Indications for the initiation of cytokines following PBCT in these three groups included a documented infection during the period of absolute neutropenia or the failure to achieve an absolute neutrophil count of 0.2 x 10^9/L by day 28 posttransplantation. Patients in Group 4 received rGM-CSF at 250 ug/$M^2$by either continuous infusion or by subcutaneous administration starting within 24 hours of PBC infusion.

Platelet transfusions were given if there was clinical evidence of bleeding or the platelet count was less than 20,000/μL. Similarly, red blood cell transfusions were given if the patient had a hemoglobin (Hg) less than 6 gm/dL or was symptomatic. The times to recover 0.5 x 10^9/L granulocytes and to become independent of red blood cell and platelet transfusions after transplantation were recorded.

Statistical Methods. Comparisons of the number of CFU-GM collected by treatment group, were made using the Wilcoxon rank-sum test. Comparisons of times to hematopoietic recovery by treatment group were made using the log-rank test. Multivariate adjustments of these comparisons for factors prognostic for engraftment were made using the multivariate survival model of Cox.

RESULTS

Patients

All patients had received considerable antitumor therapy. All four patient groups had received a median of two different combination

chemotherapy trials prior to being considered as candidates for HDT. The most common reasons for selecting peripheral blood rather than bone marrow for autografting were either marrow involvement with malignant cells in approximately 75 percent of patients or marrow hypocellularity from prior antitumor therapy, usually secondary to pelvic irradiation.

Mobilization

There was a significant difference ($p=0.0001$) in the number of CFU-GM in the collections of the mobilized and non-mobilized patients with greater numbers of CFU-GM in Groups 3 and 4 (Table 2). The number of CFU-GM were noted to increase within one to two days after the initiation of rGM-CSF for mobilization. The peak number of CFU-GM was observed five to six days after the administration of rGM-CSF and subsequently declined.

Table 2. Peripheral Blood Progenitor Cell Product Characteristics

	Median Number of CFU-GM x 10^4/Kg
Group 1 (N=86)	0.34 (0.01-13.4)
Group 2 (N=34)	1.9 (0.2-102.8)
Group 3 (N=24)	10.2 (0.30-104.3)
Group 4 (N=25)	11.0 (1.14-22.8)

Hematopoietic Recovery Following Transplantation

One hundred fifty-five of the 169 patients received high-dose chemotherapy followed by PBCT and were evaluable for hematopoietic recovery (Table 3). Nine patients in Group 1 (9%), 9 patients in Group 2 (25%), and 3 patients in Group 3 (13%) received cytokines (rGM-CSF or rG-CSF) a week or more following PBCT. The most common reason for cytokine administration post-PBCT was failure to achieve 0.2 x 10^9/L

granulocytes by day 28 after transplantation. All patients in Group 4 received cytokines (rGM-CSF) immediately following PBC transplantation.

Table 3. Hematopoietic Recovery

	Median Number of Days (D) Unit Recovery		
	ANC >0.5 x 10^9/L	Plt >20,000	Hb >8 gm/dL
Group 1 (N=80)	26 D (11-93 D)	24 D (9-266 D)	22 D (4-135 D)
Group 2 (N=31)	23 D (12-49 D)	24 D (7-102 D)	27 D (2-74 D)
Group 3 (N=23)	18 D (10-46 D)	15 D (8-41 D)	15 D (6-41 D)
Group 4 (N=21)	12 D (9-16 D)	20 D (3-70 D)	22 D (7-94 D)

The median number of days until the appearance of 0.5 x 10^9/L granulocytes in the circulation after PBCT was 26 for Group 1, 23 for Group 2, 18 for Group 3, and 12 for Group 4. There was a statistical difference in granulocyte recovery ($p=0.001$) between the non-mobilized and mobilized groups. There was also a statistical difference ($p=0.002$) observed in the patients who received mobilized PBC without cytokines posttransplant (groups 2 and 3) and patients who routinely received mobilized PBC with cytokines posttransplant (Group 4). The number of CFU-GM both prior to and following cryopreservation correlated with the rate of hematopoietic recovery. Patients with greater than 2.5 x 10^4CFU-GM/kg-patient weight had a more rapid recovery of 0.5 x 10^9/L circulating granulocytes ($p < 0.001$).

The median time to platelet transfusion independence for both Groups 1 and 2 was 24 days. Patients in Group 3 became independent of platelet transfusions at a median time of 15 days, which was statistically significant ($p=0.01$) as compared to Groups 1 and 2. The median time to platelet transfusion independence in Group 4 was 20 days, but it was not statistically different from Group 3. Results for time to red blood cell transfusion independence were similar to platelet transfusion

independence. Patients in Group 3 became independent of red blood cell transfusions at a median of 15 days as compared to 22 days for Group 1 and 27 days for Group 2. Patients in Group 4 became red blood cell independent in 22 days; however, this was not statistically different from Group 3.

DISCUSSION

Peripheral blood progenitor cells, mobilized by chemotherapy and cytokines, either alone or in combination, has permitted the administration of relatively higher doses of conventional chemotherapy. Chemotherapy has failed to mobilize PBC in patients who have received extensive, prior therapy despite the addition of hematopoietic cytokines.[3,5] Because bone marrow metastases and extensive prior chemotherapy have predicted for refractoriness to mobilization attempts, patients have been excluded from clinical trials who may have potentially benefitted from high-dose therapy.[11,12]

This report describes our continued experience with the recombinant human hematopoietic cytokine, rGM-CSF, for the mobilization of PBC in patients who were ineligible for ABMT due to the presence of bone marrow metastases and/or bone marrow hypocellularity. The patients shared common characteristic of having received extensive, prior therapy, had PBC collected in an identical manner, and received essentially the same number of mononuclear cells after administration of high-dose chemotherapy. However, this report contains additional data in regard to the effects of cytokines routinely administered following PBCT.

Mobilization solely with rGM-CSF also avoided the toxicity and the delay in initiation of PBC collection while waiting for recovery from neutropenia associated with mobilization attempts using cytotoxic agents. The transplantation of PBC mobilized with rGM-CSF resulted in a shorter period of granulocyte recovery as compared to non-mobilized PBC. In addition, the mobilization of PBC with rGM-CSF resulted in earlier platelet and RBC transfusion independence. Cytokines were not routinely administered to patients in Groups 1, 2, and 3 immediately following PBCT. Cytokines were used in less than 15 percent of these patients later in their hospital course and did not effect the rate hematopoietic recovery between the non-mobilized (Group 1) and the mobilized groups (Groups 2 and 3). However, the routine administration of hematopoietic cytokine following the transplantation of mobilized PBS resulted in a statistically significant improvement in neutrophil recovery. The post-PBC administration had no significant effect on platelet and red blood cell

recovery, although there was a trend toward the delay in platelet and red blood cell recovery as compared to the transplantation of mobilized PBC without cytokines post-PBC. Similar results have been observed in a randomized trial of cytokine administration following transplantation of mobilized PBC.[13] This data suggests that the administration of a cytokine, which primarily effects late myeloid maturation, may have preferentially effected the recovery of neutrophils over red blood cells and platelets.[14]

These trials demonstrate that rGM-CSF can effectively mobilize PBC, as measured by number of required PBC collections, CFU-GM, and hematopoietic recovery after PBCT was statistically significant as compared to non-mobilized PBC. The addition of cytokines immediately after transplantation of mobilized PBC further hastened neutrophil recovery, but has little effect on platelet and red blood cell recovery. These results were obtained in a patient population which shared important clinical characteristics of having received extensive, prior combination chemotherapy and marrow defects which precluded ABMT. These characteristics have been associated with difficulty in mobilization employing cytotoxic chemotherapy with or without cytokines. These data suggest that rGM-CSF can effectively and efficiently mobilize peripheral blood progenitor cells for transplantation following HDT.

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PERIPHERAL BLOOD STEM CELLS (PBSC) MOBILIZED WITH AND WITHOUT GRANULOCYTE-COLONY STIMULATING FACTOR (G-CSF)

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ABSTRACT

Three regimens for mobilizing PBSC were evaluated in 138 patients with hematological and non-hematological malignancies (excluding acute leukemia). A total of 155 mobilization episodes were undertaken using cyclophosphamide (Cy) 4 g/M^2 (52 procedures), Cy 7 g/M^2 (50 and Cy 5 g/M^2 + G-CSF (53). G-CSF was given subcutaneously at a dose of 5-10 μg/kg/day beginning 48-72 hours after Cy administration continuing until white cell count (WCC) >0.5 x 10^9/L. PBSC mobilization with Cy 5 g/M^2 + G-CSF results in superior CFU-GM yields, a shorter duration of neutropenia and thrombocytopenia and substantially less non-hematologic morbidity when compared to mobilization with Cy 7 g/M^2. Following high-dose chemotherapy and PBSC transplantation no differences were observed in hematopoietic recovery rates, blood product and antibiotic usage, febrile days and total hospital days when comparing transplants performed with Cy + G-CSF mobilized stem cells and those utilizing Cy alone.

INTRODUCTION

High-dose chemotherapy followed by autologous bone marrow transplantation has been shown to improve response duration and survival for patients with relapsed malignant lymphomas and acute leukemias.[1] Recently, this strategy has also been successfully employed in breast carcinoma, neuroblastoma and testicular carcinoma.[1] Progenitor cells obtained from peripheral blood can be used as an alternative to bone marrow progenitors particularly in patients in whom marrow aspiration is impossible due to tumor infiltration, marrow fibrosis or hypoplasia and previous radiation therapy. Peripheral blood stem cells (PBSC) appear to offer a number of advantages over bone marrow progenitors in the

transplant setting including a rapid rate of engraftment,[2] shorter hospitalization and thus considerable cost savings.[3] Several methods have been published concerning methods for PBSC collections including steady-state collections,[4] mobilization using chemotherapy,[5] use of hematopoietic growth factors (e.g., G-CSF, GM-CSF, IL-3, IL-1, stem cell factor, erythropoietin)[6] and a combination of chemotherapy with one or more growth factors.

This study was designed to compare three different PBSC mobilization regimens using high-dose cyclophosphamide alone at a dose of 4 or 7 g/M^2 or cyclophosphamide 5 g/M^2 followed by G-CSF. We are particularly interested in whether differences exist in posttransplantation hematopoietic recovery rates between patients given PBSC obtained by Cy alone and those given PBSC obtained by Cy + G-CSF.

STUDY DESIGN

A total of 155 mobilization procedures were performed between November 1988 and May 1994 in 138 patients (M/F - 47/108). The diseases treated included breast cancer (53 mobilizations), non-Hodgkin's and Hodgkin's lymphomas (70), multiple myeloma (13) and other solid tumors (19). Patients with acute leukemia or chronic myeloid leukemia were not included in this analysis.

Eligible patients were evaluated by physical examination, complete blood counts, blood chemistry, chest radiographs, radionuclide whole body bone scan, gated blood pool cardiac ejection fraction measurements (MUGA), pulmonary function tests, bilateral pelvic bone marrow biopsies, blood and bone marrow hematopoietic progenitor (CFU-GM) culture, psychiatric evaluation, quality of life assessments and ECOG status determination. All patients were required to be between 18-55 years of age, to have an ECOG performance status of 2 or less and an estimated life expectancy of >3 months. Patients were required to have a resting left ventricular ejection fraction (by MUGA) of 50% or more, a pulmonary spirometry of 75% or more of predicted normal value as well as adequate renal, hepatic and hematopoietic function. All evaluations were repeated immediately prior to entry into the autologus PBSCT phase and on days +28, +100 and at 6 and 12 months post PBSCT. Patients with prior history of other malignancies or those with central nervous system involvement were ineligible.

Cyclophosphamide Mobilization and Stem Cell Collection

Cyclophosphamide (Cy) was administered as previously described.[7] All patients received Mesna® and hyperhydration (3 L/M^2/day) as uroepithelial prophylaxis for 36 hours following Cy. Patients enrolled in the study after October 1992 received granulocyte-colony stimulating factor (G-CSF, Neupogen®Amgen Australia) in the dose of 5μ/kg or 10 μg/kg (for heavily pretreated patients) by daily subcutaneous injections starting 48-72 hours after the administration of Cy and continuing until the WCC exceeded 0.5 x 10^9/L. Blood stem cell collections were begun when ANC exceeded 0.8 x 10^9/L (in patients mobilized using Cy without G-CSF) or when ANC exceeded 0.5 x 10^9/L (in patients mobilized using Cy + G-CSF).

Mononuclear cells (MNC) were harvested using a continuous flow cell separator COBE "Spectra" or Fenwal CS 3000. A minimum target of 2.5 x 10^8 MNC/kg was harvested in each patient. The MNCs harvested by leukapheresis were concentrated by centrifugation and cryopreserved in liquid nitrogen using a programmed freezer. Hematopoietic precursors (CFU-GM) were quantitated using previously published methods[5] with a minimum target requirement of 15 x 10^4 CFU-GM/kg for autologous PBSCT. In our laboratory, the normal range for blood CFU-GM is 0-120 x 10^3/L and for bone marrow CFU-GM is 20-80/10^5 light density MNCs. Patients with insufficient CFU-GM collections underwent further mobilization after an interval of at least 8 weeks. A "back-up" bone marrow harvesting was not required unless the total CFU-GM yield was below the minimum requirements of 15 x 10^4 CFU-GM/kg.

From December 1992 pre-harvest peripheral blood CD34 cell counts were also determined in all patients by flow cytometry. CD34 levels were determined using two-colour immunofluorescence labelling by a whole blood method. We determined a minimum target of >5 x 10^6 CD34 cells/L as trigger for commencement of leukapheresis and a total of 4-5 x 10^6 CD34 cells/kg as the optimum number of transplantation.

High Dose Chemotherapy and PBSCT

All patients underwent restaging immediately prior to PBSC transplantation (usually between 4-6 weeks after Cy administration). Patients with progressive disease between completion of stem cell collections and planned PBSCT were removed from the study and offered alternative therapy. High-dose chemotherapy began one day after the

insertion of an indwelling double lumen central venous catheter. High-dose chemotherapy consisted of CMCp (melphalan 130 mg/M^2day -5, Cy 50 mg/kg/day on days -4 to -2 and carboplatin 400 mg/M^2/day given as a continuous IV infusion for 72 hours [day -4 to -2]) for patients with breast carcinoma and carbo-EAM (melphalan 140 mg/M^2day -8, carboplatin 400 mg/M^2/day given as a continuous IV infusion for 48 hours [day -7 and -6]), etoposide 150 mg/M^2 BD together with cytosine arabinoside 200 mg/M^2 BD for 96 hours [day -5 to -2]) for patients with lymphoma. Myeloma patients received melphalan 140 mg/M^2/day on day -5 followed by TBI over three days. Patients received Mesna® and hyperhydration (3 L/M^2/day) as uroepithelial prophylaxis if Cy was administered. Cryopreserved stem cells were thawed rapidly and reinfused on day 0. Hematopoietic growth factors were not used following stem cell infusion. Bone marrow back up was administered only after day +28 if inadequate hematopoietic engraftment was present (platelets <25,000, WCC <1,500).

Measurement of Effect

Toxicities and response evaluation following Cy mobilization and PBSCT were evaluated according to WHO criteria.[8] Neutropenia was defined as absolute neutrophil count (ANC) $<0.5 \times 10^9$/L and thrombocytopenia was defined as platelet count $<50 \times 10^9$/L. Febrile neutropenia was defined as fever >38.2°C and a ANC $<0.5 \times 10^9$/L.

Results - CY Mobilization

The morbidity of the Cy mobilization was tolerable although significant hematologic and gastrointestinal morbidity was observed in virtually all patients mobilized with Cy dose of 7 g/M^2 as previously described.[9] Patients mobilized with G-CSF experienced significantly fewer febrile days (p=0.05) and had a higher platelet nadir (p=0.03) following mobilization than did patients mobilized with 7 g/M^2Cy. The median number of CFU-GM collected following mobilization with Cy and G-CSF was greater than following mobilization with both doses of Cy alone (Table 1) although this difference failed to reach statistical significance (p=0.06). Statistically significant differences were not seen in patients mobilized with 4 g/M^2 and with 5 g/M^2 plus G-CSF. The median number of leukaphereses required to collect sufficient PBSC was similar in the three groups (median = 6, range 2-10). Patients mobilized with G-CSF required a median of 5 leukaphereses (range 2-10) compared to a median

of 6 leukapheres in patients mobilized without G-CSF (range 2-10) this difference failed to reach statistical significance (p=0.08). Six patients underwent a second mobilization because of insufficient PBSC yields following Cy + G-CSF mobilization. In two of these 6 patients sufficient stem cell numbers became available for a PBSCT as a result of the second Cy mobilization. Thus, mobilization using Cy + G-CSF results in an overall success rate for the procedure of 92% (49/53 mobilizations) which is in contrast to the 85% success rate for mobilizations using Cy alone.

CD34 cell results are available for 49 patients harvested with Cy + G-CSF. There was a wide range of CD34 collection results with a total median yield of 11.6 x 10^6 CD34 cells/kg (range 0.8-85). Twelve patients failed to achieve our minimum target of >5 x 10^6CD34 cells/kg and all but two also failed to reach the minimum total CFU-GM target of >15 x 10^4/kg (median 6.8, range 2.7-53.0). The correlation coefficient between daily CFU-GM and CD34 cell yield was r=0.89.

Table 1 summarizes the peak and total CFU-GM and total MNC yields resulting from the three separate mobilization regimens.

Table 1.

Cy Dose	No. of Phereses	MNC x 10^8/kg	CFU-GM x 10^4/kg	Peak CFU-GM x 10^8/kg	Day peak CFU-GM post Cy
4 g/M^2	6	4.5	20.7	780	17
7 g/M^2	6	4.6	41.0	1410	19
5 g/M^2 + G-CSF	5	2.7	51.9*	2640*	12

all results expressed as median

Table 2 summarizes post-mobilization hematological morbidities arising from each Cy regimen.

Table 2.

Cy Dose	Days T >38C	Days WCC <1.0	Days Plt <50	WCC Nadir	Plt Nadir	Day of WCC Nadir	Day of Plt Nadir
4 g/M^2	0	9	0	0.3	74	9	10
7 g/M^2	1	9	5	0.1	14	9	11
5 g/M^2 +G-CSF	0.5	5	1	0.2	25	8	10

all results expressed as median

Results - High-Dose Chemotherapy and PBSC Transplantation

A total of 104 transplant episodes were performed. In 61 cases transplantation was performed using PBSC mobilized with Cy alone and in 43 cases transplantation was performed using PBSC mobilized with Cy + G-CSF. Both groups received similar amounts of CFU-GM following high-dose chemotherapy (median 30.0 x 10^4/kg in patients mobilized with Cy alone, 32.0 x 10^4/kg in Cy + G-CSF patients) although the total MNC dose given was higher in the Cy alone group (3.2 x 10^8/kg) than in the Cy + G-CSF group (1.5 x 10^8/kg).

Table III summarizes the posttransplantation outcomes of the 104 transplantation episodes.

Table 3.

PBSC source	Days to Plt >50	Days to ANC	Plt Transf. Episodes	RBC Units Given	Febrile Days	Anti-biotic Days	Days in Hospital
Cy only	14	11	4	4	4	9	23
Cy + G-CSF	14	10	6	3	4	9	22

all results expressed as median

DISCUSSION

PBSC can be successfully mobilized into the circulation with the use of high-dose Cy in >85% of cases. Mobilization is far more effective when a higher dose of Cy is used (i.e., 7 g/M^2) but unfortunately despite better CFU-GM yields this regimen is substantially toxic to patients as reported previously.[9] The addition of G-CSF to Cy has resulted in comparable yield of CFU-GM and at the same time a significant reduction in hematologic morbidity with fewer febrile episodes, higher WCC and platelet nadirs and fewer days of Grade 3/4 neutropenia and thrombocytopenia. Cy + G-CSF mobilization also allows for better scheduling of leukaphereses as the majority of patients will begin their stem cell collections 9-10 days after the administration of Cy with a peak CFU-GM 12 days after the administration of Cy.

Following high-dose chemotherapy and PBSC transplantation, we have observed no differences in the rates of hematopoietic recovery, antibiotic or blood product usage according to the source of stem cells used. We have also seen similar non-hematolopoietic morbidities in the two groups with virtually identical duration of hospitalization. We therefore believe that mobilization with Cy + G-CSF is superior to

mobilization with Cy alone (4 or 7 g/M^2) providing a reliable and safe means of PBSC mobilization in 92% of all patients.

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LONG TERM HEMATOLOGIC RECOVERY AFTER AUTOLOGOUS PERIPHERAL BLOOD PROGENITORS OR BONE MARROW TRANSPLANTATION FOR ADVANCED LYMPHOMAS

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INTRODUCTION

The prognosis for relapsed and refractory lymphoma is poor with conventional chemotherapy and has been improved with high-dose therapy and autologous stem cell transplantation (ASCT). In the past 10 years two major improvements were observed: The use of mobilized peripheral blood progenitor cells (PBPC) and the use of hematopoietic growth factors.[1,2]

PBPC mobilized after myelosuppressive chemotherapy with G-CSF allows faster hematopoietic recovery when reinfused alone without any bone marrow rescue.[3] However, long-term hematopoietic reconstitution has been questioned as early recovery is related to committed progenitors.[4] Seventy-eight with relapsing or refractory lymphomas and Hodgkin's disease receiving high-dose therapy followed by PBPC were compared with 72 patients receiving autologous bone marrow transplantation (BM). The purpose of this report was to compare failure free-survival in these two groups and to compare early and late hematologic recovery.

PATIENT CHARACTERISTICS

Between October 1986 and December 1993, 150 patients with advanced lymphomas received high-dose therapy and autologous stem cell transplantation in our institution. Patient characteristics are summarized in Table 1.

Bone marrow was harvested and cryopreserved by routine techniques. PBPC were collected by three leukaphereses during hematologic recovery following mobilizing chemotherapy as previously described.[3] Mobilizing chemotherapy was the second line regimen administered to reduce tumor bulk before high-dose therapy. These

regimens were heterogeneous, but they all included either high-dose cyclophosphamide (2 to 7 g/M^2) or ifosfamide (4.5 to 7.5 g/M^2).

Table 1. Patient Characteristics

	Group I - PBPC	Group II - BM
Number	78	72
- Age (median)	40 years	34 years
- Aggressive NHL	35 pts	30 pts
- Low grade NHL	24	4
- Hodgkin's	19	38
- BM involvement	18	4
- CT regimen >2	22 (28%)	26 (37%) p:NS

Hematopoietic Growth Factors

PBPC collection: Granulocyte colony stimulating factor (G-CSF) at 5 μg/Kg/day was given to 36 patients (46%) from day 6 after chemotherapy until the last leukapheresis. They were performed when the while blood cell count reached 2,000/μL after neutropenia and if platelet count was >50,000/μL.

G-CSF after transplantation: G-CSF has been started the day after reinjection at 5 μg/Kg/day in 60 patients until the white bloood cell (WBC) count was >1 x 10^9/L for 3 days.

Conditioning Regimens

Chemotherapy: Three conditioning regimens were given: BEAC (BCNU - 300 mg/M^2, etoposide - 800 mg/M^2, aracytine - 800 mg/M^2, cyclophosphamide - 6 g/M^2) in 36 patients. CBV (cyclophosphamide - 6 g/M^2, BCNU - 300 mg/M^2, etoposide - 1 g/M^2) in 35 patients. BEAM (BCNU - 300 mg/M^2, etoposide - 800 mg/M^2, aracytine - 800 mg/M^2, melphalan - 140 mg/M^2) in 45 patients.

Chemotherapy: 34 patients received total body irradiation (TBI) delivered at 12 grays in 6 fractions and associated with cyclophosphamide: 120 mg/Kg and etoposide 900 mg/M^2.

Study Definition

Hematologic reconstitution was defined by two parameters: a) The median time to reach a neutrophil count >500/μL, and b) The median

time to have a platelet count >50,000/μL without transfusion. These results were analyzed according to the stem cell graft (bone marrow versus PBPC) and according to whether hematopoietic growth factors (G-CSF) were used or not after transplantation. Hematologic reconstitution was also assessed at three months (97 pts), six months (94 pts) and one year (87 pts) for patients responding to high-dose therapy and without further treatment.

RESULTS

Table 2 summarizes the conditioning regimen and the hematologic recovery after ASCT, patients who died early are excluded from this table.

Table 2. Hematologic Reconstitution

	Group I - PBSC	Group II - BM
- Conditioning regimen		
- TBI	33%	11%
- BEAC/CBV	29%	61%
- BEAM	38%	28%
- G-CSF post reinfusion	43 pts	16 pts
Number of day to recover:		
- Neutrophils >500/μL	13 days	23 days
With G-CSF	11 days (43 pts)	19 days (16 pts)
Without G-CSF	16 days (34 pts)	24 days (51 pts)
- Platelets >50,000/μL	18 days	26 days

Patients received a more intensive conditioning regimen with TBI in the PBPC group. Despite these differences, we observed a significant reduction in the number of days to recover neutrophils and platelets. The only factor found to influence significantly the hematologic recovery was the presence of TBI in the conditioning regimen. This factor was only found in the PBPC group but very few patients received TBI in the bone marrow group (5 pts).

Blood samples were collected from three months to one year after ASCT in 97 patients in persistent complete remission and not receiving radiotherapy after ASCT. The majority of the patients maintained a normal blood count (Table 3). Four patients in the PBPC group and 2

patients in the bone marrow group needed platelet transfusions at 3 months. No patient was being transfused at one year. TBI negatively influenced long-term platelet recovery, and there was a significant difference in the median platelet count at 3,6 and 12 months after ASCT.

Table 3. Long Term Hematologic Recovery

PBPC-CCR Pts	46 patients (3 months)	43 patients (6 months)	36 patients (12 months)
Platelets recovery:			
Median Plts x 10^9/L			
+ G-CSF	108 (7-239)	146 (6-269)	131 (66-397)
- G-CSF	158 (10-272)	155 (22-353)	220 (34-358)
+ TBI	101 (7-203)	136 (6-269)$_{S.}$	117 (4-30)$_{S.}$
- TBI	171 (20-272)	182 (30-353)	220 (60-397)
+ All BM pts (5 TBI)	145 (55-448)	157 (32-323)	196 (40-420)
Neutrophil recovery:			
Median WBC x 10^9/L			
+ G-CSF	5,5 (1,8 - 8,9)	5,6 (1,7 - 13)	5,5 (4,2 - 8,6)
- G-CSF	4,1 (1,2 - 8,2)	4,6 (2,3 - 7,8)	5 (3 - 3,7)
+ TBI	5,3 (1,2 - 8,2)	4,1 (1,7 - 8,3)	5,3 (3,7 - 6,9)
- TBI	5,4 (3 - 8,9)	6,6 (3,3 - 13)	5,6 (3 - 8,7)
+ All BM pts (5 TBI)	4,6 (1,5 - 9,8)	4,2 (0,9 - 7,9)	5,1 (1,3 - 13,6)

The follow-up was longer for the bone marrow group. Results are summarized in Table 4. No differences were found in the number of relapses in either group. The number of deaths was much higher in the bone marrow group, however, in the PBPC group not all relapsing patients are dead. All deaths were related to lymphoma or secondary leukemia.

Table 4. Follow-up after High-Dose Therapy and ASCT

	Group I (PBSC) N = 78	Group II (BM) N = 72
Follow-up:		
Median time follow-up	30 months	50 months
Relapses	30 (38%)	24 (33%)
Median time for relapse	10.6 months	11 months
Continuous CR	35 (45%)	33 (46%)
Deaths	24 (31%)	35 (49%)
Median survival	15 months	19 months

CONCLUSION

In conclusion, PBPC transplantation is a safe procedure. After high-dose therapy, hematopoietic recovery was significantly accelerated for neutrophils and platelets as compared with bone marrow transplantation. This rapid hematologic engraftment has been associated with a low toxicity and a reduction in duration of hospital stay. The engraftment persisted in long-term studies and has permitted further treatment. Only TBI affected platelets recovery. We did not observe any difference in failure free-survival between PBPC and bone marrow grafts as reported previously.[5] Forty-five percent of patients with advanced lymphomas in both groups were in complete remission at two years after high-dose therapy and ASCT.

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MOBILIZED STEM CELLS IN LEUKEMIA

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INTRODUCTION

It is now well known that transplants of peripheral blood stem cells (PBSC) restore hematopoiesis as completely and permanently as bone marrow-derived cells. As opposed to autologous bone marrow transplantation (ABMT), the use of autologous blood stem transplantation (ABSCT) has some advantages, such as a faster speed of engraftment and less infectious morbidity.[1] In acute myeloblastic leukemia (AML), ABSCT has shown a disease-free survival (DFS) similar to that achieved by ABMT with lower morbidity and mortality due to a faster hematopoietic recovery.[2-6] We report the results obtained with ABSCT in 37 AML patients in first remission after ablative chemotherapy combination of busulfan (BU) and cyclophosphamide (CY) as conditioning regimen.

PATIENTS AND METHODS

Patients

From November 1989, all adult AML patients less than 60 years of age without HLA-identical sibling donors who achieved complete remission following conventional induction chemotherapy were considered eligible for ABSCT. A total of 37 patients were entered in the protocol. Their main characteristics are shown in Table 1. In the first 25 patients, PBSC were collected after induction and after consolidation, and the ABSCT was performed after consolidation (Group A). Since January 1992, in an attempt to decrease the relapse rate, we performed PBSC collections only after consolidation, and a third chemotherapy course (early intensification) was added pretransplant. The patients included in this new protocol are the remaining 12 (Group B).

Table 1. ABSCT in AML. Patient Characteristics

Characteristic	Group A	Group B
Sex		
Male	14	7
Female	11	5
Age (years)		
Median	44	47
Range	14-62	31-58
FAB subtype		
M0	2	0
M1 + M2	12	7
M3	1	0
M4 + M5	8	3
M6 + M7	2	2
Interval Diagnosis-ABSCT (months)		
Median	5	6
Range	3-7	5-8

Peripheral Blood Stem Cell Collection

Leukaphereses were undertaken during bone marrow recovery postchemotherapy when total leukocyte count achieved 0.8-1x10^9/L and there was monocytosis as previously described.[7] In Group A, daily leukaphereses were performed during 3-5 subsequent days following each course of chemotherapy. In Group B, PBSC collections were performed daily during 5 consecutive days only after consolidation. Figure 1 shows the therapeutic approach followed in both groups. Technical aspects of collection, pocessing and cryopreservation have been described elsewhere.[7] Granulocyte-macrophage progenitor cells (CFU-GM) and the number of CD34+ cells in the mononuclear cell collection were analyzed in each harvested cell suspension.

Bone Marrow Collection and Processing

Backup bone marrows were harvested and cryopreserved by standard techniques about one week after the last leukapheresis.

Blood Stem Cell Transfusion

Cells were thawed rapidly in a 38°C waterbath and immediately injected into a central venous line without removal of the DMSO.

Induction/Consolidation Treatment and Pretransplant Regimen

In 23 out of 25 patients of Group A, the chemotherapy regimen employed for remission induction was daunorubicin (DNR) 60 mg/M^2/d x

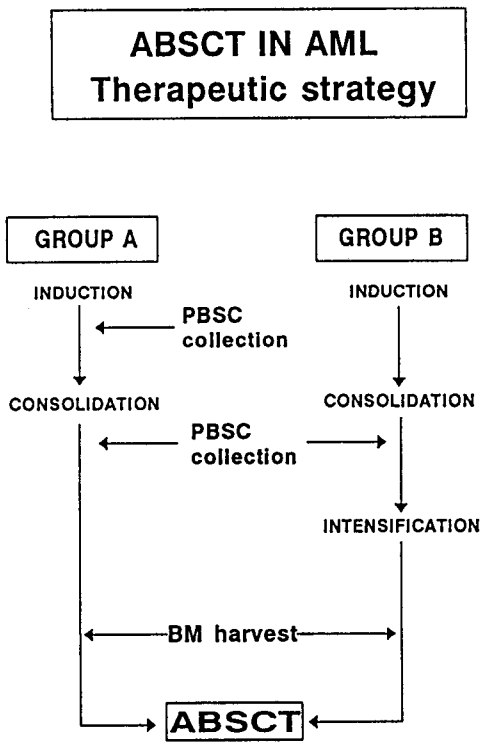

Figure 1. *ABSCT in AML patients in first remission. Therapeutic approach followed in the two study groups.*

3 days and cytarabine (Ara-C) 200 mg/M^2/d in continuous infusion x 7 days. One patient with AML-M3 received DNR alone for induction (2 mg/kg/d x 5 days). The only patient older than 60 years of age was treated with mitoxantrone (10 mg/M^2/d x 3 days) and cytarabine (150 mg/M^2/d in continuous infusion x 7 days). Consolidation consisted of an additional course of the same chemotherapy combination used for induction, except for the patient with AML-M3 who was administered adriamycin (45 mg/M^2/d x 7 days), Ara-C (150 mg/M^2/d x 7 days) and thioguanine (200 mg/M^2/d x 7 days). In Group B, four out of 12 patients received idarubicin (12 mg/M^2/d x 3 days) and Ara-C (200 mg/M^2/d in continuous infusion x 7 days) as induction and consolidation chemotherapy. In the remaining eight patients the chemotherapy regimen for remission induction employed was DAV (DNR 45 mg/M^2/d x 3 days; Ara-C 100 mg/M^2/12h x 7 days; VP-16 100 mg/M^2/d x 5 days). Consolidation consisted of DNR 45 mg/M^2/d x 2 days; Ara-C 100 mg/M^2/12h x 5 days; VP-16 100 mg/M^2/d x 5 days. In every patient in Group B a third chemotherapy course (early intensification) consisting of Ara-C 1 g/M^2/12h x 3 days and mitoxantrone 12 mg/M^2/d x 3 days was administered pretransplant. Conditioning regimen with BU 16 mg/kg and CY 200 mg/kg was given as previously described.[7] After two days of rest, transplantation was performed on day 0.

Supportive Therapy

All patients had a right atrial catheter inserted and were nursed in reverse barrier isolation. Prophylactic administration of oral ciprofloxacin, fluconazole, nystatin, cotrimoxazole, acyclovir and anti-cytomegalovirus hyperimmune gammaglobulin has been described elsewhere.[7,8] Infectious complications management and transfusion policy were those commonly employed when treating patients with acute leukemia. All cellular blood products were irradiated to 1.5 Gy and filtered prior to infusion.

Statistical Analysis

Clinical and laboratory data were analyzed according to standard statistical methods using the BMDP statistical software.[9] Time to relapse was calculated from the time between ABSCT and relapse. Survival was considered as the time elapsed between ABSCT and death due to any cause, and DFS as the time from transplantation to relapse or death. The Kaplan-Meier product limit method was used for plotting actuarial relapse, survival and DFS curves. Statistical comparisons between different actuarial curves were based on log-rank tests. Actuarial curves were

calculated from the day of ABSCT (day 0) and were analyzed as of May 31, 1994.

RESULTS

PBSC Collection

The total volume processed per run was 7-10 liters. The median number of MNC, CFU-GM and CD34+ cells administered are shown in Table 2.

Table 2. ABSCT in AML. Progenitors Administered

	Group A	Group B*
MNC x 10^8/Kg		
Median	5.6	8.3
Range	3.1-15	3.7-34.8
CFU-GM x 10^4/Kg		
Median	11	73
Range	1.5-141.6	0-683.1
CD34+ x 10^6/Kg		
Median	8.6	16.3
Range	1.5-30.7	1.3-142.2

**G-CSF was administered to some patients after consolidation as part of a double-bind randomized study.*

Hematopoietic Reconstitution

Engraftment was observed in 23 out of 25 Group A patients. Graft failure was observed in the remaining two patients in whom great differences in the number of CFU-GM (1.5 and 29.9 x 10^4/kg) and CD34+ cells (1.9 and 18.9 x 10^6/kg) infused were observed. Backup marrow was administered in both patients on days +41 and +61, respectively. One died before recovery of marrow function and complete hematological reconstitution occurred in the remaining patient. In the only patient in Group B, who developed graft failure the dose of progenitors administered was 0.0 x 10^4 CFU-GM/kg and 1.5 x 10^6/kg CD34+ cells. This patient achieved complete hematological recovery after backup marrow infusion. Mean time to attain 0.5 x 10^9 PMN/L in both groups was 13 days (range 10-17 days in Group A and 11-22 days in Group B). Mean time to 1 x 10^9 PMN/L was 15 days in both groups (range 10-26 days and 11-30 days respectively). Mean time to reach a platelet count over 20 x 10^9/L was 21 days (range 6-94) in Group A, and 26 days (range 8-171 days) in Group B. Median time to achieve 50 x 10^9 platelets/L was 19 days in Group A, and 13 days in Group B. The median hospital stay was 28 days. No patient developed late graft failure.

ABSCT-Related Complications

Every patient developed mild or moderate mucositis (26 Grade 1 and 11 Grade 2) requiring parenteral nutrition and narcotic drugs. Other toxicities were mild and uncommon. Fever was seen in 32 patients (86%). Infection was microbiologically documented in 12 cases (8 with bacteremia), clinically documented in 13 and possible in seven. Six patients (16%) developed hepatic venoocclusive disease (VOD). Two of them developed multiorgan failure and died on day +31 and +40 respectively. These two patients and another with graft failure, who finally developed a fatal infection by *Pseudomonas aeruginosa*, were the only patients who died as consequence of treatment-related complications.

DFS and Clinical Status after ABSCT

The overall DFS at 4 years is 37%. The DFS in each group is 30% (Group A) and 69% (Group B) respectively (Figure 2). Fourteen patients (52%) in Group A relapsed from two to 40 months after ABSCT. Eight remain in continuous complete remission +7 to +31 months after transplant. In Group B, with a shorter follow-up, three patients (25%) relapsed between one and seven months and the remaining nine patients are in complete remission between +4 and +16 months. The actuarial risk of relapse in each group was 66% and 31% respectively (Figure 3). The overall actuarial survival is 52% at 4 years.

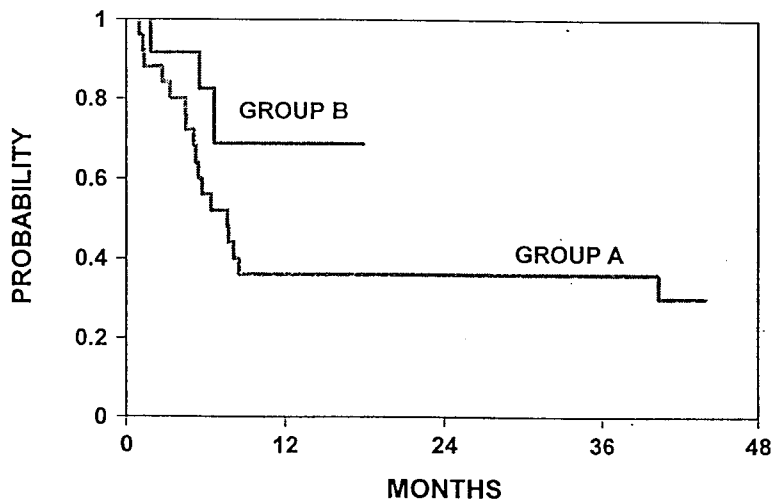

Figure 2. ABSCT in AML patients in first remission. Comparison of the actuarial disease-free survival between the two study groups.

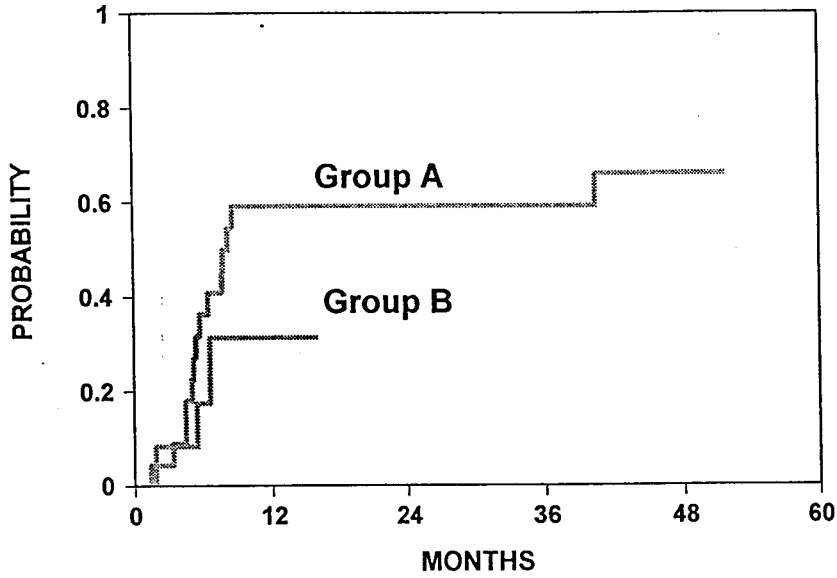

Figure 3. ABSCT in AML patients in first remission. Comparison of the actuarial risk of relapse between the two study groups.

DISCUSSION

Our study shows that standard AML remission induction and consolidation chemotherapy mobilizes adequate number of PBSC that ensures, in the majority of the patients, a rapid, complete and sustained hematopoietic recovery after transplant. Three graft failures were observed and in two, could be attributed to a low number of stem cells. However, the remaining graft failure occurred in a patient infused with a much greater number of CFU-GM and CD34+ cells than the median of the series and, consequently, other possible mechanisms for graft failure such as cryopreservation injury or bone marrow microenvironment damage must be considered.

Hematopoietic recovery is faster after ABSCT than after ABMT. Median time to recover 0.5 PMN x 10^9/L has ranged from 10-13 days after ABSCT,[2-6] 22-39 days after unpurged ABMT,[10-12] and 28-29 days after purged ABMT.[2,13] Likewise, platelet recovery is also significantly enhanced. In our series, median time to reach a stable platelet count over $20x10^9$/L ranged between 21-26 days contrasting with an uniformly longer time in purged or non-purged ABMT series (46 and 71 days respectively).[2,10,12,13] This faster hematological reconstitution in ABSCT patients decreases the risks related to granulocytopenia and thrombocytopenia, and makes ABSCT a safer procedure than ABMT.

Hepatic VOD is the most common life-threatening complication of preparative regimens for bone marrow transplant, ranging from 1-2% to over 50%.[14] In our series, VOD was observed in six cases (16%) and was fatal in two of them. Renal, cardiac, pulmonary or other complications that could be unequivocally attributable to drug-related toxic effects were not observed in the posttransplant period. This low incidence of hematological and non-hematological complications, apart from VOD, after transplant was associated, as in other series, with a brief hospital stay, shorter than that observed after ABMT.[2,5]

Leukemic relapses remain as the most frequent cause of treatment failure in patients with AML. In our series, the first administering BU plus CY at a dose of 200 mg/kg as conditioning regimen before ABSCT, the overall actuarial DFS at 4 years was 37%. Other authors[2] reported a two-year DFS rate of 35% using CY and total body irradiation as preparative regimen. The EBMT group[4] reported an estimated DFS at three years of 39% for AML patients in first remission autografted with PBSC. Similar results have been reported by Szer et al[5] with an actuarial probability of survival and of DFS at 26 months of 34.9% and 38% respectively, in 36 transplanted patients with AML in first remission. Preliminary non-randomized studies between ABMT and ABSCT in the same cohort of patients have shown similar antileukemic efficacy and DFS.[2,4] These results remained when both groups were matched for age, FAB subtype and interval between remission and transplantation.[4] Recently, Reiffers et al[15] have reported the preliminary results of a prospective randomized study of the BGMT group between ABMT and ABSCT in AML patients in first remission, showing no differences in terms of DFS and relapse rate.

Two potential origins of AML relapse following ABSCT should be considered, AML in the host surviving the intensified pretransplant therapy or contaminating AML cells in the blood autograft. Thus, possible approaches to decrease the incidence of relapse might be the intensification of post-remission chemotherapy and/or the conditioning regimen,[16] the use of purging techniques[17-19] to reduce the risk of infusion of leukemic cells, and the modification of the posttransplantation immune response by using cyclosporine,[20] or cytokines.[21,22] To decrease the relapse rate observed in Group A patients, we developed a new therapeutic approach with the following modifications: the administration of a third chemotherapy course pretransplant (early intensification) in an attempt to reduce residual disease in the host, and performing PBSC collections only after consolidation trying to decrease possible leukemic contamination in the PBSC collections. Although very preliminary, the results of this new

protocol are encouraging with a DFS and a probability of relapse at 7 months of 69% and 31% respectively, not associated with a higher transplant-related toxicity.

In conclusion, this study clearly confirms the feasibility of ABSCT in AML and shows some of the advantages of this procedure over ABMT. The two modifications we made in Group B patients were PBSC collection only after consolidation and the use of early intensification before transplantation. The rate of engraftment following ABSCT was not affected by those facts and DFS tended to be longer in these patients. However, the recruitment of more patients and a longer follow-up are clearly needed to confirm these preliminary observations.

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PERIPHERAL BLOOD STEM CELL AUTOGRAFTS IN THE TREATMENT OF PEDIATRIC SOLID TUMORS

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For the Childhood Working Parties of the Japanese Cooperative Study Group of PBSCT (JCSG/PBSCT)

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INTRODUCTION

The popularity of autografts with peripheral blood stem cells (PBSCT) is based on the ease of collection[1,2], more rapid engraftment[3,4], and the theoretical possibility that peripheral blood stem cells (PBSC) contain fewer cancer cells than marrow. Mobilized PBSC offer the opportunity of more intensive treatment regimens for high-risk childhood cancer, with an improved safety margin for malignant disorders for which a sharp dose-response relationship exists with chemotherapy. However, despite the wide-spread use of PBSCT in the treatment of cancer in adult patients, only a few comprehensive studies have been performed to test its efficacy in the treatment of childhood cancer.[5-10] In this report, we will summarize our clinical and laboratory experience with PBSCT for the treatment of childhood solid tumors based on studies performed by the Childhood Working Parties of a Japanese national study group.

PATIENTS AND METHODS

Between May, 1988 and May, 1994, 50 children with various types of malignant solid tumors underwent PBSCT following cytoreductive chemotherapy at transplant centers participating in the Japanese Cooperative Study Group of PBSCT (Table 1). The characteristics of these patients are shown in Table 2. The median age of the patients at PBSCT was 6 years (range 1-18 years). Twenty-four patients had neuroblastoma (NB: 22 stage IV disease), 10 had rhabdomyosarcoma (RS: 6 group IV disease), 6 had brain tumors (BT) and 10 patients had other tumors. The median age of patients at PBSCT was 4 years (range 1-10 years) in NB and 12 years (range 3-18 years) in RS. Fifteen NB patients were in complete clinical remission (CR) when

they underwent PBSCT, 7 were in partial remission (PR), and 2 were in the refractory stage of the disease (PD). Six patients with RS (n=4), BT (n=1), or sarcoma of the bone (n=1) underwent PBSCT twice for very high-risk features. The risks of the treatment protocols were explained in detail and written consent was obtained from the children's guardians.

In all cases, PBSC were harvested by 2 to 9 aphereses during bone marrow recovery after chemotherapy with or without G-CSF, and subsequently cryopreserved.[11] The standard procedure has been reported elsewhere.[1,2]

Table 1. Participating Institutions and Investigators in the Childhood Working Parties of the Japanese Cooperative Study Group of PBSCT (JCSG/PBSCT)

INSTITUTION	INVESTIGATOR
Akita University	A. Watanabe
Fukushima Medical College	A. Kikuta
Iwate Medical College	M. Endo
Kanazawa University	S. Koizumi
Kurume University	H. Eguchi
Kyoto Medical College	T. Matsumura, A. Sawada
National Children Hospital	Y. Sekine
National Medical Center Hospital	T. Matsushita
National Okayama Hospital	T. Koyama
Sapporo Medical College	T. Kudo
Shikoku Cancer Center Hospital	T. Shimokawa
Shizuoka Children's Hospital	Y. Horikoshi
Tenri Yorozu Hospital	K. Shimizu
Tokushima University	Y. Takaue, Y, Kawano
Yamaguchi University	H. Kawasaki

Cytoreductive Regimen

In patients with NB or RS, the primary cytoreductive regimen was "high MEC", which consists of CBDCA (400mg/m^2) and VP16 (200mg/m^2) on days -7 through -4, and L-PAM (90mg/m^2) on days -3 and -2. Most other regimens were variants of"high MEC" and the use of total body irradiation (TBI) was limited to 4 patients. In patients with BT, ACNU or MCNU was used instead of L-PAM. PBSC were rapidly thawed at 37-38°C and promptly infused into the patients through a central

venous catheter without any additional post-thaw washing following these cytoreductive regimens.

Table 2. Characteristics of the 50 patients with solid tumors who underwent cytoreductive chemotherapy and PBSCT.

Diagnosis	No.	Age Median (range)	State at Transplant (no.)	
Total	50	6 (1-18)		
Neuroblastoma	24	4 (1-10)	1st CR	15
			1st PR	7
			PD	2
Rhabdomyosarcoma	10	12 (3-18)	1st CR	6
			1st PR	2
			6th CR	1
			PD	1
Brain Tumor	6	5,9,10,13,17,18	1st CR	4
			2nd CR	1
			3rd CR	1
Other Tumors	10	1,3,3,5,8,9, 16,17,18,18		

CR = complete remission
PR = partial remission
PD = progressive disease

Statistical Analysis

The numbers of PBSC harvested in each disease were compared by the Mann-Whitney U test. Short-term hematopoietic recovery curves were plotted using the Kaplan-Meier technique and compared using a log-rank test. The disease-free interval was measured from PBSCT to relapse, death from any cause, or date of last follow-up. The proportion of disease-free patients was estimated by Kaplan-Meier procedures, and comparisons among subsets of patients were determined by a log rank analysis.

RESULTS

Collection Data

At Kurume University, the mean numbers of CFU-GM were 33.0 X 10^4/kg in patients with leukemia/lymphoma (n=25) and 19.4 X 10^4/kg in those with solid tumors (n=50), with no significant difference (Fig. 1). The mean numbers of PBSC harvested were similar among all of the diseases; 18.7 X 10^4/kg in NB (n=20), 21.1 X 10^4/kg in RS (n=17), and 18.4 X 10^4/kg in BT (n=13). The minimum requirement of 10.0 X 10^4/kg of CFU-GM for safe PBSCT could be collected by a single apheresis in 72% of patients with liquid disease and 60% of those with a solid tumor, with no significant difference.

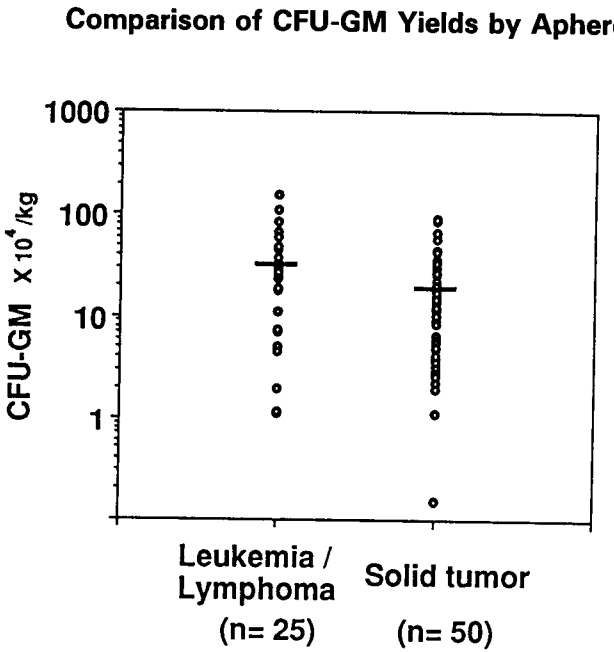

Figure 1. *PBSC collection at Kurume University. The mean numbers of CFU-GM (horizontal bars) were 33.0 X 10^4/kg in patients with leukemia/lymphoma (n=25) and 19.4 X 10^4/kg in those with solid tumors (n=50), with no significant difference. The minimum requirement of 10.0 X 10^4/kg of CFU-GM for safe PBSCT could be collected by a single course of apheresis in 72% of patients with liquid disease and in 60% of those with a solid tumor, with no significant difference.*

Toxicities and Complications

We previously reported that transient, but significant, toxicity associated with the infusion of PBSC autografts is rather common in children.[12] In our series, 6 patients died of complications of the cytoreductive regimens. One patient with NB, in whom the affected kidney had been resected in a delayed primary operation, died of cardiac and renal failure at day 7 of PBSCT. Pre-PBSCT renal function, as evaluated by creatinine clearance and glomerular filtration rate by renogram, was near normal. Another patient with recurrent intracranial germinoma, who had received whole brain irradiation with 40Gy during induction treatment, died of suspected intracranial hemorrhage at day 7. Another patient with medulloblastoma died of interstitial pneumonitis, probably due to ACNU or MCNU, at day 45 after the 2nd PBSCT. Three other patients died of sepsis (days 7 & 38) and shock of unknown cause (day 36). One patient with sarcoma of the bone suffered from renal dysfunction with a slow recovery.

Hematological Engraftment

Hematological recovery was evaluated in 53 courses of PBSCT. Engraftment failure was observed in three patients. Engraftment was successful in the 50 remaining courses and the median number of days to achieve a WBC count $>1.0 \times 10^9$/L and an AGC count $>0.5 \times 10^9$/L was, respectively, 11 days (7-34 days) and 11 days (7-34 days). No difference was observed in the speed of leukocyte recovery between patients with liquid and solid disease. The median time required for platelets to rise above 50×10^9/L was 34 days (10-168 days). In 6 patients who underwent two courses of PBSCT, hematopoietic recovery after the 2nd PBSCT was comparable to that after the 1st PBSCT. Based on the preliminary results of our study in children with lymphoid disease, no major difference appears to exist between immunological reconstitution in BMT and PBSCT.[13]

The current strategy for enhancing hematopoietic engraftment after BMT involves the use of recombinant cytokines. There is a possibility that the additional use of G-CSF may further enhance the already rapid rise of hematopoietic recovery after PBSCT. However, in our study we found that growth factor activities, such as those of G-CSF, can be identified in the serum of transplant recipients.[14] Thus, these endogenously secreted CSFs may provide sufficient stimulation of hematopoietic engraftment without the need for an exogenous supply of

cytokines.[15] Prospective randomized trials which might confirm this notion are pending.

Clinical Results

Only the data of the 24 patients with NB could be subjected to a meaningful analysis. Three of these patients died of complications of the procedure (cardiac and renal failure, sepsis, shock). Nine developed relapse 6 to 41 months after transplant: 5 subsequently died of the disease, and the remaining 4 are currently surviving 5 to 31 months after the relapse. One patient who was transplanted during the refractory stage of the disease died of disease progression without remission. Currently, 11 patients are surviving disease-free at 11 to 48 months after PBSCT. The overall survival (OS) rate and disease-free survival (DFS) rate are 55% and 32%, respectively. The prognosis of the patients who were transplanted during CR is superior to that of patients who were not transplanted during CR (OS rate, 71% vs 30%, $p<0.05$. DFS rate, 57% vs 0%, $p<0.05$) Figs. 2A, 2B).

Among the patients with RS, 4 underwent PBSCT twice. Three patients who underwent single PBSCT in their 1st CR, 6th CR and PD developed relapse and died of the disease. In the 4 patients who underwent PBSCT twice, two developed relapse after the 2nd PBSCT (6, 18 mo). The OS rate and DFS rate are 41% and 26%, respectively (Fig. 2C).

Among the patients with brain tumor, 2 died of regimen-related toxicities (days 7, 45). One patient with a 2nd recurrent medulloblastoma is surviving disease-free at 10 months after PBSCT without any additional radiation therapy.

DISCUSSION

The risks of life-threatening infections increase as cytopenia persists after ABMT. The rapid recovery of hematopoiesis observed after PBSCT may make this procedure suitable for even wider use in treating patients with solid tumors[3,4], even those in whom the possibility of cancer cell contamination into the infused graft is negligible. However, initial trials with PBSCT have been performed mainly in adult patients and there have been few reports in small children. We have been testing the clinical value of a PBSCT program for children with various types of cancer and

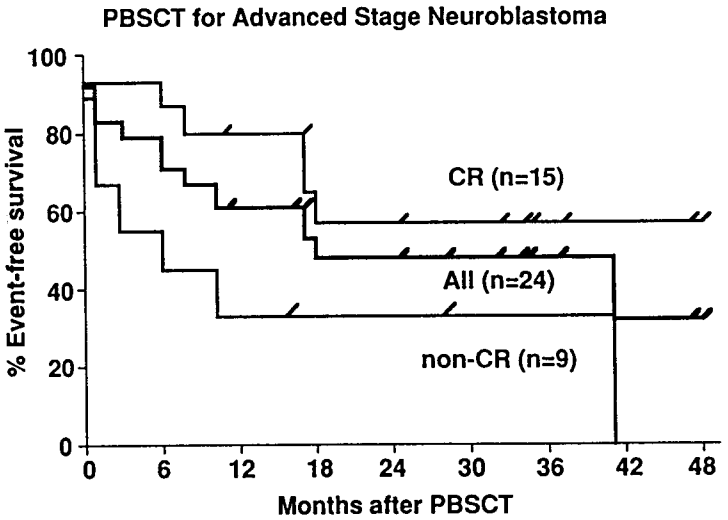

Figure 2A.

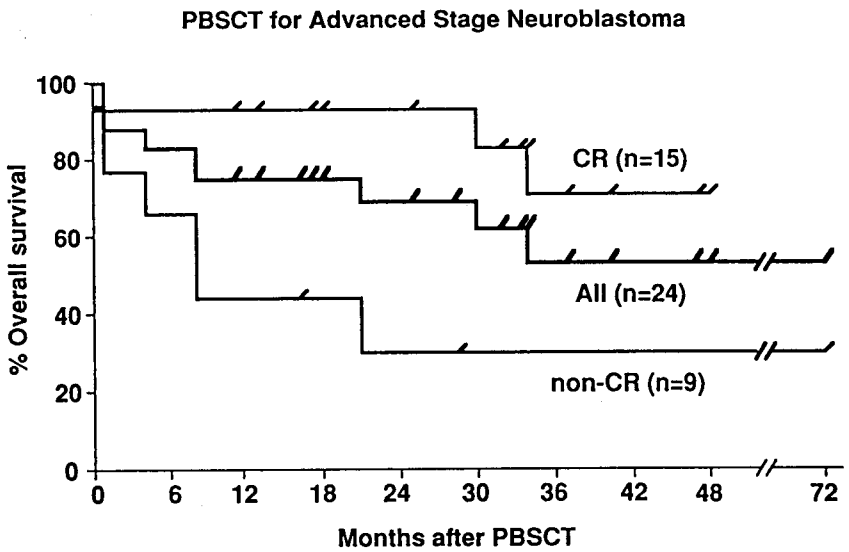

Figure 2B.

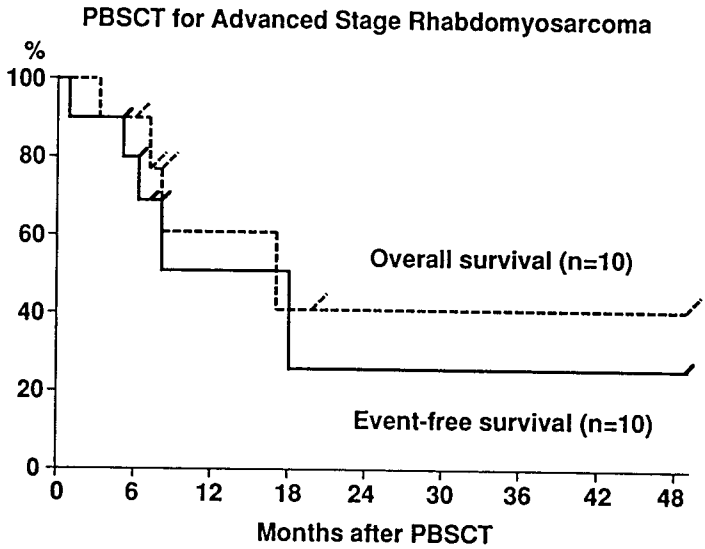

Figure 2. *Event-free survival and overall survival curves after PBSCT for 24 patients with advanced neuroblastoma (Figure 2A,2B) and 10 patients with rhabdomyosarcoma (Figure 2C). The prognosis of patients with neuroblastoma transplanted during CR was superior to that in patients who were not transplanted during CR.*

have suggested that at least some groups of children with acute lymphoblastic leukemia may benefit from high-dose therapy and PBSCT.[5]

Potential problems related to apheresis in small children include access to blood and tolerance of extracorporeal circulation. However, our experience suggests that, with certain adjustments, this procedure is simple and effective even in very small children with active malignancy.[1,2]

The issue of PBSC contamination with cancer cells remains controversial.[16-18] However, we believe that this may be important only when more effective anticancer cytoreductive regimens are developed. In any event, laboratory protocols we have developed can be used to evaluate possible contamination by surface marker and molecular biology techniques.

Our research has been hampered by the small number of patients with heterogeneous disease entities and a short follow-up. Nevertheless, our very preliminary clinical results in a limited number of patients may provide support for the incorporation of this program into the salvage protocol for children with high-risk tumors, as in a phase III group-wide

prospective protocol study. The next step in our research is to consider PBSCT for a larger number of patients. We are currently testing the possibility that a reduced total therapeutic period and a resultant decrease in the toxicity associated with prolonged use of toxic drugs may be achieved with an initial application of PBSCT. A randomized clinical trial is required to define the exact role of current therapeutic options in these patients and a cost/benefit analysis of the PBSCT procedure, as compared to conventional chemotherapy and auto-BMT, will be performed.

PBSC has mainly been tested in transplant settings, in which high-dose therapy was administered over a short period to time. However, it is possible that intensified repetitive chemotherapy can be administered for a prolonged period of time with the support of PBSC infusion. Serially engrafting patients with repetitive, closely timed courses of high-dose therapy might be particularly effective for treating patients with solid tumors.

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PEDIATRIC AUTOLOGOUS BMT IN ITALY: 10-YEAR EXPERIENCE

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ABSTRACT

Data from the Italian Association of Pediatric Hematology/Oncology (AIEOP-FONOP) BMT Registry were reviewed. The results refer to the period between 1984-1993. Five hundred and forty-six autologous BMTs (ABMTs) were registered from 13 Italian Pediatric BMT Centers. Most patients were transplanted for acute leukemias (163 ALL, 138 AnLL). Neuroblastoma (NB) patients were the most consistent group (n=123) among children who underwent ABMT for solid tumor. After a median follow-up time of 44 months, overall survival and DFS were 35.4% (Standard Error, SE 2.7) and 32.8% (2.2), respectively. The 6-year DFSs in the different diseases were as follows: ALL in 2nd CR, 27.9% (SE 3.7); AnLL in 1st CR, 42.5% (6.0), AnLL in 2nd CR, 40.1% (9.9); NB stage IV, 39.6% (7.6); non-Hodgkin's lymphomas, 44% (at 36 mos), Hodgkin's lymphoma, 50% (at 36 mos); Wilms' tumor, 38% (at 48 mos); Ewing's sarcoma, 22% (at 24 mos); soft tissue sarcomas, 16% (at 24 mos); CNS tumors, 14% (at 12 mos). Among the most interesting results, we report a 66.3% (9.2) DFS in patients autografted for early extramedullary relapse of ALL.

Starting from 1993, data on autologous peripheral blood stem cells collection and transplantation (APBSCC&T) have been collected (n=24 patients, as of December 1993).

Most patients were transplanted according to cooperative protocols, especially the conditioning regimens.

The AIEOP-BMT Registry provides an important and functional tool with which to collect and analyze data.

INTRODUCTION

Since 1984, a cooperative group for pediatric BMT has been active in Italy. The aims of this group (Italian Association of Pediatric Hematology/Oncology, AIEOP-FONOP BMT Group) are as follows: 1) to standardize the BMT procedure in the centers; 2) to develop prospective studies; and 3) to collect data on the BMTs performed. The AIEOP-FONOP BMT Registry was initiated in 1986. A total of 1166 BMTs had been registered as of December 31, 1993.

Data from 546 autologous BMTs were reviewed and are reported in this paper along with the results in terms of disease-free survival (DFS), relapse rate (RR), and no-relapse related mortality (no-RRM) as for acute lymphoblastic leukemia (ALL), acute nonlymphoblastic leukemia (AnLL), and neuroblastoma (NB) patients.

PATIENTS

From a total of 1166 marrow transplants reported in the AIEOP-FONOP BMT Registry, 546 (47%) were ABMTs.

The diagnoses for ABMT were as follows: ALL (n=163), AnLL (n=138), non-Hodgkin's lymphoma (NHL, n=32), Hodgkin's lymphoma (HD, n=16), secondary AnLL (n=5), chronic myeloid leukemia (n=1), NB (n=123), soft tissue sarcomas (STS, n=19), Wilms' tumor (WT, n=16), central nervous system tumors (CNS, n=13), Ewing's sarcoma (ES, n=12), peripheral neuroectodermic tumor (PNET, n=3), hepatoblastoma (n=1), Langerhans cell histiocytosis (LCH, n=4).

The number of ABMTs per year were as follows: 1984=7, 1985=36, 1986=75, 1987=53, 1988=62, 1989=69, 1990=68, 1991=60, 1992=67, 1993=49. There were 208 females and 338 males.

The median age at diagnosis was 5.6 years (range 1 mo - 18 yrs) and at transplant, 8 years (7 mos - 21 yrs). Regarding the most frequent diagnosis (ALL, AnLL and NB), the median age (and range) at diagnosis was 5 years (1 mo - 5 yrs), 7.5 years (1 mo - 17 yrs), and 3 years (1 mo - 10 yrs) respectively, and at transplant 9 years (2 mos - 21 yrs), 8.5 years (7 mos - 18 yrs), and 4 years (1 mo - 11 yrs), respectively. Regarding disease status, ALL patients underwent ABMT in first complete remission (CR-1) (n=14 high risk patients), in CR-2 (n=91), in >2CR (n=54) or in relapse (n=4).

AnLL patients were in CR-1 (n=83), in CR-2 (n=43), in >CR-2 (n=9) or with disease (n=3) when they were autografted. Fifty-five stage

IV-NB patients underwent ABMT while in CR-1 or in very good partial remission (VGPR), and 68 children had more advanced disease.

STATISTICAL METHODS

Patient data were collected from reporting forms compiled by a physician in charge at the BMT center. All information was stored, controlled and analyzed by VENUS, an integrated system of software facilities running on an IBM mainframe at the North-East Italian Interuniversity Computing Center (CINECA).

The overall survival (SUR), the DFS, the RR and the non-RRM were estimated by the Kaplan-Meier method, as of May 31, 1994.

The follow-up time and the time to a terminal event were calculated from the day of transplantation. We considered terminal events to be: death for SUR, death or relapse (whichever came first) for DFS, relapse for RR analysis and death not due to disease relapse for non-RRM.

RESULTS

ALL. The overall DFS at 6 years of ALL patients who underwent ABMT during the decade 1984-1993 was 27.9% (Standard Error, SE 3.7). Figure 1 shows the DFS for disease status (CR-1 vs CR-2 vs >CR-2 + on relapse patients). Considering the relapse site, the patients autografted in CR-2 were divided into 2 groups: 1) patients who underwent ABMT following a marrow +/- other(s) site(s) (n=63), and 2) patients who experienced an isolated extramedullary relapse before ABMT (n=28, 20 of whom in the CNS). Figure 2 shows the DFS of these 2 groups of patients.

The RR resulted 41.3% (14.2), 64.5% (5.5), and 80.5% (6.1) in patients transplanted in CR-1, CR-2 or >CR-2/with disease, respectively; the non-RRM was 14.3% (9.3), 9.0% (3.0) and 15.5% (5.1) in the same subgroups of patients, respectively.

AnLL. The overall DFS at 6 years was 40.2% (4.8). As shown in Figure 3, there was no difference between patients autografted in CR-1 or CR-2.

The RR was 50.7% (5.8) in patients transplanted in CR-1 and 51.3% (11.3) in patients who underwent ABMT in CR-2. Their respective non-RRM were 13.7% (6.6) and 16.7% (6.5).

NB. The overall DFS at 6 years was 28.4% (4.6). Figure 4 shows the DFS of children who were autografted in CR-1/VGPR and of all the

remaining children transplanted in the more advanced disease status (first or second partial remission, CR-2).

In those two subgroups of NB children, the RR resulted 56.3% (8.2) and 78.2% (5.5), and the non-RRM was 9.3% (4.0) and 9.4% (4.4) respectively.

Other diseases. The DFS of patients autografted for other diseases was as follows: non-Hodgkin's lymphoma, 44% (at 36 mos), Hodgkin's lymphoma, 50% (at 36 mos); Wilms' tumor, 38% (at 48 mos); Ewing's sarcoma, 22% (at 24 mos); soft tissue sarcomas, 16% (at 24 mos); CNS tumors, 14% (at 12 mos).

DISCUSSION

The data on pediatric BMTs collected in the AIEOP-FONOP BMT Registry constitute an important and large amount of material for study. Such a database allows retrospective studies and surveys able to give an overview of the present situation, the knowledge of which is essential for planning prospective trials. The extensive data can provide a valid rationale for clinical strategies. For example, in ALL patients in CR following an early isolated extramedullary relapse ABMT could be considered the treatment of choice.

Moreover, based on such data analyses, it is possible to improve medical, technological, organizational policies and give information for politicians involved in health care decisions.

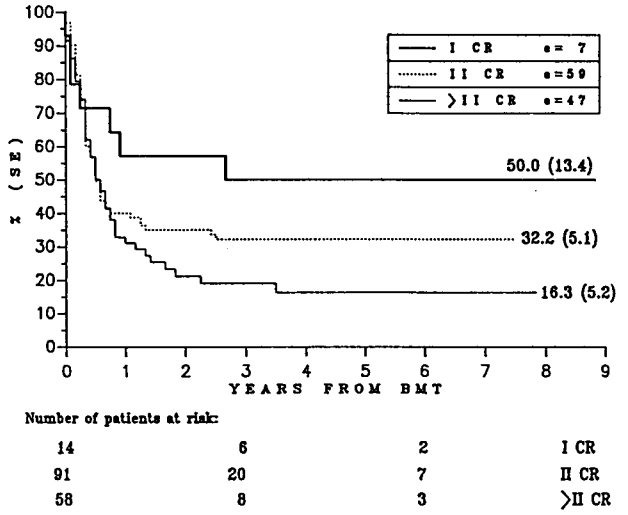

Figure 1.. *DFS of ALL patients according to disease status (e= no. of events).*

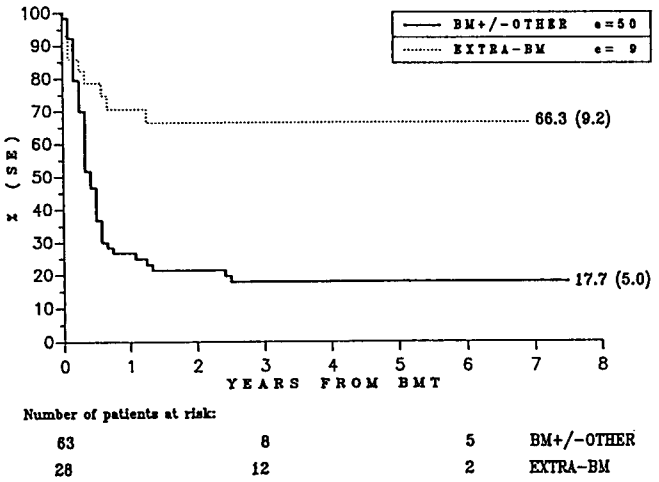

Figure 2. *DFS of ALL patients according to site of first relapse (bone marrow +/- other site vs isolated extramedullary).*

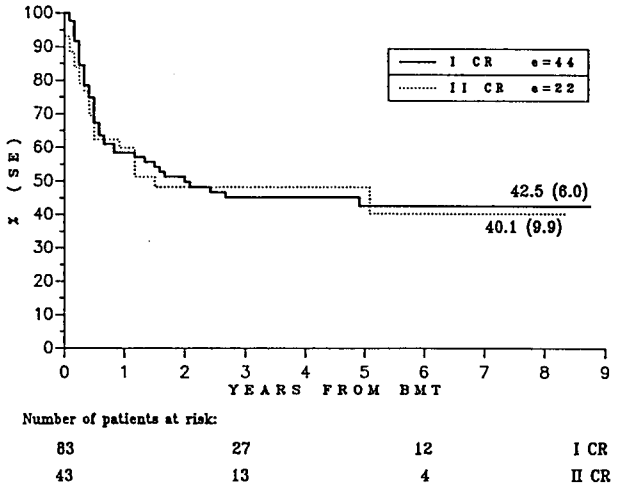

Figure 3. *DFS of AnLL patients according to disease status.*

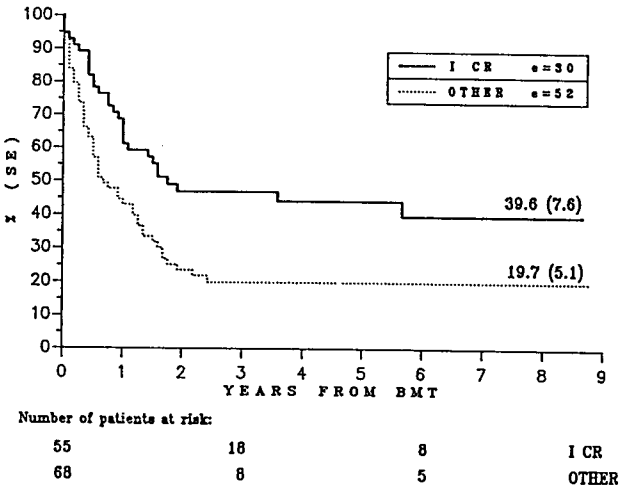

Figure 4. *DFS of NB patients according to disease status.*

PERIPHERAL BLOOD PROGENITOR CELL (PBPC) SUPPORT IN LOW- AND INTERMEDIATE-GRADE NON-HODGKIN'S LYMPHOMA (NHL)

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ABSTRACT

Since July 1992, 42 patients with NHL (35 centrocytic-centroblastic [follicular]), 4 centrocytic, and 3 lymphocytic NHL, according to the Kiel classification were enrolled onto a dose-escalated treatment protocol. There were 21 males and 21 females with a median age of 42 years (range 26-55). At the time of entry, 27 patients had achieved complete or partial remission following first-line therapy, 6 patients were in second or higher remission, and 9 patients had active disease (tumor progression or relapse). Patients received high-dose Ara-C and mitoxantrone, supported by recombinant human G-CSF (filgrastim, 300 μg/day, sc.). The therapy combined an effective antineoplastic treatment with a PBPC mobilization modality. During leukocyte recovery, PBPC were collected by a median of two leukaphereses (range 1-7). In 31 patients with measurable disease, therapy with HAM resulted in a response rate of 74%. The pretransplant conditioning therapy consisted of total body irradiation ([TBI] 14.4 Gy, 4 days) and cyclophosphamide (200 mg/kg). Two patients who were not eligible for TBI received the BEAM-protocol. Rapid hematological reconstitution was achieved in all patients in less than 19 days (ANC $\geq 0.5 \times 10^9$ and an unsupported platelet count $\geq 20.0 \times 10^9$/L). One patient died of cardiac failure 19 days posttransplantation, and one patient died due to vasculitis of unknown etiology 188 days posttransplantation. Four patients with follicular NHL relapsed between 5 and 13 months post-grafting. Three were autografted in second or higher remission with a median duration of disease of 47 months (range 9-62). It is of note that 3 patients were autografted with harvests containing PCR-detectable t(14;18) positive cells (1 patient not known). On the other hand, 9 patients who were transplanted with PCR-positive leukapheresis products converted to PCR-negativity in samples from bone marrow and peripheral blood between 3 and 13 months

posttransplantation. Sequential high-dose therapy in patients with low- and intermediate-grade lymphoma is safe, since PBPC autografting results in a short duration of aplasia. The role of purging or positive selection of CD34+ cells as well as the significance of persisting PCR-detectable cells remain open.

INTRODUCTION

Earlier reports have shown the efficacy of autologous bone marrow transplantation (ABMT) in the treatment of patients with low- and intermediate-grade non-Hodgkin's lymphoma (NHL).[1-3] Since the majority of patients with advanced-stage disease have bone marrow involvement, purging techniques have been proposed to reduce the amount of contaminating tumor cells.[4] This included the application of monoclonal antibodies directed against various B-cell epitopes plus complement or immunomagnetic beads. Peripheral blood provides an alternative source of hematopoietic stem cells for the support of dose-escalated cytotoxic therapy.[5] Previously, we have shown that peripheral blood progenitor cells (PBPC) mobilized by cytotoxic chemotherapy including recombinant human granulocyte colony-stimulating factor (G-CSF) contain an extremely small proportion of early CD34+/CD19+ lymphoid progenitor cells and a relatively low percentage of CD19+ B cells.[6,7] We therefore decided to use blood-derived hematopoietic progenitor cells to hematologically rescue patients with low- and intermediate-grade NHL following high-dose therapy with total body irradiation (TBI). In this report we summarize our experience with the first 42 patients autografted.

METHODS AND MATERIALS

Patients

Forty-two patients with low- and intermediate-grade NHL were enrolled onto this study (Table 1). According to the Kiel-classification, thirty-five patients had centrocytic-centroblastic NHL, four patients had centrocytic NHL and three patients had lymphocytic NHL. There were twenty-one males and twenty-one females with a median age of 42 years (range 26-55). The disease status at the time of entry into the study was as follows: twenty-seven patients had achieved complete (CR) or partial remission (PR) following first-line therapy. Six patients were in second or higher remission, while nine patients had active disease (tumor progression

or relapse). Independent of the disease status, patients received high-dose cytarabine (2 g/M^2 every 12 hours on days 1 and 2) and mitoxantrone (10 mg/M^2/d on days 2 and 3) followed by recombinant human G-CSF (filgrastim 300 μg/day, sc.). The cytokine-supported chemotherapy (HAM) served as consolidation or salvage therapy and was found to be an efficient PBPC mobilization modality.

Table 1. Patient Characteristics

	Follicular NHL	Centrocytic NHL	Lymphocytic NHL	Total
Number of patients	35	4	3	42
Age (years)				
median	41	48	43	42
range	26-55	46-51	34-48	26-55
Males/females	18/17	1/3	2/1	21/21
BM involvement prior to PBPC mobilization	11	3	2	16
Number of cycles				
median	6	6	6	6
range	0-21	4-9	3-7	0-21
Radiotherapy prior to PBPC mobilization	4	0	0	4
Disease status prior to mobilization				
1st remission	21	4	2	27
2nd and higher remission	6	0	0	6
active disease	8	0	1	9
Disease status at the time of autografting				
CR	25	2	2	29
PR	10	2	1	13

The blood-derived progenitor cells were harvested during the rebound phase of marrow recovery as soon as a distinct population of CD34+ cells could be identified by direct immunofluorescence analysis.

The study was performed under the ethical guidelines of the Joint Committee of Clinical Investigation of the University of Heidelberg. Informed consent was obtained from each patient. The cut-off date of this report is June 30, 1994.

Collection of PBPC and Cryopreservation. Harvesting was performed with a Fenwal CS 3000 (Baxter Deutschland, München, Germany). For each leukapheresis, 10 1iters of blood were processed at a flow rate of 50-70 ml/min. The aphresis product of 50 ml cell suspension was mixed with the same volume of minimal essential medium (MEM),

containing 20% dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany). The final 100 ml cell suspension was transferred into freezing bags (Delmed, New Brunswick, NJ) and frozen to -100°C with a computer-controlled cryopreservation device (Cryoson BV-6; Cryoson Deutschland, Germany). The frozen cells were transferred into the liquid phase of nitrogen and stored at -196°C.

Hematopoietic Progenitor Cell Assessment. The blood-derived hematopoietic progenitor cells were determined using a semi-solid culture assay and direct immunofluorescence analysis. Both methods have been described in detail elsewhere.[6]

Pretransplant Conditioning Regimen and Intensive Care Posttransplant. Pretransplant conditioning therapy consisted of TBI (14.4 Gy hyperfractionated over 4 days) and cyclophosphamide (200 mg/kg over 4 days) in 40 patients, while two patients received a protocol consisting of carmustine 300 mg/M^2, etoposide 1,200 mg/M^2, ara-C 800 mg/M^2, and melphalan 140 mg/M^2 (BEAM). All patients received prophylactic partial gut decontamination. Antibiotic combination therapy was administered for fever of more than 38.5°C, and amphotericin B was added for documented fungal infection or persistent fever. A platelet count of more than 20 x 10^9/L was maintained by human leukocyte antigen (HLA)-A-B-matched platelet transfusions and packed red blood cells were administered when the hemoglobin level was less than 8 g/dL.

RESULTS

Cytotoxic Chemotherapy with High-Dose Cytarabine and Mitoxantrone. The patients with non-Hodgkin's lymphoma presented here differ with respect to histological subtype, previous cytotoxic therapy and disease status at the time of entry into the study. Common denominator is the diagnosis of a B-cell malignancy which is only rarely cured by conventional chemotherapy. Our treatment protocol is aimed at a stepwise increase of dose-intensity including TBI. Filgrastim served as adjuvant of the conventional chemotherapy, while peripheral blood progenitor cells were used to support the dose-escalated regimen.

The first aspect relates to the efficacy of HAM. Thirty-one patients had measurable disease including 16 patients with histopathological bone marrow involvement. They received a total of 61 cycles of HAM which were associated with non-hematological toxicity not exceeding Grade 2 (WHO-classification). The response rate was 74%

reflecting the therapeutic efficacy of HAM as second-line regimen for B-lymphoid malignancies.

In all patients, PBPC were collected during filgrastim-enhanced leucocyte recovery. A median number of two leukaphereses (range 1-7) were necessary to obtain a median number of 6.0 x 10^6CD34+ cells per kg body weight (range 2.9 x 10^6 - 24.9 x 10^6), while the corresponding number of CFU-GM/kg harvested was 25.7 x 10^4(median, range 3.8 x 10^4 - 111.1 x 10^4). The harvests of patients in first remission was comparable to those of patients with history of relapse or progressive disease. Patients with more than six cycles of previous chemotherapy tended to have lower numbers of CD34+ cells per leukapheresis.

High-Dose Therapy with PBPC Support. The pretransplant conditioning regimen consisted of high-dose cyclophosphamide and total body irradiation. Two patients with previous radiotherapy were not eligible for TBI and received the BEAM protocol. The PBPC were reinfused one day after the last dose of cyclophosphamide had been administered. No additional bone marrow or growth factor support was given. All patients achieved complete engraftment in less than 19 days. Correspondingly, the median stay in hospital - following PBPC - was 19 days. The cumulative frequency of hematological reconstitution is shown in Figure 1.

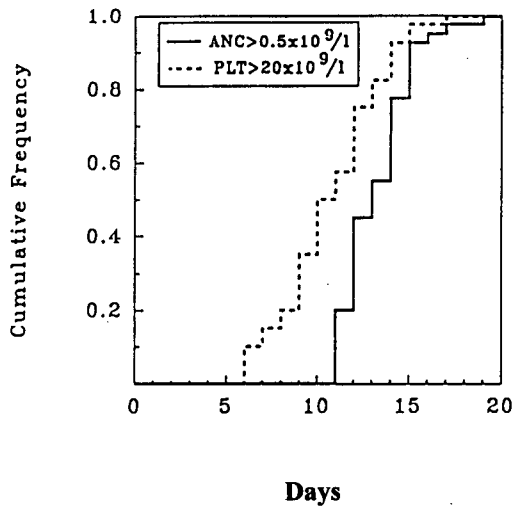

Figure 1. *Cumulative frequency of hematological reconstitution. (----) days to reach unsubstituted platelet count ≥20 x 10^9/L. (___) days to reach absolute neutrophil count ≥0.5 x 10^9/L*

One patient died of cardiac failure 13 days posttransplantation. There was also one late treatment-related death due to vasculitis of unknown etiology with renal and musculocutaneous involvement. Despite immunosuppressive treatment in addition to dialysis and plasmapheresis, the patient died of multiorgan failure 188 days posttransplantation.

There were four patients with centrocytic-centroblastic NHL who relapsed between 5 and 13 months post-grafting. Three of these patients were included into the transplant protocol after they had achieved second or third remission. It is of note that recurrence of disease involved the bone marrow in three of the patients, whereas only one patient relapsed at nodular sites that were previously affected. With a median follow-up time of 12 months (range 1-24), 36 patients are alive and in remission. This translates into a probability of event-free survival of 78% at 2 years (Figure 2).

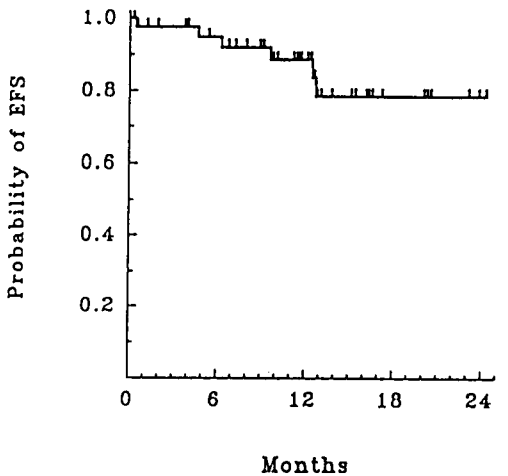

Figure 2. *Probability of event-free survival in months posttransplantation*

DISCUSSION

In this report we present the data of 42 patients with low- and intermediate-grade NHL who underwent sequential high-dose therapy including a TBI-containing conditioning regimen. Rapid hematological recovery was observed in all patients, since the number of CD34+ cells

reinfused exceeded a threshold quantity of 2.5 x 10^6/kg. As a result of the fast recovery, the risk for bleeding or infectious complications is low. Of note, the two deaths occurred due to toxic-allergic organ failure. Dose limitations are now imposed by organ impairment rather than hematological toxicity. The treatment-related mortality of 5% is comparable to autologous bone marrow transplantation.[1-3] Considering that the therapeutic benefit of dose-escalated therapy has not yet been proven, the treatment-related risk remains an important factor in the decision-making process.

Four patients relapsed between 5 and 13 months posttransplantation. Three of them had advanced-stage disease and were autografted in second or higher remission. The prognosis of these patients being treated with conventional chemotherapy would have been bad as well. A recent report indicates a median survival of 2.4 years in patients with a remission duration less than one year following first-line therapy.[8] On the other hand, the treatment failures following PBPC autografting clearly demonstrate either the inability of high-dose regimens to eradicate malignant cells or that reinfused tumor cells have caused or contributed to recurrence of disease. To minimize the amount of tumor cells transplanted, one may consider purging methods or the positive selection of CD34+ cells.[4,9] Both approaches are feasible in patients with autografts containing sufficient numbers of CD34+ cells. This is an important prerequisite, keeping in mind that every *ex vivo* procedure is associated with a loss of hematopoietic progenitor cells. Detection of residual disease could be the rationale for applying *ex-vivo* methods. As PCR analysis revealed, the autografts of three relapsed patients contained t(14;18)-positive cells, while for one patient no PCR data are available.

Gribben et al[10] could show that patients autografted with bone marrow containing PCR-positive cells and PCR-positive samples post-grafting had a remarkably high relapse rate. On the other hand, presence of PCR-positive cells in a bone marrow or blood-derived autograft might merely reflect a critical size of the *in vivo* tumor burden indicating a higher risk of tumor cells escaping the cytotoxic effects of high-dose therapy. As we have shown more recently, there were also 6 patients with centrocytic-centroblastic NHL who converted to PCR-negativity following autografting with PCR-positive leukapheresis products.[11] The significance of a positive PCR-finding in the setting of PBPC-supported high-dose therapy remains therefore open. The impact of purging procedures or CD34+ cell selection is worthwhile to be addressed prospectively. For

patients in second or higher remission being at an increased risk of relapse one may also consider a maintenance therapy with alpha-interferon.[12]

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MOBILIZATION OF PERIPHERAL BLOOD PROGENITOR CELLS (PBPC) WITH RECOMBINANT HUMAN G-CSF (FILGRASTIM) DURING STEADY-STATE HEMATOPOIESIS AND POST-CHEMOTHERAPY

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SUMMARY

Recombinant human G-CSF (filgrastim, R-metHUG-CSF, 300 μg/day) can be administered either during steady-state hematopoiesis or post-chemotherapy to mobilize peripheral blood progenitor cells (PBPC). Using two-color immunofluorescence, we compared the effect of both mobilization modalities on quantity and phenotype of blood-derived CD34+ cells. Leukapheresis products (Lps) of patients with hematological malignancies (acute leukemia [AL], Hodgkin's disease [HD], non-Hodgkin's lymphoma [NHL], multiple myeloma [MM]) were analyzed. Nineteen patients (2 AL, 2 HD, 8 NHL, 7 MM) received filgrastim during steady-state hematopoiesis (82 Lps), and 128 patients (17 HD, 87 NHL, 24 MM) following cytotoxic chemotherapy (477 Lps). The chemotherapeutic regimens used for PBPC mobilization were Dexa-BEAM, cytarabine/mitoxantrone and high-dose cyclophosphamide. The mean number of CD34+ cells collected post-chemotherapy was 5.7-fold greater in comparison with mobilizing during steady-state (2.27 vs 0.40 x 10^6/kg bw). Post-chemotherapy, the mean proportion of early CD34+ progenitor cells was substantially lower (1.29% vs 3.24% CD34+/HLA-DR- and 0.74% vs 6.25% CD34+/CD38-). However, due to the greater number of mobilized CD34+ cells, the absolute yield of CD34+/HLA-DR- cells (per kg bw) was 2.3-fold increased post-chemotherapy. The proportion of lineage-committed CD34+/CD33+ cells was significantly increased post-chemotherapy compared with steady-state (83.7% vs 64.6%). LP collected during steady-stage administration contained between 1% and 10% CD19+ cells, whereas less than 1% CD19+ cells were found in the majority of Lps post-chemotherapy. Of note, in 20% of these Lps virtually no B-cells were detected. Treatment with G-CSF following cytotoxic chemotherapy results in a higher yield of CD34+ progenitor cells compared with steady-state administration, compensating for the smaller proportion of early hematopoietic progenitor cells. The content of B-cells

that may contain clonogenic lymphoma cells is substantially lower in Lps collected post-chemotherapy.

INTRODUCTION

By administration of cytokines and hematopoietic growth factors like granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3), hematopoietic progenitor cells are mobilized into the peripheral blood and can be collected to support high-dose cytotoxic therapy in patients with hematological malignancies.[1-3] During recovery from chemotherapy, the number of circulating hematopoietic progenitor cells is also increased.[4] Successful autografting of PBPC collected after chemotherapy without administration of cytokines has been described previously.[5] Mobilization of PBPC with growth factors post-chemotherapy is therefore likely to be more effective compared with treatment during steady-state hematopoiesis. Since hematopoietic reconstitution following PBPC autografting not only depends on the number of progenitor cells in the autograft, but also on their degree of differentiation and lineage-commitment, differences in the composition of the PBPC are of particular interest.[6]

Using monoclonal antibodies to the CD34 antigen, the yield of hematopoietic progenitor cells in the leukapheresis product can reliably be assessed by immunofluorescence analysis. It could be demonstrated in previous studies that the number of CD34+ cells transplanted is inversely correlated to the duration of aplasia following high-dose conditioning therapy and PBPC support.[7] Lineage-committed as well as early, pluripotent hematopoietic progenitors are positive for CD34. Analysis of the co-expression of the differentiation-associated antigens CD38, HLA-DR and CD33 provides a means of distinguishing more primitive from committed progenitor cells, since more primitive CD34+ hematopoietic progenitor cells lack expression of these antigens.[8]

We analyzed number and phenotype of blood-derived CD34+ PBPC of patients with hematological malignancies mobilized with filgrastim (R-metHuG-CSF) either during steady-state hematopoiesis or post-chemotherapy. We also assessed the co-expression of B-lymphoid antigens (CD19, CD10) and of the transferrin receptor (CD71). CD71 is expressed on erythroid and proliferating progenitors of all lineages. Expression of the transferrin receptor is of special interest, since a virus-free system based on transferrin receptor mediated gene transfer may be used for introducing DNA into hematopoietic progenitor cells.[9] The

majority of the patients of this study suffered from B-cell lymphoma. In this entity, circulating clonogenic lymphoma cells are presumably contained in the CD19+ cell fraction. We therefore analyzed the content of B-cells (CD19+) in the leukapheresis products.

PATIENTS AND METHODS

Patients

Eighty-two Lps of 19 patients were collected during the administration of filgrastim starting in steady-state hematopoiesis, and 477 Lps of 128 patients during the administration following cytotoxic chemotherapy. The patient characteristics are shown in Table 1.

Table 1. Patient Characteristics

	Steady-state	Post-chemotherapy
Patients	19	128
Age (median, range)	31 (16-49)	43.5 (18-60)
Male/female	11/8	71/55
Number of Lps	82	477
Diagnosis:		
- Acute leukemia	2	-
- Hodgkin's disease	2	17
- Non-Hodgkin's lymphoma	8	87
- Multiple myeloma	7	24

Chemotherapeutic regimens (Table 2) were chosen according to diagnosis and course of the disease. Most of the patients with non-Hodgkin's lymphoma received HAM, with Hodgkin's disease DexaBEAM, and with multiple myeloma high-dose cyclophosphamide. Filgrastim (R-metHuG-CSF, Neupogen®, Amgen, Thousand Oaks, CA USA) was given during steady-state hematopoiesis and continued at the same dose (300 µg/kg sc. per day) until PBPC harvesting was completed. Daily leukaphereses (except for weekends) began at least 3 days after starting the cytokine treatment, using a Fenwal CS 3000 blood cell separator (Baxter Deutschland, München, Germany). The median white blood count at the day of leukapheresis was 20.9 x 10^9/L. A median of 4 leukaphereses per patient were performed (range 1-7). In patients receiving previous chemotherapy, filgrastim (300 µg sc. per day) was started one day after the end of chemotherapy. PBPC were collected when

a WBC of ≥5.0 x 10^9/L was reached. Cytokine treatment was continued until the PBPC collections were completed. A median of 3 (range 1-10) leukaphereses were performed. The median WBC at the day of leukapheresis was 16.6 x 10^9/L. For the assessment of circulating CD34+ cells, 59 samples of peripheral blood were obtained during steady-state mobilization and 350 following filgrastim-supported chemotherapy. The study took place under the guidelines of the ethical committee of the University of Heidelberg. Informed consent was obtained from each patient.

Table 2. Chemotherapeutic Regimens

High-dose cyclophosphamide (2, 4 or 7 g/M^2)	24 patients
High-dose ara-C/mitoxantrone	81 patients
Dexa-BEAM (Dexamethasone, BCNU, etoposide, Ara-C, melphalan)	15 patients
CHO(E)P or COP-BLAM	6 patients
IEV (Ifosfamide, epirubicin, etoposide)	2 patients

Immunofluorescence Staining and Flow Cytometry

For dual-color immunofluorescence analysis, 1 x 10^6 mononuclear cells (MNC) or 20-50 ul of whole blood were incubated for 30 min at 4°C with the phycoerythrin (PE)- or fluorescein (FITC)-conjugated monoclonal antibody (moAb) anti-CD34 (HPCA-2, Becton-Dickinson, Heidelberg, Germany) and one of the following conjugated moAbs: anti-CD45 FITC, anti-CD38 PE, anti-HLA-Dr PE, anti-CD19, anti CD10, anti-CD71 (HLe-1, Leu17, Clone L243, Leu12, CALLA, and TfR, Becton-Dickinson, Heidelberg, Germany), anti-CD33 PE (MY9, Coulter, Krefeld, Germany). Isotype-identical antibodies served as controls: IgG1, IgG2a (FITC/PE-conjugated, Becton-Dickinson, Heidelberg, Germany). The cells were analyzed with a Becton-Dickinson FACScan. We used a forward scatter characteristics (FSC) versus CD45 fluorescence dot plot to discriminate between a small lymphohematopoietic cell population and erythrocytes or debris. The CD34+ cell population was analyzed in a fluorescence (FL)

versus side scatter characteristics (SSC) dot plot. Cells with lymphoid or lymphoblastoid characteristics (low SSC) were counted as CD34+ cells. Following the subtraction of false positive events in the negative control, the percentage of CD34+ cells was calculated as the ratio of CD34+ cells to CD45+ cells and multiplied by 100. Subset analysis of CD34+ cells was performed on cells acquired in a CD34-fluorescence/SSC gate. Cells with a fluorescence intensity >99.5% of that in the isotype-specific control were counted as positive. To evaluate CD34+ cells/ul of peripheral blood, the percentage of CD34+ cells was multiplied by the WBC.

Statistical Analysis

The results are given as mean ± standard error of the mean (SEM). Statistical significance was tested using Student's t-test for independent samples; n.s. = statistically not significant.

RESULTS

Number of circulating CD34+ cells in peripheral blood and PBPC yield. During administration of filgrastim in steady-state hematopoiesis, the number of CD34+ cells on the days of leukapheresis was between 0 and 31.5 /ul (median 4.0 /ul). Peak levels of CD34+ cells during cytokine-enhanced recovery post-chemotherapy were found approximately two weeks following the start of chemotherapy. The number of circulating CD34+ cells on the days of leukapheresis varied between 0 and 810.9 /ul (median 21.5/ul). After chemotherapy, the average number of circulating CD34+ cells was 11.0-fold higher in comparison with filgrastim treatment during steady-state. Due to the greater number of circulating progenitor cells after filgrastim-supported chemotherapy, a 5.0-fold greater proportion of CD34+ cells was found in the leukapheresis products in comparison with steady-state mobilization ($1.39 \pm 0.09\%$ vs $0.28 \pm 0.04\%$, $p<0.001$). The cell number collected per leukapheresis during steady-state hematopoiesis was not significantly different from post-chemotherapy (12.2 ± 0.9 vs $10.9 \pm 0.3 \times 10^9$ mononuclear cells/LP, n.s.). Therefore, the yield of CD34+ cells per leukapheresis post-chemotherapy was also 5.7-fold higher. CD34+ progenitor cells collected per leukapheresis are shown in Figure 1. Interestingly, the number of circulating CD34+ cells analyzed on the days of leukapheresis was predictive of the yield in the corresponding leukapheresis product. An identical regression curve was found for the

mobilization with filgrastim during steady-fast hematopoiesis and post-chemotherapy (Figure 2).

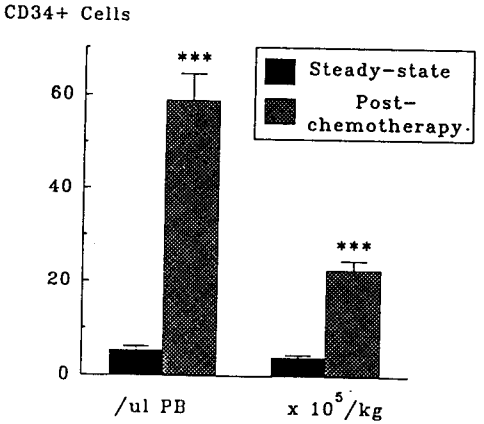

Figure 1. *Number of filgrastim-mobilized CD34+ cells circulating in the peripheral blood and CD34+ cell yield in the corresponding leukapheresis products. Post-chemotherapy, a significantly higher CD34+ cell number and yield was obtained.*** $p<0.001$*

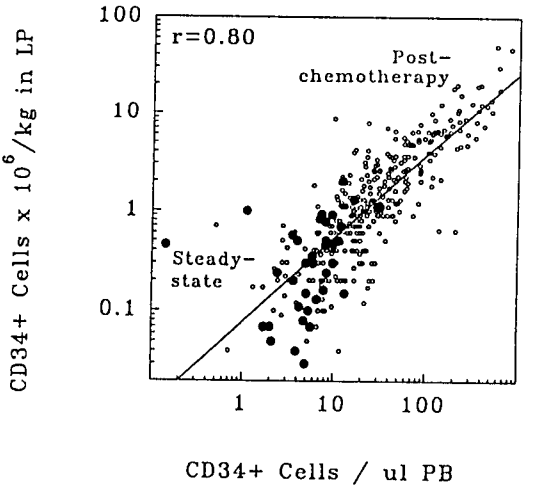

Figure 2. *Correlation between the number of CD34+ PBPC in the peripheral blood and the yield in the leukapheresis products. An identical regression curve was found for filgrastim-mobilized PBPC with (open symbols) and without preceding chemotherapy (filled symbols).*

Antigenic profile of the CD34+ cells. Co-expression analyses are available for 272 (HLA-DR), 179 (CD38), 469 (CD33), 162 (CD71), 458

(CD19), and 89 (CD10) leukapheresis products. CD34+ cells collected post-chemotherapy had a different antigenic profile compared with CD34+ cells mobilized with filgrastim during steady-state (Figure 3). We focused on co-expression analysis of antigens indicating lineage-commitment as described for HLA-DR, CD38, and CD33. The subpopulation of CD34+/HLA-DR- cells was significantly smaller post-chemotherapy. However, absolute numbers of CD34+/HLA-DR- cells per kg bodyweight were 2.3-fold higher compared with administration of filgrastim in steady-state (2.20 ± 0.27 vs $0.94 \pm 0.27 \times 10^4$/kg, n.s.). Co-expression of CD38 was comparable with the results obtained for HLA-DR. The proportion of CD34+/CD33+ cells was significantly greater post-chemotherapy, whereas no substantial difference was found for co-expression of the transferrin receptor CD71. The relative number of B-lymphoid progenitor cells (CD34+/CD19+ and CD34+/CD10+) tended to be lower post-chemotherapy. For CD34+/CD10+ progenitors, the difference was statistically significant.

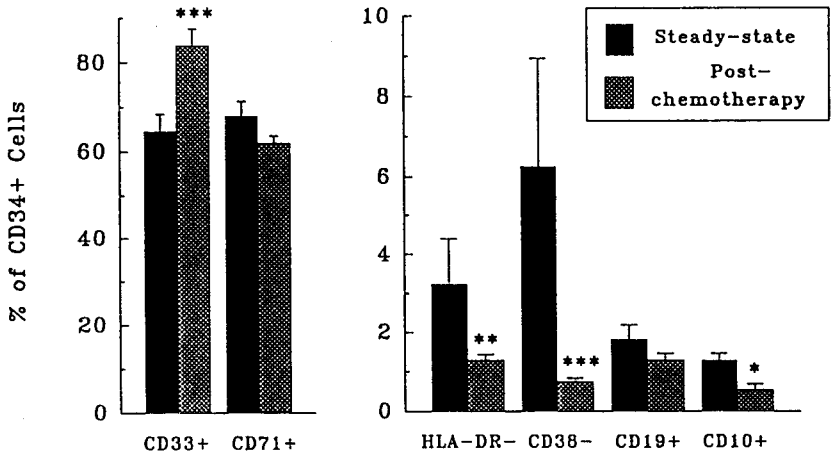

Figure 3. *Co-expression analysis of CD34+ cells in the leukapheresis product. *p<0.05, **p<0.01, **p<0.001*

B-cell content in PBPC autographs. The majority of the leukapheresis products collected during administration of filgrastim without preceding cytotoxic therapy contained between 1 and 10% CD19+ cells (Figure 4). There were no leukapheresis products with less than 0.3% CD19+ cells. The proportion of CD19+ cells in the leukapheresis products harvested post-chemotherapy was substantially lower, since in

most of the Lps less than 1% B-cells were found. Of note, 20% of the Lps collected after chemotherapy contained virtually no CD19+ cells.

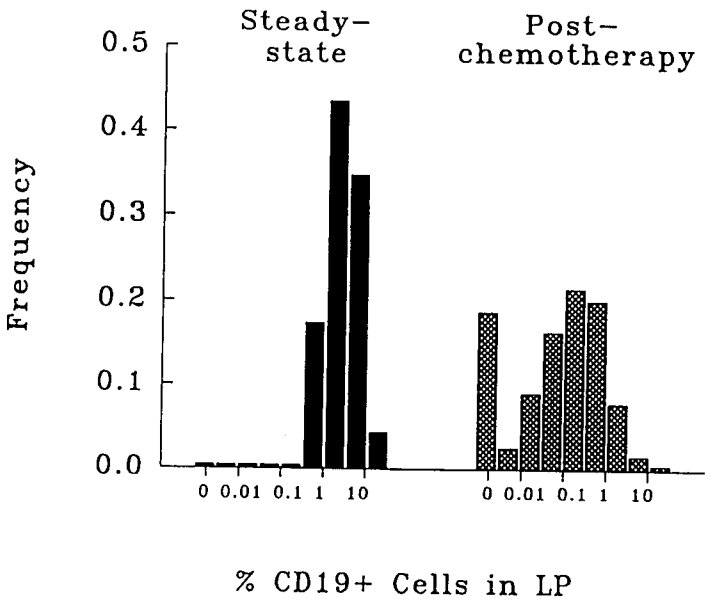

Figure 4. *Relative frequency of the proportion of B-cell in the leukapheresis products. Most of the Lps collected during filgrastim-treatment without preceding chemotherapy contained between 1% and 10% B-cells. Post-chemotherapy, the proportion of B-cells in most of the Lps was less than 1%. Nearly 20% of these Lps contained no B-cells as measured by immunofluorescence.*

DISCUSSION

This report is based on the analysis of 559 leukapheresis products and clearly demonstrates that filgrastim mobilizes more CD34+ cells when administered after cytotoxic chemotherapy. The relative proportion of early progenitor cells (CD34+/HLA-DR-) within the CD34+ population was significantly lower post-chemotherapy. However, the absolute number of CD34+ cells lacking HLA-DR was more than 2-fold higher compared with cytokine treatment alone. This subset represents early hematopoietic stem cells with self-renewal capacity and high proliferative potential.[8] Stable long-term hematopoiesis is likely to arise from these cells rather than from committed progenitors. In a previous animal study, G-CSF mobilized higher levels of more primitive progenitor cells when administered after chemotherapy.[10]

The results of our study show that committed CD34+ cells mainly contribute to the higher number of circulating progenitors after filgrastim-supported chemotherapy, which is reflected by the greater proportion of CD34+/CD33+ cells. Expression of HLA-DR, CD38 and CD33 found on the vast majority of these cells clearly indicates lineage-commitment.[8,11,12] Interestingly, the proportion of CD71 co-expression is comparable in leukapheresis products mobilized with filgrastim alone or post-chemotherapy. This finding might be important for gene transfer studies using cytokine-mobilized PBPC. The expression of CD71 on the majority of the CD34+ cells independent of the mobilization modality allows the use of a transferrin receptor mediated gene transfer that does not require viruses for studies of DNA transfer into CD34+ hematopoietic progenitor cells.[9] In this system, DNA conjugated with transferrin-polycations can be introduced into the cells by endocytosis.

We suggest that in patients with hematological malignancies, PBPC should preferentially be mobilized by cytokine-supported chemotherapy rather than using cytokines alone. In this setting, the lower proportion of more primitive progenitor cells is at least compensated by the substantially higher yield of CD34+ cells. Furthermore, the smaller number of CD19+ cells in the leukapheresis products might be of advantage for patients with B-cell lymphoma, since contaminating tumor cells are contained in this subset. However, the correlation of B-cell content and probability of contaminating tumor cells in the leukapheresis product remains open. In the setting of allogeneic transplantation, mobilization of PBPC without previous cytotoxic therapy could be an alternative to the bone marrow harvest. In spite of the lower CD34+ yield, the greater proportion of early, pluripotent hematopoietic progenitor and stem cells could allow successful engraftment and stable long-term hematopoiesis.

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PROGRESS IN THE DETECTION OF MINIMAL LYMPHOMA AND NEW APPROACHES TO THE TREATMENT OF MINIMAL DISEASE

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INTRODUCTION AND OVERVIEW

Increasingly, it is clear that histologically or cytologically normal appearing bone marrow or blood stem cell harvests of non-Hodgkin's lymphoma (NHL) patients can be contaminated with tumor cells at levels below detection by morphological examination or Southern analysis of DNA for the presence of clonal populations of lymphoid cells.[1] Lymphoma cells at these low frequencies can, however, be detected by either culture of the harvest or by the polymerase chain reaction (PCR) for tumor specific DNA sequences.[2] Lymphoma cells detected by these techniques at very low frequencies (approximately 1 in 10^3 to 10^5 nucleated cells) are however, clinically significant. In our most recent analysis of 65 consecutive NHL patients, the five-year disease-free survival of recipients receiving a tumor negative bone marrow was 50% as compared to 17% for recipients of a histological normal appearing but minimally contaminated harvest. This confirms our earlier observations.[3] Our studies hint that in patients with low grade lymphoma, both the bone marrow and blood stem cell harvests are more likely to be contaminated with lymphoma than in patients with intermediate and high grade lymphoma.[4] However, there appear to be some discrepancies between PCR detection of lymphoma and its growth in culture in that such harvests may be more likely to be PCR positive than culture positive although, not at the very high levels reported in some small series in the literature. This raises the question as to whether PCR techniques can be too sensitive and detect cells which are not clinically relevant. Alternatively, the culture technique may only be detecting tumor cells with a significant capacity for clonal growth, perhaps because of altered *bcl-2* or other anti-apoptotic gene expression.

Interestingly, in comparisons of lymphoma detection by both Southern analysis and culture growth, our studies suggest that blood stem cell harvests are either less frequently contaminated than marrow harvests

or the level of tumor cell contamination is generally lower and thus below the minimum sensitivity of the detection method. Present methods do not permit a distinction between these alternatives. Gribben et al[5] noted that detection of lymphoma cells by PCR in blood is less predictive for relapse in patients with follicular lymphoma than is detection in marrow. Furthermore, when patients with histologically positive marrows at the time of transplant were transplanted with tumor negative blood stem cell harvests, their five-year disease-free survival was 64% which was not different from those recipients of tumor negative marrow harvests (50%), but much better than recipients of even minimally contaminated marrow harvests (17%). This was not, however, a randomized study. These data, when taken together with the observations that recipients of minimally contaminated marrow harvests have a much poorer outcome, strongly suggest that a significant proportion of relapses/disease progression, perhaps 35-50% of cases, arise from reinfused minimal lymphoma. This conclusion is strengthened by the observations[6,7] of relapses arising from genetically marked cells. This raises the question of the need for purging and/or positive selection of CD-34 positive cells even though analysis of registry data currently shows no benefit of purging in lymphoma.[8]

Finally, it is clear that even those patients transplanted with a tumor negative harvest and who achieved a CR at 100 days posttransplant are still at high risk of relapse, i.e., 35-50% presumably from minimal disease present following the transplant and most likely arising from lymphoma cells which survived the high-dose therapy. This suggests that additional therapy in CR, when the patient has a minimal disease status, may be beneficial. Since the lymphoma cells being targeted survived high-dose therapy, an approach which employs a therapy that is mechanistically non-cross reactive with chemotherapy would be preferable. Furthermore, since the patients are clinically well, such therapies should be minimally toxic and preferably have non-overlapping toxicities with prior agents to which the patient has been exposed.

We summarize below the strategies that we are pursuing to address these various questions. A multicenter prospective randomized trial is in progress to compare bone marrow versus blood stem cells as support for high-dose therapy for intermediate and high grade lymphoma. The centers involved and clinical investigators are: University of Nebraska Medical Center - J. Vose, MD (study coordinator); University of Hawaii - Kevin Loh, MD; University of Indiana - Craig Nichols, MD; Mayo Clinic - Dave Inwards, MD. In association with this trial, a comparison of the molecular detection of minimal disease before and after

therapy of the patient and in the harvests using patient specific PCR probes and culture methods is being undertaken.[2] Oligonucleotides complementary to the mRNA (antisense) of genes whose expression is associated with lymphoma cells and which may be non-toxic to hematopoietic stem cells are being evaluated as potential lymphoma purging agents.[9] As regards, posttransplant therapy of patients in CR but with suspected minimal disease, immunotherapeutic strategies are being evaluated in pre-clinical studies[10] together with systemic antisense oligonucleotide strategies. Radiolabelled antibodies may also be effectively applied although hematopoietic toxicity is a concern.

Comparison of Culture Methods and Patient Specific PCR Probes for the Detection of Minimal Lymphoma

The serendipitous observation that placing histologically normal appearing bone marrow and cytologically normal apheresis harvests into long-term culture with a potent stimulus of lymphoid cell growth (PHA conditioned human spleen cell medium) has provided a sensitive method of detecting minimal lymphoma.[3] The presence of minimal lymphoma at an estimated frequency of 1 in 10^3 or 10^4 normal cells in such harvests[11] is clinically predictive of relapse/progression. The basis of this association is not yet clear. It is likely, but not formally proven, that inadvertent reinfusion of lymphoma cells with the harvest is the cause of some relapses.

However, some puzzling observations remain to be explained, such as fact that the relapses which occur, in virtually all cases, do so at sites of prior disease not in a systemic pattern.[3] At our institution only a few very high grade lymphomas have relapsed with a systemic, leukemic-like pattern. In the long-term cultures, when lymphoma outgrowths occur, they arise in association with the attached stromal cells. Initially small round cells, presumed to be lymphoma, are attached to the surface of the stromal cells. Their number increases and eventually some are released into the supernatant. Few such cells are observed in areas of the flask where there is no stroma. Such cultures can be passaged by scraping up the stroma and attached lymphoma cells intact, but not via the supernatant alone. Only a small proportion of cultures of high grade Burkitt's type or some T cell lymphomas can be passaged from the supernatant cells only. It is tempting to speculate that this is the type of lymphoma which, if reinfused, would cause a systemic pattern of relapse. The majority of lymphomas appear to require an association with stroma/stromal factors

for their optimum growth and survival. Clearly the definition of such factors is an important future objective.

A further factor in the ability of lymphomas to grow in culture, which might also be associated with their growth and/or resistance to therapy *in vivo*, is the associated expression of various oncogenes/cell cycle associated genes such as *bcl-2* in follicular and some intermediate grade lymphomas, *p53* in intermediate grade lymphomas and *myc* in high grade lymphomas. Studies have only recently begun to determine the relationship, if any, between the expression of these genes and the growth of the lymphoma cells *in vitro,* as well as their response to therapy *in vivo*. Potentially, these genes may be prognostic indicators as well as important therapeutic targets (see below).

Because the culture method is cumbersome and time consuming we have evaluated other approaches which provide material which can be used not only to assess clonality of lymphoid populations but also probe for rearrangements of other genes such as *bcl-2* and *myc*. During the generation of lymphoid populations, rearrangements of the germ line segments occurs, creating V-D-J rearranged segments. In the normal lymphoid system many such rearrangements should be generated (polyclonal) and the frequency of any single clone should be very low. Note that this may not be the case in some patients with immunodeficiency states or in the regenerative phase following transplantation. Flanking the rearranged DNA sequences are framework regions which have relatively conserved DNA sequences. Consequently, it is possible to construct consensus PCR primers which employ the adjacent complementarity determining sequences as the template and amplify across the rearranged DNA sequences. Starting with the original biopsy samples, either frozen or prepared as paraffin blocks (but not if fixed in B5 fixative) it is possible in the majority of instances, but not all, to amplify the rearranged sequences. If there is no clonal population present at a higher than normal frequency, when the PCR product is run out on a gel, multiple faint bands, constituting a smear on the gel, and representing the polyclonal normal lymphoid populations is obtained. If however, a significant clonal population representing lymphoma is present, in theory a clear band is obtained. This band can then be cut from the gel and its DNA sequenced. Based on this DNA sequence a second set of primers and/or a clone-specific probe can be generated. These reagents can be used to reprobe the original tumor sample to confirm the presence of tumor and verify the specificity of the probe/primers. In addition, these reagents can be used to probe hematopoietic harvests for minimal lymphoma and patient samples

post-therapy to identify those patients with minimal residual disease. Potentially such patients are candidates for additional therapies, although at this time the ability of this general approach to predict relapse/progression has not yet been established for other than follicular lymphomas.[5]

In model studies using Namalwa cells added to normal hematopoietic harvests, this approach can detect one lymphoma cell in 10^4 to 10^5 normal cells, i.e., about one long more sensitive than the culture method. Interestingly, combining culture and tumor specific PCR probe methods is even more sensitive, but the ultimate sensitive of a combined approach is yet to be determined. At the present time, it is not known whether the lymphoma cells detected by the tumor-specific PCR probe method are clinically significant. If other factors such as stromal cell dependence/factors are important in lymphoma growth *in vivo*, the culture method may be clinically predictive, but the tumor-specific PCR probe approach may not. For this reason these methods are being compared in a multi-institutional study (UNMC, University of Hawaii, University of Indiana, and Mayo Clinic). To date, 16 patients have been entered into the study and the culture method applied to 10 patients with 1 of 10 patients (10%) having a positive harvest. The tumor specific PCR probe method has been applied so far to 6 patients and the success rate has been to obtain probes for 5 patients (83%). The failure was a B5 fixed sample. Using these probes 2/6 (33%) of the harvests were positive including the harvest that was culture positive.

It is estimated that this study will take 3-4 years to complete. Too few cases have been analyzed to date to provide detailed cost/efficiency comparisons for the two methods, although it looks as if the tumor-specific PCR probe approach will be feasible as a specialist pathology laboratory service.

Evaluation of Antisense Oligonucleotides for *ex vivo* Purging of Lymphoma

If the circumstantial evidence which indicates minimal lymphoma reinfused with hematopoietic harvest leads to relapse/disease progression is confirmed, then the impetus to purge hematopoietic harvests of lymphoma will be significantly increased. Current analyses indicate no benefit to purging for lymphoma[8] but this may be due to the inclusion of many uninformative cases in the analysis. For example, patient selection, i.e., patients with more advanced disease underwent purging and may have

been compared to unpurged patients who might be expected to have a better outcome and this will mask the effectiveness of the procedure. Alternatively, current purging protocols may be ineffective. In theory, the purging method should be tumor-specific and non-toxic to hematopoietic stem/progenitor cells. Based on the hypothesis that specific oncogenes are rearranged and abnormally expressed in lymphoma cells, we have begun to evaluate oligonucleotides (ODNs) complementary to the mRNA of various genes: *myc*, *myb*, *p53* and *bcl-2* to date, for their ability to eliminate lymphoma cells without toxicity to hematopoietic stem/progenitor cells.[9] These ODNs are selectively toxic for lymphoma cells and appear to kill, at least in part by inducing apoptosis. Anti-*myc* ODNs kill OMA BL1 aggressive Burkitt's lymphoma cells, and anti-*myb* ODNs kill Raji cells whereas anti-*p53* has little effect on these cells. Anti-*bcl-2* ODNs kill some follicular lymphoma cells and T cell lymphomas. Anti-*p53* and anti-*bcl-2* ODNs have been shown to be cytotoxic to primary lymphoma cells from 6 of 6 patients with advanced follicular lymphoma. In most instances, specific down regulation for the target gene has been demonstrated. However, it is not clear that this is the mechanism of the cytotoxicity because other processes such as superinduction of *p53* and aptomer effects may be occurring. For example, the anti-*myb* ODN is a "4G" oligonucleotide.[12]

All of these oligonucleotides at their effective concentrations do not appear to be unduly toxic to normal hematopoietic stem/progenitor cells. This property makes antisense ODNs attractive candidates as tumor purging agents. The extent of killing of aggressive tumor cell lines, which is about one log at micromolar concentrations, is less than obtained with many chemotherapeutic agents or antibody purging methods. However, if this occurs without toxicity to hematopoietic stem/progenitor cells, the therapeutic ratio may be equivalent. Currently, too little is known about the mechanism of these effects to predict the outcome with any lymphoma and additional molecular information, especially as regards mechanism(s) is needed.

Potential Therapeutic Strategies Applicable Posttransplant in Patients with Minimal Residual Lymphoma

As noted earlier, even patients who achieve a CR posttransplant face a 50% risk of relapse/progression. These patients can potentially be identified early posttransplant using the tumor-specific PCR probes, consequently it is appropriate to consider possible therapeutic strategies

for these patients. The same oligonucleotides that may be useful in purging lymphoma from harvests *ex vivo* might be useful as systemic therapy. One such anti-*p53* ODN has completed a phase I trial in AML without evidence of systemic toxicity.[13] However, at the present time, the same reservations concerning the prediction of response and the mechanisms of action as noted for the *ex vivo* use of these agents exist.[14]

Alternatively, immunotherapeutic strategies are attractive. There is a strong suggestion that recipients of blood stem cell harvests have a better outcome than recipients of bone marrow harvests and the lower rate of relapse in the first 2-3 years post-therapy is still present for the former group even after potential differences in tumor contamination are accounted for. This suggests that an immunotherapeutic effect, albeit minimal, may be occurring. In pre-clinical models, the use of IL-2 posttransplant has been shown to improve immunological reconstitution and prolong survival.[10] What needs to be worked out is the protocol to produce the optimal effects of such agents with minimal toxicity. Currently, unresolved questions include whether the use of such agents during the collection or processing of the harvest is useful and how soon after transplantation of the harvest the administration of the interleukin is necessary or safe. Interleukins other than IL-2 including IL-7 and IL-12 are now being evaluated. A concern with all of these agents is that, in addition to their positive effects on normal immune cells, is whether they can expand minimal residual lymphoma. Consequently, in all of these approaches it will be necessary to carefully monitor minimal lymphoma and the methods described earlier may be useful for this task.

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PRELIMINARY RESULTS USING FLOW CYTOMETRY TO MEASURE TUMOR CELLS IN PROGENITOR SOURCES OF SOLID TUMOR PATIENTS

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ABSTRACT

We have developed a technique for immunofluorescent labeling of cytokeratin-positive cells using whole marrow or whole blood, thus minimizing the loss of pertinent cells through laborious separation procedures. Large numbers of cells, $>1.2 \times 10^5$, can be quickly analyzed via flow cytometry with a background of 0.0%. Aspirates from 95 breast and lung cancer patients were stained for cytokeratin and analyzed with flow cytometry and traditional pathology. Using flow cytometry, 27% (26/95) were determined to be positive with a median tumor frequency of 1 in 20,000 cells. Pathology detected malignant cells in 10% (9/95) of the marrow smears, all of which were positive using our method. The median tumor frequency in the samples found positive by both flow and pathology was 1 in 10,000 cells. Our method detected positive cells 3x more often and had a maximum sensitivity of 1 in 120,000 cells over pathology alone with a maximum sensitivity of 1 in 17,000 cells. Samples from 151 bone marrow harvests were also examined with flow cytometry and 19% (28/151) of them were found to be positive with a median tumor frequency of 1 in 25,000 cells. This translates into 1.6×10^5 contaminating tumor cells in an outpatient harvest of 4×10^9 mononuclear cells (MNC). Our initial studies of peripheral stem cell collections have also identified circulating cytokeratin-positive cells in some solid tumor patients. In 26 pheresis products, 35% tested positive with a median tumor frequency of 1 in 60,000. This translates into 1.3×10^5 contaminating tumor cells in 7.5×10^9 pheresed MNC. Our method is extremely simple and more sensitive than pathology alone. Used in conjunction with visual confirmation, minimal residual disease can be more accurately identified.

INTRODUCTION

Clinical concern persists regarding the presence of micrometastases in the marrow and bloodstream of breast and lung cancer

patients. A simple, sensitive and reproducible assay for detecting minimal residual disease (MRD) in these patients could supply additional information for staging and identify patients who could benefit from adjuvant therapy. Response to treatment might also be monitored and help determine optimal times for stem cell harvesting. Light microscopy is estimated to detect 1 tumor cell in 2000 nucleated marrow cells. Sensitivity can be increased with immunohistochemical staining, as indicated in this study, where pathology detected as few as 1 tumor cell in 17,000. Model systems of serially diluted tumor cell lines and normal cells have predicted sensitivities ranging from 1 in 10^4 to 1 in 10^6 using immunocytochemical and immunofluorescent antibody techniques.[1] Over the past 8 years, numerous studies have employed polyclonal and monoclonal antibodies, singly and in combination, to membrane tumor antigens and cytoplasmic molecules to increase the sensitivity of identifying MRD in the marrows of solid tumor patients.[1] Results vary considerably since cells may be lost during separation and concentration steps, antibodies to tumor antigens vary in their reactivity, reviewing large numbers of cells on slides is laborious, and fixation techniques can destroy cell morphology. The procedure reported here utilizes cytokeratin staining since this molecule is ubiquitous to all epithelial cells and is absent in cells of hematopoietic origin. Red cell lysis, membrane permeabilization and fixation is accomplished in a single step, thereby minimizing the loss of pertinent cells and preserving cell morphology for visual confirmation. Immunofluorescent labeling of cytokeratin allows the analysis of large numbers of cells in a few minutes using flow cytometry.

MATERIALS AND METHODS

Bone Marrow Aspirates

Bone marrow aspirates were performed on 95 breast and lung cancer patients using a Jamshidi Tray (Baxter). Two mls of marrow were aspirated from a single bone puncture. One ml was placed in a tube containing EDTA and sent for cytokeratin labeling and flow cytometric analysis. The second ml was examined for spicules, smeared on ten slides and sent to pathology for morphologic analysis and immunohistochemical staining of cytokeratin. A portion of marrow was placed in B5 fixative containing 10% formaldehyde to promote clot formation for sectional analysis by pathology.

Bone marrow Harvests

Under local anesthesia, 480 mls of bone marrow was harvested, 60 mls at a time, from 8 different bone sites on one side of the iliac crest.[2] Pooled marrow was buffy coated to remove platelets and red blood cells. Concentrated white cells were counted, tested for sterility, frozen, and a sample stained for cytokeratin with flow cytometric analysis.

Blood Samples and Peripheral Stem Cell Pheresis

Before begining chemotherapy, 2 cc of whole blood was collected in EDTA, counted and sent for cytokeratin labeling and analysis with flow cytometry. On days 12-16, after chemotherapy (Cytoxan 3 gm/M^2 and Platinol 90 mg/M^2 or Etoposide 750 mg/M^2 and Platinol 90 mg/M^2) and growth factor support (Neupogen 5 μg/kg sq daily) or on days 4-5 with growth factor only, patients with circulating levels of CD34+ stem cells >8 x 10^4/ml were eligible for peripheral stem cell (PSC) pheresis. Using their double lumen Groshong catheter, or their single lumen Groshong and a venous access, patients were pheresed at 20 mls/min on a CS3000 (Fenwal) for a total processed volume of 4-5 liters. A 1 ml sample was removed from the final product for counting and cytokeratin staining with analysis by flow cytometry. The remainder was frozen and stored.

Labeling and Analysis of Cells

Whole blood, whole marrow, or collected progenitor cells (4 x 10^6 cells) were incubated for 15 minutes in 5 mls of 1x Facslyse (Becton Dickinson) at room temperature. After 2 washings in phosphate buffered saline (PBS), cells were split between two tubes and incubated with 15 μl of Cam5.2 anti-cytokeratin FITC antibody or an IgG2 FITC isotype control (Becton Dickinson) at room temperature in the dark for 45 minutes. A positive control (MCF7) was run concurrently with patient specimens and was always >65% positive with fluorescence in the 3rd and 4th decades, clearly distinct from background in the 2nd decade. After 2 washings in 1 ml of PBS, cells were resuspended in 1 ml of PBS and 1.2 x 10^5 were analyzed on an Epics Profile flow cytometer (Coulter Corp.). The entire mononuclear population was live gated. Markers and quad stats were set so the isotypic control background was 0.0%. Specimens were considered positive if the mean fluorescent channel of "positive events" in the sample was at least twice the background channel of the control. Median increases in fluorescence above background are reported. Results are also expressed as the median # of tumor cells/1.2 x 10^5 and as the tumor frequency; the ratio of 1 tumor cell : negative cells.

RESULTS

Bone Marrow Aspirates

Bone marrow aspirates were performed on 95 patients and sent for cytokeratin staining with flow cytometric analysis and pathological examination. Of the 95 samples tested, 27% (26/95) were found positive with flow cytometry (Table 1) compared with 10% (10/95) determined to be positive by pathology (Table 2). The 9 samples that were found positive with both methods had a tumor frequency ranging from 1 in 3000 to 1 in 17,000. The 17 samples which were positive with flow cytometry alone, had a tumor frequency between 1 in 24,000 and 1 in 120,000. Ranges of fluorescence intensity did not vary between samples found positive with both methods (3-23x) compared to samples found positive by flow only (2-15x). There was one sample which was negative with flow, and pathologically negative on the smears, however a clot section did indicate malignant cells. Initially, samples from acute myelogenous leukemia and ovarian patients were stained and run on the flow cytometer (data not shown); always exhibiting negative results.

Table 1. Results for Bone Marrow

	Aspirates	Harvests
% Positive	27 (26/95)	19 (28/151)
Median # positive cells	6 (1-119)	5 (1-27)
Tumor frequency (Range)	1/20,000 (1,000-120,000)	1/25,000 (4,400-120,000)
Median increase in fluorescence	6x (2-23x)	5x (2-47x)

Table 2. Sensitivity of Cytokeratin Staining

Flow Cytometry	Pathology
68 negative samples	negative pathology
9 positive samples	positive pathology
17 positive samples	negative pathology
1 negative sample	negative smear; positive clot
27% positive (26/95)	10% positive (10/95)

Bone Marrow Harvests

Of 151 harvest procedures performed on 65 patients who had negative aspirates, 19% (28/151) were found to contain cytokeratin-positive cells (Table 1). The median tumor frequency was 1 in 25,000

which would predict 1.6 x 10^5 potential tumor cells in a median harvest of 4 x 10^9 MNC. The median increase in fluorescence was 5x (2-47x); consistent with the 6x increase reported for marrow aspirates. The fact that the 28 positive specimens came from 21 patients (7 were positive in both iliac crests) is alarming because it means that cells were not detected in 32% (21/65) of the patient's aspirates, using either method, prior to marrow harvesting.

Blood and Peripheral Stem Cell Harvests

Initial results with whole blood and peripheral stem cell (PSC) harvests indicate 38% (5/13) and 35% (9/26) positive respectively (Table 3). The tumor frequency in blood is surprisingly high, 1 in 8500, with a small median increase above background intensity of 3x; indicating possible interference and the need to analyze larger numbers of samples. The median tumor frequency in PSC harvests is 1 in 60,000 with a median increase of 15x (2-86x) above background intensity. A median PSC harvest of 7.5x10^9 mononuclear cells could therefore be contaminated with 1.3 x 10^5 tumor cells.

Table 3. Results for Blood and Peripheral Stem Cells

	Blood	Peripheral Stem Cells
% Positive	38% (5/13)	35% (9/26)
Median # positive cells	15 (6-21)	9 (1-24)
Tumor frequency	1/8500	1/60,000
(Range)	(7,000-20,000)	(5,700-120,000)
Median increase in fluorescence	3x (2-9x)	15x (2-86x)

DISCUSSION

Cells from breast and lung cancer patients were prepared in a single step, cytoplasmically labeled for cytokeratin using an immunofluorescent antibody, and analyzed with flow cytometry. In a direct comparison of marrow aspirates, we detected cytokeratin positive cells 3x more often with our method (27%) versus immunocytochemical staining and patholgy (10%). This is most likely the result of analyzing larger numbers of cells, yielding a sensitive tumor frequency of 1 in 120,000 cells using flow versus 1 in 17,000 cells with pathology.

We also detected positive cells in 19% of our bone marrow collections even though a negative aspirate, using both flow and pathology, was prerequisite for marrow harvesting. In other words, 32%

of the patients with negative aspirates had positive harvests; 7 of the 21 patients were positive from both iliac crests. This is most likely because eight or more sites are sampled during a marrow harvest suggesting that sensitivity is greatly compromised, regardless of the assay employed, when a sample is aspirated from a single unilateral site. Bilateral or multiple site aspirations might increase the chances of locating micrometastases; and are being considered for future testing. It is also possible that very small numbers of tumor cells might not only escape pathology, but could also elude detection with flow cytometry if they persist as aggregates or remain attached to spicules throughout processing; flow can only analyze single cells. Enzymatic digestion prior to permeabilizing cells might promote a single cell suspension, should not interfere with cytoplasmic labeling of cytokeratin, and could yield a more accurate estimation of tumor contamination.

Initial results with whole blood are premature, with only 13 samples analyzed. The high frequency of 1 positive cell in 8500 cells and the low increase (3x) in fluorescence above background has prompted a blind study of blood from normal individuals versus solid tumor patients. Staining and flow cytometry of 26 PSC phereses showed tumor contamination in 35% of the final products. The median tumor frequency was lower than marrow; 1 in 60,000 versus 1 in 25,000 respectively, with a median increase in flourescence 15x background. Since larger numbers of MNC are typically collected during pheresis, a median of 7.5×10^9 at our center, there is a potential for 1.3×10^5 contaminating tumor cells. The clinical significance of these cells needs to be evaluated; especially since we have found that 50% of the positive cells in marrow and PSC can survive freezing and thawing procedures.

We have begun evaluating our method as an additional means of monitoring patient response to therapy and determining optimal times for stem cell harvesting. Two breast cancer patients that initially tested positive in their marrow with large tumor frequencies, 1 in 3100 and 1 in 4300, received 4 courses of chemotherapy. Follow-up cytokeratin testing indicated a negative marrow for one patient, who opted for no further treatment, and a reduction to 1 positive cell in 60,000 cells for the other. After another three courses, the latter patient had a negative aspirate and proceeded to marrow harvesting which also tested negative.

As the sensitivity for detecting MRD in blood and marrow increases, so does the debate over the clinical significance of small numbers of tumor cells, especially in the absence of other prognostic factors. Recent studies have correlated *in vitro* clonogenic growth with

immunocytochemical detection of breast cancer cells in marrow and peripheral stem cells.[2] Immunocytological detection of cytokeratin-positive cells in the marrow of operable non-small cell lung cancer patients correlated with a 66.7% relapse rate 13 months after primary surgery compared with a 36.6% relapse rate when the marrow tested negative.[3] Long-term culture techniques have identified viable, occult metastases in the cellular material filtered during marrow harvesting of breast cancer patients who had normal biopsies and aspirates.[4] Converesely, a study of metastatic breast cancer patients receiving high dose chemotherapy and autologous transplant showed no correlation between the presence of malignant cells in the marrow and relapse rate, time to disease progression or overall survival.[5]

In the future, we will analyze larger numbers of cells ($1x10^6$) using a more sensitive flow cytometer with sorting capabilities so positive cells may be selected and verified under microscopy. Multiple sites will be tested prior to harvesting and steps employed to break up aggregates of cells and facilitate flow cytometric analysis.

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GRAFT ASSESSMENT OF CYTOKINE-MOBILIZED PERIPHERAL BLOOD PROGENITORS BY CD34+ CELL ENUMERATION

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INTRODUCTION

Within hematopoietic tissue, expression of the CD34 antigen is restricted to primitive progenitor/stem cells that constitute 1-3% of normal bone marrow mononuclear cells.[1,2] Purified CD34+ cells are capable of reconstituting the entire hematopoietic system in both experimental animals and humans,[3] suggesting that CD34+ cells are responsible for both initial rapid engraftment from committed progenitors, and long-term reconstitution of hematopoiesis from true stem cells.[4] CD34+ cells can also be mobilized from the marrow to the peripheral circulation by combinations of chemotherapy and cytokines and peripheral blood stem cell autografts are increasingly used to reconstitute hematopoiesis after intensive, potentially marrow-ablative therapy.[5,6]

A common approach to assess the quality of peripheral blood stem cell (PBSC) grafts relies on methylcellulose-based colony assays (that take 10-14 days) for lineage-committed hematopoietic progenitors such as CFU-GM. However, a reliable and rapid means to assess more primitive cells that mediate long-term reconstitution, is lacking. A safe PBSC dose is thus difficult to determine and may depend upon a number of factors including the malignant diagnosis and extent of prior chemotherapy. Due to differences in the way these assays are performed between different centers, including widely differing culture conditions (e.g., leucocyte conditioned medium versus recombinant growth factors), it is difficult to make meaningful comparisons on data from different institutions (reviewed in 7).

Graft assessment by quantitation of primitive stem/progenitor cells bearing the CD34 antigen has several potential advantages over colony-forming assays in that it takes less than 2 hours to perform, the CD34+ population encompasses the earliest stem cells and maturing, lineage-committed progenitors, and should generate data which are more directly comparable between different transplant centers.[7] Such a procedure would

also be suitable for the determination of optimal timing for apheresis collections. Finally, when 'positive selection' techniques are to be employed to purify CD34+ cells,[3,8,9] the ability to accurately enumerate them as a percentage of total nucleated white blood cells is of critical importance in calculating the efficiency and yield of the purification technique. Unfortunately, different investigators use different CD34 antibodies and some CD34 antibodies, especially those that detect carbohydrate-associated epitopes, fail to detect CD34+ cells in some clinical samples.[(2, unpublished observations)] Furthermore, flow cytometric enumeration using CD34 staining only, or CD34 staining together with either light scatter gating, or side scatter gating rarely correlates with estimates obtained by fluorescence microscopy. Furthermore, isotype matched control antibody staining of minor populations of cells can often mask true staining of rare populations. Finally, sample variability is a significant problem with respect to numbers of red cells, nucleated red cells, platelets, nonspecifically stained adherent cells and cellular debris, all of which may be faithfully recorded by flow cytometers. Since there is no standardized way to enumerate CD34+ cells by flow cytometry,[7] failure of some clinical investigations to show a correlation between CD34+ cell number and rate of engraftment may in part be related to these problems.[10] Here we describe a simple, rapid and reliable flow technique for the accurate enumeration of CD34+ cells in a variety of normal and abnormal hematopoietic tissues.

METHODS

Cells and Antibodies

A light density mononuclear cell (MNC) fraction was isolated from normal consenting blood donors on a Ficoll gradient (density 1.077 g/cm^3, Pharmacia, Piscataway, New Jersey). Peripheral blood was obtained from a patient with multiple myeloma following mobilization with cyclophosphamide (4 g/M^2) and GM-CSF according to local Institutional Review Board approved protocols. GM-CSF was administered at 5μg/kg/day subcutaneously (sc.) when the granulocyte count reached 0.1 x 10^9/L and apheresis was performed when the white cell count reached 1 x 10^9/L.

Phycoerythrin conjugates of the IgG_1 CD34 antibody 8G12[11] and the IgG_1 CD34 antibody QBEnd10[1,9] were obtained from Becton Dickinson Canada (Mississauga, Ontario) and AMAC (BIO/CAN Scientific, Mississauga, Ontario), respectively. Both antibodies are

equally efficient at detecting CD34+ cells in all normal and clinical samples tested to date (unpublished observations). The IgG_2 monoclonal antibody 4B2 (American Type Culture Collection, Rockville, Maryland) that detects a 'framework' epitope on all isoforms of the CD45 family of molecules was affinity purified on Protein A-sepharose and conjugated to fluorescein isothyocyanate (FITC) using standard procedures.

Cells were simultaneously stained with CD45 FITC and CD34 PE, and analyzed by fluorescence microscopy on a FACScan flow cytometer (Becton Dickinson Instrument Systems, San Jose, California). Four data parameters were collected in Listmode files: linear forward scatter, linear side scatter, log FITC and log PE fluorescence. Compensation settings were established using CalBrite beads (BDIS) and confirmed using lymphocytes stained with Simultest CD3 FITC/CD19 PE (BDIS). Off-line analysis was performed using the Lysis II software as supplied by BDIS.

RESULTS

Enumeration of CD34+ Cells in PB of a Cytokine-Treated Myeloma Patient

In preliminary studies of several patients undergoing cytokine-induced mobilization of PBSCs, the circulating CD34+ cells in these patients, even when present in numbers too low to accurately count by fluorescence microscopy, were uniformly brightly stained by CD34 PE. Moreover, they exhibited a much less heterogeneous size distribution compared to normal bone marrow CD34+ cells. In other samples containing higher numbers of CD34+ cells, flow cytometry using single parameter (gating on CD34+ events only) or dual parameter (light scatter versus CD34 staining, or side scatter versus CD34 staining) failed to generate data comparable to observations with fluorescence microscope.

To address these problems, some samples were simultaneously stained with CD45 FITC which stains all nucleated white blood cell types with the exception of some plasma cells. Red blood cells, their nucleated precursors, and other cellular debris, that variably contaminate low density MNC preparations, and can be recorded by flow cytometers, are thereby excluded from the analysis. Thus, apheresis products from a myeloma patient were stained with CD45 FITC and CD34 PE and the number of CD34+ cells assessed to be about 10% by fluorescence microscopy. As had been noted with the other samples described above, the CD34+ cells in this sample were remarkably uniform with respect to their intermediate size and bright CD34 fluorescence. When CD45 fluorescence was

examined under FITC filters, CD34 PE-stained cells were still visible due to the broad bandwidth of the filters in the microscope. CD45 fluorescence was not detectable on these CD34+ cells due to this breakthrough of the PE staining indicating that if CD45 was expressed on CD34+ cells, it was relatively weak. Although small lymphocyte-like cells and monocyte-like cells were stained brightly by the CD45 FITC reagent, granulocyte-series cells were only stained weakly. Red blood cells and their nucleated precursors were not detectably stained with CD45 FITC, in keeping with known characteristics.[12]

When the same sample was processed on the FACScan, two populations of positive cells (Figure 1, top two rows) were detected after CD45 FITC staining (box A, region R1) as well as an unstained population (which by light scatter analysis appeared to consist mainly of red blood cells and their nucleated precursors). CD45+ cells contained in R1 were back scattered for CD45 staining versus side scatter (granularity) and major populations of lymphocytes (bright CD45 staining, low side scatter), monocytes (bright CD45 staining, intermediate side scatter) and granulocytes (low CD45 staining, high side scatter) were resolved (box C) as recently described for this type of gating analysis.[13] When the CD45+ cells (region R1) were analyzed for CD34 PE staining, a distinct population of positive cells could be detected (box B, region R2). The cells contained in the CD34+ fraction R2 were similarly back scattered and the majority of the events in the CD34+ region R2, clustered in an area characterized by uniform low side scatter and weak CD45 staining (box D, region R3). In keeping with observations by microscopy, region R3 cells exhibited a relatively restricted range of (medium) sizes, and were found within the generic blast/lymphocyte gate (R4) when analyzed by side scatter and forward scatter parameters (box E). Statistical analysis of the cells gated by R1, R2, R3 and R4 (=G4) showed the number of CD34+ cells to represent 9.14% of the total nucleated white blood cell fraction identified in region R1 (=G1 in gate statistics). CD34+ events were not detected in the $CD45^-$ fraction of this sample nor in that of many other similar analyses (data not shown), confirming that all subsets of CD34+ cells express CD45 molecules, albeit at relatively low levels.

Enumeration of CD34+ Cells in Normal Steady-State Blood Samples

The CD34+ cell content of steady-state peripheral blood from normal subjects has been estimated to be in the 0.02-0.05% range by a variety of techniques including limiting dilution analysis for long-term culture initiating-cells.[14] To assess the sensitivity of the above procedure,

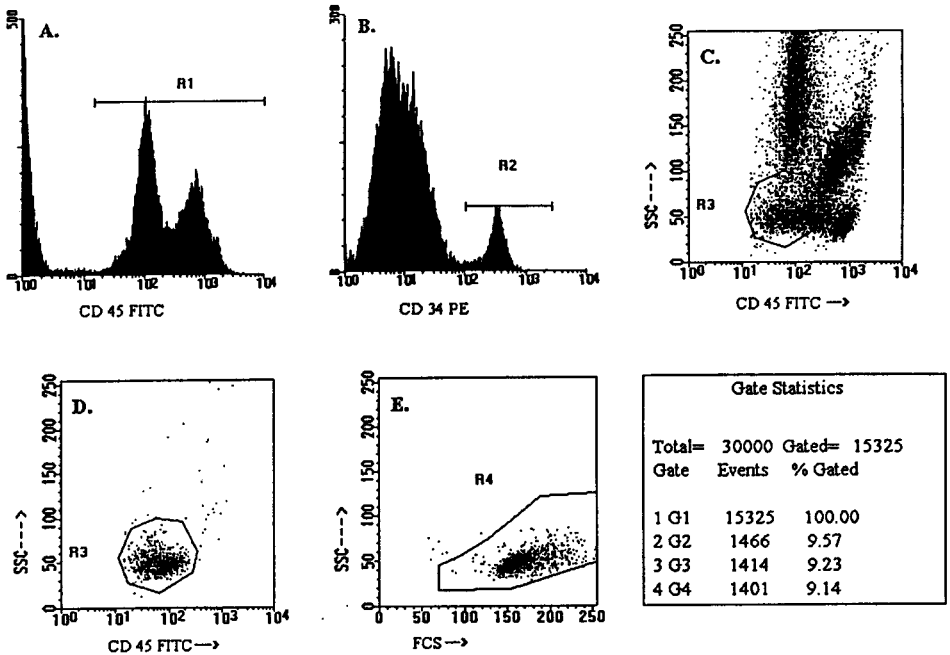
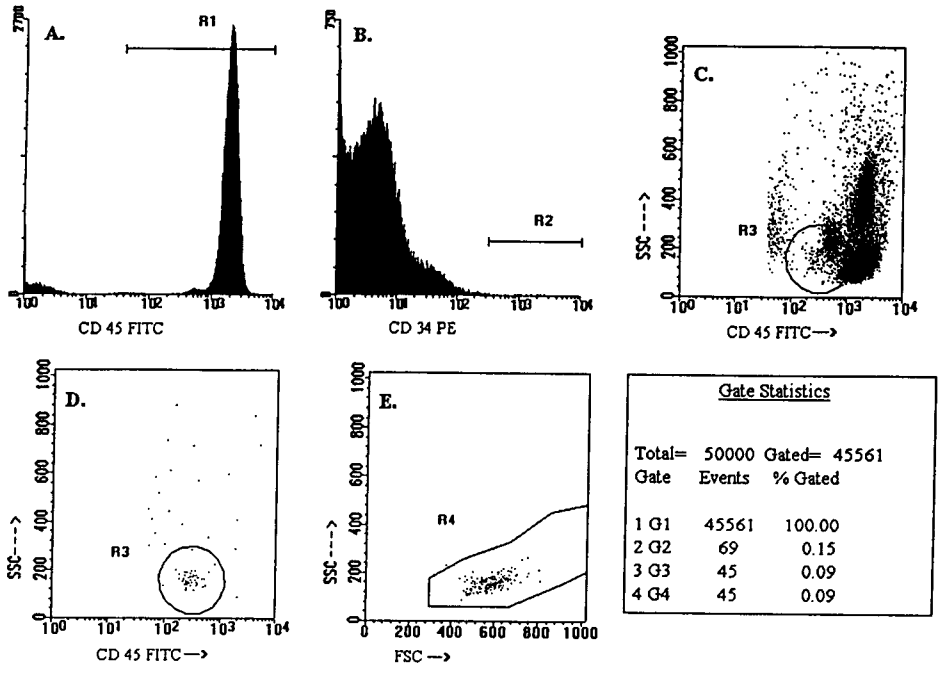


Figure 1: ***Top 2 rows*** *- Analysis of CD34+ cells in peripheral blood (apheresis sample) of GM-CSF treated patients. Box A; Sample stained with anti-CD45 FITC. B; anti-CD34 PE staining of cells gated in region R1. Region R2 represents CD34+ events. C; CD45 versus side scatter analysis of events gated in region R1. D; CD45 versus side scatter analysis of CD34+ cells gated by R1 and R2. E; light scatter (LS) properties of cells gated by R1, R2 and R3. Region R4 represents a blast/lymphocyte region into which most bone marrow-derived CD34+ cells scatter. Gate statistics of analysis showing that of 30,000 events collected, 15,325 were nucleated white blood cells as defined by CD45 staining (R1). Gate statistics of cells gated in R1 (G1), R1 plus R2 (G2), R1 to R3 (G3), and R1 to R4 (G4).*



Lower 2 rows *- Analysis of CD34+ cells in normal peripheral blood sample. Staining, analysis and enumeration performed exactly as described for apheresis sample.*

we enumerated CD34+ cells in the peripheral blood of a number of normal individuals. As shown in the lower two rows of Figure 1, the CD34+ cell number of one such sample was found to be 0.09% of the total nucleated white blood cells. Similar to those in cytokine-mobilized blood, true CD34+ cells formed a tight cluster on CD45 staining/side scatter analysis (region R3, box D), underscoring the merit of back-scattering the events gated into the putative CD34+ region R2 (box B). Similar to the cytokine-mobilized example described above, and umbilical cord blood CD34+ cells, the CD34+ cells in normal blood exhibited a more restricted range of size and granularity compared to normal marrow.[15] Assessment of CD34+ cells in six normal blood samples generated values of 0.03%-0.09%.[15]

Correlation of % CD34+ Cells with White Cell Count and CFU-GM in Cytokine-Treated Patients.

To determine if the CD34+ cell enumeration procedure was of use in determining the optimal timing of apheresis collections, patients with lymphoma or myeloma undergoing chemotherapy with cyclophosphamide (4 g/M^2) were studied. Five days after chemotherapy, patients received IL-3 (2.5 μg/kg sc. for five days) followed by GM-CSF (5 μg/kg sc. until apheresis was completed). In the example shown in Table 1, CD34+ cells were enumerated in a myeloma patient as described above on days 10, 11, 14-18 post-chemotherapy and the numbers obtained were compared with the corresponding white blood cell count. Apheresis was performed on days 14 through 18 and assessed for CD34+ cells and the number of CFU-GM per kg body weight. In the peripheral blood samples, a good correlation between increase in CD34+ cell numbers and total white blood cell count is noted. Similarly, in the apheresis products there is good correlation between CD34+ cell numbers and the levels of CFU-GM/kg. In patients who failed to show an increase in CD34+ cell numbers in response to cytokines, both the white cell count and the CFU-GM levels remained low.

Table 1. Enumeration of CD34+ Cells in Peripheral Blood and Apheresis Products of Cytokine-Treated Myeloma Patient.

	Days Post-Chemotherapy								
	10	11	12	13	14	15	16	17	18
Peripheral Blood									
WBC	0.3	0.2	0.3	0.4	0.9	1.6	2.0	2.7	5
% CD34+	0.03	0.04	--	--	0.07	0.29	0.34	0.29	0.3
Apheresis									
% CD34+	--	--	--	--	0.12	0.17	0.37	0.44	0.35
CFU-GM/kg ($x10^4$)	--	--	--	--	0.68	2.46	5.65	4.76	4.13

Footnote: Myeloma patient ER given cyclophosphamide (4g/M^2) on day 0 followed by IL-3 (2.5 μg/kg) days 5-9, and GM-CSF (5 μg/kg) days 10-18. Apheresis was performed on days 14-18.

DISCUSSION

We have developed a sensitive and specific multi-parameter flow cytometric assay for the enumeration of CD34+ progenitors in a variety of normal and abnormal hematopoietic tissues. This technically simple procedure utilizes fluorescein-conjugated CD45 antibody to reliably quantify total nucleated white blood cells. Red blood cells, their nucleated precursors, platelets and cell debris which can drastically inflate the number of CD34$^-$ events and whose numbers are highly variable in clinical samples, are thereby excluded. Simultaneous staining by phycoerythrin-conjugated CD34 antibody (8G12 or QBEnd10) then defines an approximate number for the CD34+ stem/progenitor fraction. When starting CD34+ cell numbers are low (0.01%-0.1%), as in some cytokine-mobilized PBSC samples from heavily pretreated cancer patients undergoing evaluation for autologous peripheral blood progenitor cell transplants, nonspecific staining of other leukocytes makes accurate enumeration impossible. However, when the CD34+ fraction is analyzed for CD45 expression versus side scatter, true CD34+ blast cells form a discrete cluster exhibiting low density CD45 expression and low side scatter characteristics.

Although CD45 gating using 'framework' antibodies has been used in the past to identify subsets of cells in normal[12] and leukemic[13] bone marrow samples by flow cytometry, the key observations that; a) CD34+ cells express only low levels of CD45, and b) have generally low side

scatter, have allowed us to separate true CD34+ blast cells from other cell-types that display similar or partially overlapping light-scattering characteristics. Thus, lymphocytes that exhibit characteristics of side and forward scatter similar to some true CD34+ blasts, can now be separated from CD34+ cells since they stain brightly for CD45.[12,13] Similarly, monocytes that can exhibit light scatter characteristics that partially overlap those of true CD34+ blast cells, can be distinguished by their high CD45 expression and increased side scatter. Neutrophils, eosinophils and their precursors, which stain weakly, but nonspecifically with some CD34 antibodies, can also be distinguished from true CD34+ cells by high side scatter. Using this technique, we can reproducibly detect CD34+ cells in the 0.01%-0.1% range, free of contaminating events, in steady-state peripheral blood from normal subjects as well as umbilical cord blood (range 0.33%-1.98% in 25 samples), normal bone marrow aspirates[15] and cytokine-mobilized PB from cancer patients.[(in preparation)] While isoform-specific CD45 antibodies (CD45 RA, RB, RO) have also been used in the subset analysis of CD34+ cells,[10,11] expression of CD45 isoforms varies depending on whether the CD34+ cells are from BM, cord blood, G-CSF- or GM-CSF-mobilized PBSC.[16] Thus, for routine analysis of clinical samples, additional sample variations are obviated by the use of framework CD45 antibodies as described in this report, and the CD34+ cell number is always expressed as a percentage of the nucleated white blood cell count.

When enumerating CD34+ cells in bone marrow specimens that tend to be rich in nucleated red cells, it may be useful to utilize a readout of CD34+ cells as a percentage of total nucleated cells, since nucleated red cells are included in the total nucleated cell count of routine hematology instruments. In such cases, total nucleated cells are identified by staining with the nucleic acid stain LDS-751 that can be detected in the third fluorescence channel of the FACScan.[17] In such cases, the LDS-751+ cells are gated first, followed by gating on CD45+ events, CD34+ events, and side scatter as described above. CD34+ cells then can be expressed as a percentage of either total nucleated cells (LDS-751+) or nucleated white blood cells (CD45+) (data not shown).

The technique outlined herein can be performed on a single laser instrument, using routine instrument settings and only basic software is required for data analysis, making it suitable for routine FCM analysis in immunopathology laboratories. It can be performed rapidly, the analysis is simple in most cases and it is thus suitable for the real-time analysis of apheresis products.

In summary, we have shown that flow cytometry, using a combination of appropriate antibodies to CD45 and CD34, and side scatter analysis represents a sensitive, specific and reproducible means of enumerating CD34+ cells in hematopoietic samples.

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INFLUENCE OF THE "MATURITY PROFILE" OF CD34+ CELLS ON ENGRAFTMENT

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INTRODUCTION

A better definition of the degree of differentiation and stimulation of the cells constituting grafts is a longstanding goal in transplantation; improved comprehension of the individual role of each subset of cells is of special interest if grafts are manipulated prior to being injected, as in expansion *ex-vivo*, purging, and stem cell enrichment which are all currently pursued. Adding momentum to this issue may be the pending regulations and efforts for standardization put into action by governmental institutions.

Except for grafts with enriched stem cells, mature cells are always present in abundance. Among them, only monocytes and eventually helper T-cells may play a role in recuperating hematopoiesis, as after stimulation they are a potent source of cytokines,[1] while other lymphocytes and residual granulocytes and thrombocytes can be considered as "neutral" in this respect. All the more, the quantitative and qualitative composition of the hematopoietic cell compartment, which comprises only about 0.5-5% of all grafted cells, is of utmost importance for repopulating hematopoiesis after ablative treatment.

While cells of this compartment are very inhomogenous with respect to size, light scatter, and buoyant density, they are indistinguishable from mature mononuclear blood cells by these criteria. As opposed to the latter, however, they all express the CD34 surface antigen,[2] thus providing a means to determine the total size of this compartment by cytofluorimetry. If subsets among them with different maturity or lineage commitment are to be distinguished, they have to be put into appropriate culture conditions and stimulated, or their coexpression of maturity or lineage markers have to be determined.

Efforts in recent years have led to improved data regarding requirements of grafts in terms of CFU-GM or CD34+ cell numbers for each source of grafting material; with regard to subsets of human hematopoietic cells, however, less is known. Based on initial observations

of early and late engraftment failure, it was inferred that advanced progenitors enact the early phase of recovery, while more immature stem cells, which also *in-vitro* need more time for reaching the advanced progenitor state, take over production and output of mature cells during the following phase.[3] In addition, there may exist a very small fraction of "repopulating stem cells" which long term, sustain cell production of all three blood lineages and immunocytes, but this issue is known better in mice than in man (Figure 1).

If this model applies, it can be expected that the more advanced progenitors are present in a given type of graft, the better and faster short term recovery will proceed. Grafts of different origin, like bone marrow or mobilized peripheral blood stem cells, usually contain different total amounts of hematopoietic cells, and clinical experience shows faster recovery with the latter. A recent study demonstrates dependence of aplasia time from the number of grafted CFU-GM (which represent a fraction of committed progenitors) in the range below threshold values, and interestingly the data stemming from grafts of different origin fit in the same plot.[4] It can now be asked, whether this scope can be extended to the entire compartment of hematopoietic cells, which comprises the whole spectrum from the most immature stem cells to the most advanced progenitors. As the relative size of different CD34+ cell subsets can now be determined, it seems timely to relate them to recovery data and see if they are compatible with the model.

THE MATURITY PROFILE BASED ON *IN VITRO* FEATURES OF CD34+CELLS

A) The subset of early CD34+ cells

The operational definition of these cells is that they are unable to form colonies in short term culture containing late acting growth factors. They grow only on supporting feeder layers under elaborate conditions, or in the presence of stem cell factor in combination with other growth factors, and a time laps of at least 5 weeks is required until they have developed into advanced progenitors able to generate colonies of mature cells after final stimulation.

Several studies show that cells at this immature stage do not express the surface marker CD38, whereas during further maturation they start expressing it, independent of their lineage commitment.[5] This makes it possible to assign the entire fraction of earlier hematopoietic cells to the CD38 subset of CD34+ cells. (Further resolution of this subset with other

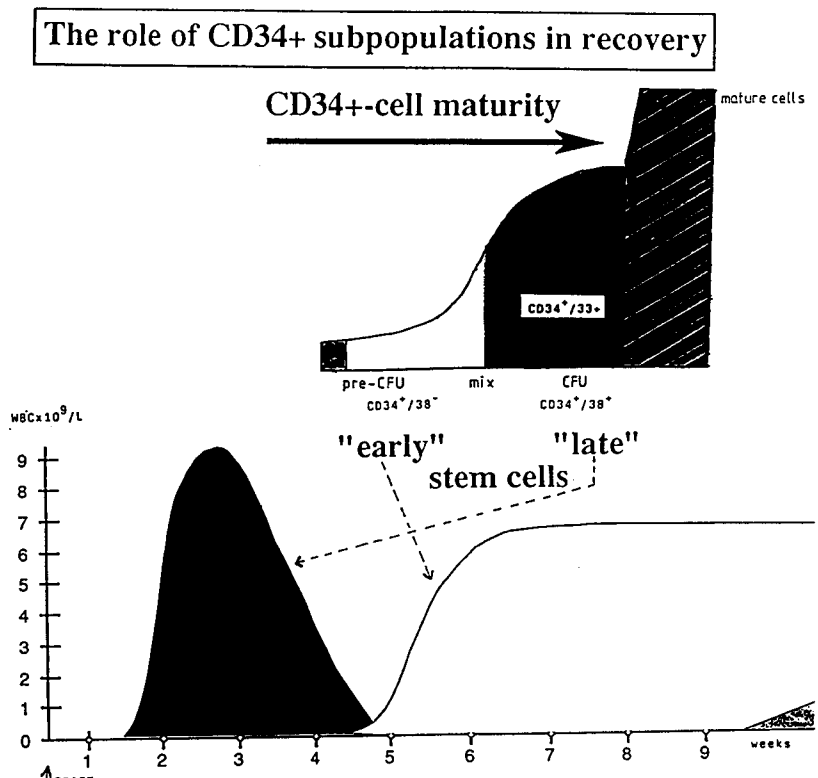

Figure 1. *Model of early and long term recovery related to advanced progenitors and "early" stem cells, respectively.*

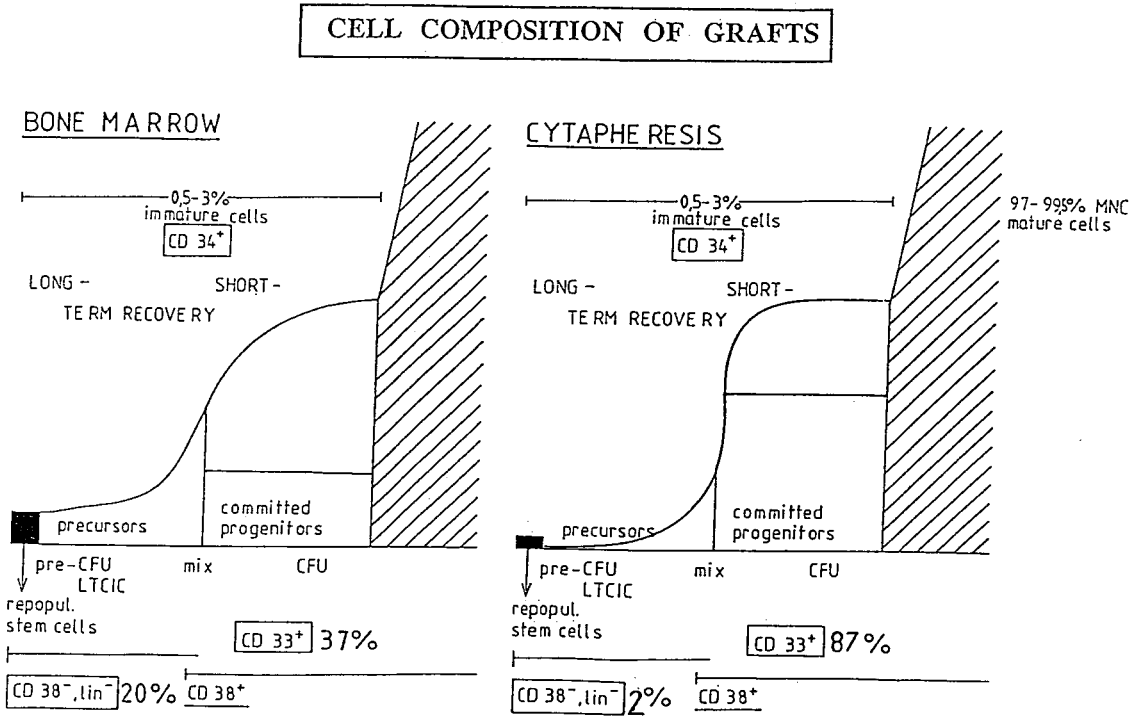

Figure 2. *Maturity profiles of hematopoietic cells in bone marrow (left) and cytapheresis products after HDC-mobilization (right; schematic presentation.*

early markers as C-Kit Thy l, or MDR-l/Rhodamine depend on extremely rare cell frequencies, which necessitate prior enrichment steps for determination).

B) Subsets of advanced progenitors

According to the above, the complementary CD34+/CD38+ subset contains cells of all different lineage commitments and is quantitatively preponderant The most important parameter for estimating the general maturity of a given graft type is the total size. Technically, further resolution into subsets of cells committed to a particular single lineage would be possible, since enough cells of these subsets are present. It was previously proposed to use the CD33 marker for this purpose.[6] Recently, however, the meaning of this marker, initially thought to indicate progenitors with commitment to the granulomonocytic lineage, was questioned for several reasons. Several groups, including our own, failed to find a correlation between the CD34+/CD33+ subset and CFU-GM in short term cultures. Moreover, Fritsch found among these cells, after flow-sorting and stimulation, some red cell colonies.[7] Finally, Terstappen (this meeting) has found, using a special cytofluorimeter with considerably increased sensitivity, and mAB's with very high affinity, that the CD33 marker is also expressed at a low level on CD38- cells.

Nevertheless, the relative yield of CD34+/CD33+ cells (as conventionally determined) is increased in mobilized peripheral stem cells in comparison to bone marrow cells (see below), and even more so if growth factors for mobilization (in addition to high dose cytostatics), like GM-CSF are applied. In conclusion, it appears more prudent to consider CD33 as a marker for maturation, which indicates preferentially the white cell lineage.

RELATIONS OF THE MATURITY PROFILE TO GRAFT SOURCE AND RECOVERY KINETICS

In the suggested simplified scheme, CD34+ subsets are arranged from left to right with increasing degree of maturity; accordingly, boosting mature elements in grafting materials is recognized by a right-shift, with decrease of the CD38- subset and increase of the total CD38+ committed progenitor subset.

Phenotype analysis of grafts taken from bone marrow showed 16-20% CD34+ cells lacking CD38, while in HDC (Melphalan) mobilized circulating blood stem cells only 1.8% belong to this subset (n=9). So, there is an impressive difference within the early stem cell subset: it is

relatively small after mobilization, with an overwhelming preponderance for the lineage committed advanced progenitors, indicated as a strong right-shift towards mature hematopoietic cells from 80% in bone marrow to 98.2% in mobilized stem cells. Among these cells, in addition, the CD33+ subset increases from 52.2% (average) in bone marrow to 87.5% (average significance level of the difference p=0.037; Whitney Man two tailed test).

In order to correlate graft outcome with hematopoietic cell doses, the frequency of cell subsets must be calculated. In bone marrow grafts an average of $0.9x10^6$ CD34+ cells/kg were identified (containing $3.9x10^4$ CFU-GM/kg), while in CSC 2.4 x 10^6 CD34+ cells/kg (containing $22.5x10^4$ CFU-GM/kg), and $4.2x10^4$ CD34+/38- cells/kg were obtained. So, in our series there were 2-4 times more early stem cells provided in the bone marrow grafts, although 2.5 times less total CD34+ cells were given than in CSC grafts. The lowest CD34+/38- cell dose given was $1x10^4$/kg, and there was no late recovery failure observed.

In assessing the advanced progenitors, the CD34+/33+ cell dose was about 4 times higher in CSC than in bone marrow ($2.1x10^6$/kg vs. $0.47x\ 10^6$/kg), and in terms of CFU-GM there was nearly a 6-fold excess in the former. In comparison, duration of aplasia (WBC >1000/mm^3) was 18 days (average) for bone marrow grafts, and 11.7 days for CSC grafts. This result is compatible with a functional relationship between this subset and short time recovery.

CONCLUSION

Unlike in laboratory animals, functional analysis of graft kinetics in patients cannot be performed by simply purifying subsets of hematopoietic cells and transplanting them separately.

Nonetheless, differences in aplasia time from grafts of different sources provide a key for assigning short time recovery to phenotypically defined subsets, in the event that the proportion of total CD34+ cells is significantly different. The data on two series of bone marrow and CSC, revealing significant differences ind the duration of aplasia, showed a corresponding difference in levels of CD34+/38+ and CD34+/33+ subsets and also CFU-GM content: the CSC grafts had about 6-fold more CFU-GM than the bone marrow grafts, and fell into the threshold range for optimal recovery time, while the bone marrow grafts were in the suboptimal range.

These data support the contention, that the degree of maturity of transplanted hemopoietic cells corresponds to the speed of early recovery. This contention is substantiated by the experience of the Nebraska group with grafting circulating stem cells from steady-state blood; such grafts contain around 60% early CD34+/38- cells and show a "left"-shift, immature profile; aplasia times are above 28 days. On the other hand, CSC mobilized with HDC plus growth factors contain more advanced and less immature cells, indicating a "right"-shift, and aplasia is reduced to a minimum of 9 days. Taken together, the evidence is compatible with the model of recovery phases that correlate with subsets of CD34+ and in addition, give indications of compartment sizes in absolute numbers.

For the immature CD34+/38- subset, there is no corresponding quantitative change in long term recovery, which suggests that in all grafts sufficient numbers of these cells are present. So, with all necessary precaution, a first estimate can be derived, that in an adult about one million of these cells in a graft appear to be sufficient. This is an upper limit estimation, and certainly, extension of the database on this figure will be beneficial before applying it to manipulated grafts; also extended efforts for standardization of this demanding measurement method between different laboratories will be necessary to integrate data coming from different transplantation centers.

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A PROSPECTIVE, RANDOMIZED PHASE III STUDY USING THE CEPRATE® SC STEM CELL CONCENTRATOR TO ISOLATE CD34+ HEMATOPOIETIC PROGENITORS FOR AUTOLOGOUS MARROW TRANSPLANTATION AFTER HIGH DOSE CHEMOTHERAPY

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INTRODUCTION

Autologous bone marrow transplantation (ABMT) has been used to treat patients with a wide variety of hematological malignancies and solid tumors. Serious and life-threatening toxicities can occur with autologous marrow infusion. The potential for acute complications, especially cardiovascular events, may be greater than previously recognized because patients generally have not been monitored or have remained asymptomatic. The incidence of cardiac complications may increase as more aggressive chemotherapy regimens are utilized. For example, a higher incidence of heart block was observed by Styler et al among patients who had received prior therapy with cyclophosphamide or vinca alkaloids.[1] Peritransplant cardiac and respiratory problems may also be exacerbated by the marrow infusion, which could have a major adverse effect on the patient's transplant course. In addition, children undergoing chemotherapy are considered to have inadequate circulatory and renal reserves and may be at increased risk for serious toxicities due to marrow infusion.[2] The therapeutic utility of the CEPRATE® SC Stem Cell Concentration System is its ability to positively select CD34+ cells for engraftment while reducing the amount of DMSO, contaminating cells, cellular debris, and volume of infusate. The therapeutic benefit would be threefold: 1) reduce infusional toxicities, 2) reduce requirements for monitoring during graft infusions and reduce need to treat infusional toxicities, and 3) achieve a practical advantage for long-term marrow storage with smaller volumes.

STUDY DESIGN

A prospective, randomized, multicenter Phase III study was performed using the CEPRATE SC Stem Cell Concentration System to concentrate hematopoietic progenitors for hematopoietic support after high-dose chemotherapy. The control arm was standard buffy coat preparation and infusion for hematopoietic support. The study had two primary endpoints, one for safety and one for efficacy. The primary safety endpoint was to demonstrate equivalent hematological recovery in patients who received marrow concentrated with the CEPRATE SC Stem Cell Concentration System compared to patients who received marrow processed by standard buffy coat preparation. The primary efficacy endpoint was to demonstrate reduced cardiovascular side effects from marrow infusion in patients who received marrow concentrated with the CEPRATE SC Stem Cell Concentration System compared to patients who received marrow processed by standard buffy coat preparation. Secondary endpoints were to measure the capacity of the CEPRATE SC Stem Cell Concentration System to concentrate CD34+ hematopoietic progenitor cells, to compare additional safety parameters, and to evaluate other efficacy parameters.

Ninety-eight patients were entered in the study at five different clinical sites. Patients were women with a diagnosis of Stage II, III or IV breast cancer who had not received more than two prior chemotherapy regimens, including adjuvant chemotherapy; who showed no evidence of progressive disease at time of study entry; and who had not received myelosuppressive chemotherapy within 14 days of marrow harvest. Patients were excluded if they had received previous pelvic radiotherapy, previous treatment with mitomycin-C or carmustine, or if there was bone marrow involvement with tumor at the time of marrow harvest. Of the 98 patients entered in the study, 92 patients were eligible and randomized after marrow harvest to Arm A (CEPRATE) or Arm B (buffy coat). The median age of patients in Arm A (CEPRATE) was 44 years (range 31-58) and in Arm B was 42 years (range 30-55). Demographics were similar in both arms with 65% and 67% of the patients having stage IV breast cancer in Arm A (CEPRATE) and Arm B, (buffy coat) respectively. Of the 92 patients eligible and randomized into the study, 89 were infused with marrow. Three of the patients, who were not infused, are not included in the secondary analyses.

PROCESSING RESULTS

Stem cell processing results are summarized, with medians and ranges, in Table 1. Prior to the processing with the CEPRATE SC Stem Cell Concentration System, the median number of CFU-GM/10^5 was 30, compared to 1781 in the CD34+ selected fraction after processing. The median number of CFU-GM/10^5 cells was 5 in the unadsorbed cell fraction that passed through the CEPRATE SC Stem Cell Concentration System (CD34-depleted). The selection and concentration by the CEPRATE SC Stem Cell Concentration System in this trial resulted in a 46-fold enrichment of CD34+ cells with approximately 60-fold enrichment of CFU-GM cells.

Table 1. Summary of Phase III Cell Processing Results

Analyses	Arm A (Ceprate)
% CD34+, starting	1.7
(Buffy Coat)	(0.4 - 3.5)
% CD34+ after processing	78
(Adsorbed cells)	(16 - 92)
% CD34+ depleted fraction	0.9
(Unadsorbed cells)	(0.1 - 2.4)

PRIMARY SAFETY ENDPOINT RESULTS

Successful neutrophil engraftment by Day 20 post marrow infusion was 91% (40/44) for Arm A (CEPRATE) compared to 88% (42/48) for Arm B (buffy coat). Of the 92 patients in the above "intent to treat" analysis, three patients, two in Arm A (CEPRATE), one in Arm B, were not infused. Of the two patients in Arm A (CEPRATE), one patient withdrew from the study prior to infusion and the other patient died nine days after harvest but prior to infusion. The patient in Arm B (buffy coat) was discontinued from the study due to progressive lung metastases detected after harvest, but prior to infusion.

PRIMARY EFFICACY ENDPOINT RESULTS

The mean maximum systolic blood pressure (BP) increase for the Arm A (CEPRATE) patients (n=42) was 9 mmHg compared to 22 mmHg for the Arm B (buffy coat) patients (n=47). Similarly, the mean maximum diastolic BP increase for the Arm A (CEPRATE) patients was 7 mmHg

compared to 16 mmHg for Arm B (buffy coat) patients. In addition, the Arm A (CEPRATE) patients had a mean maximum heart rate decrease of 9 beats/min compared to 22 beats/min for the Arm B (buffy coat) patients. Thus, all cardiovascular primary endpoints for infusional toxicity were significantly reduced in Arm A (CEPRATE) compared to Arm B (buffy coat) (p value <0.001 for all three endpoints).

SECONDARY EFFICACY RESULTS

The number of Grade 3 or 4 adverse events and interventions occurring on the day of marrow infusion and Day 1 posttransplant are listed in Table 2.

Table 2. Number of Grade 3 or 4 Adverse Events and Interventions Occurring Day 0 or Day 1 After Marrow Infusion

Analyses	Arm A (CEPRATE)	Arm B (Buffy Coat)
Number of Grade 3 or 4 events	16	33
Number of Interventions	21	43

Approximately half as many Grade 3 and 4 adverse events occurred after marrow infusion in Arm A (CEPRATE) patients compared to Arm B (buffy coat) patients. Twelve patients in Arm A (CEPRATE) had Grade 3 or 4 adverse events compared to 19 patients in Arm B (buffy coat). Major clinical adverse events related to marrow infusion were limited to Arm B (buffy coat) patients and included severe hypertension, gross hematuria, acute renal failure, acute respiratory failure, and an anaphylactoid reaction.

Less than half as many medical interventions were required due to adverse events posttransplant in Arm A (CEPRATE) patients compared to Arm B (buffy coat) patients. Sixteen patients in Arm A (CEPRATE) and 20 patients in Arm B (buffy coat) required medical intervention related to adverse events on Day 0 or 1. Interventions on Day 0 and 1 of clinical significance occurred exclusively in Arm B (buffy coat) patients. One patient in Arm B (buffy coat) required endotracheal intubation and mechanical ventilatory support during the marrow infusion. Five patients in Arm B (compared to no Arm A [CEPRATE] patients) required a decrease in the infusion rate of their marrow due to toxicities ranging from severe nausea and vomiting, headaches, an anaphylactoid reaction, and acute respiratory failure. Four patients required intravenous fluids for

gross hematuria. One patient required anti-hypertensive medication for significant hypertension associated with a severe headache.

Three patients in Arm B (buffy coat) and none of the patients in Arm A (CEPRATE), developed potentially life-threatening toxicity associated with the marrow infusion.

IMMUNE RECONSTITUTION RESULTS

The study was designed originally to follow patients through 100 days posttransplant. However, immunophenotyping data were collected at the six month follow-up time point for a small subset of patients. Immune function was also examined on a subset of patients through the use of lymphocyte proliferation assays.

Lymphocyte enumeration was accomplished using fluorescent-labeled monoclonal antibodies and flow cytometric analysis to determine levels of total T-cells (CD3+), total B-cells (CD19+), helper/inducer T-cells (CD4+), suppressor/cytotoxic T-cells (CD8+), and natural killer cells (CD16+ and CD56+), as well as the ratio of CD4+ cells to CD8+ cells (CD4+/CD8+). There were no significant differences between the study arms in absolute counts for total lymphocytes, CD3+, CD4+, CD8+, CD19+, or CD16+ and CD56+ lymphocyte subsets at six months or one year posttransplant. The median absolute lymphocyte count for both arms was below the normal range prior to transplant (See Figure 1). At 100 days, six months, and one year posttransplant, the median absolute lymphocyte count for both arms remained below the normal range. There was no significant difference between the arms at any time point in the median absolute lymphocyte counts.

Quantification of immunoglobulins was accomplished using class specific antibodies and rate nephelometry to determine levels of IgA, IgG, and IgM. There were no significant differences between the study arms for any of the specific immunoglobulins at six months or one year posttransplant.

Determination of cell-mediated immunity was accomplished using lymphocyte proliferation assays that measure the incorporation of ^{3}H-thymidine into newly synthesized DNA to indicate the level of T- or B-cell proliferation in response to mitogens. Kidney bean lectin phytohemagglutinin (PHA), plant lectin concanavalin A (ConA), and pokeweed mitogen (PWM) were used in lymphocyte proliferation assays to assess general cell-mediated immunity. A patient's stimulation index (SI) to each mitogen was calculated using the formula listed below:

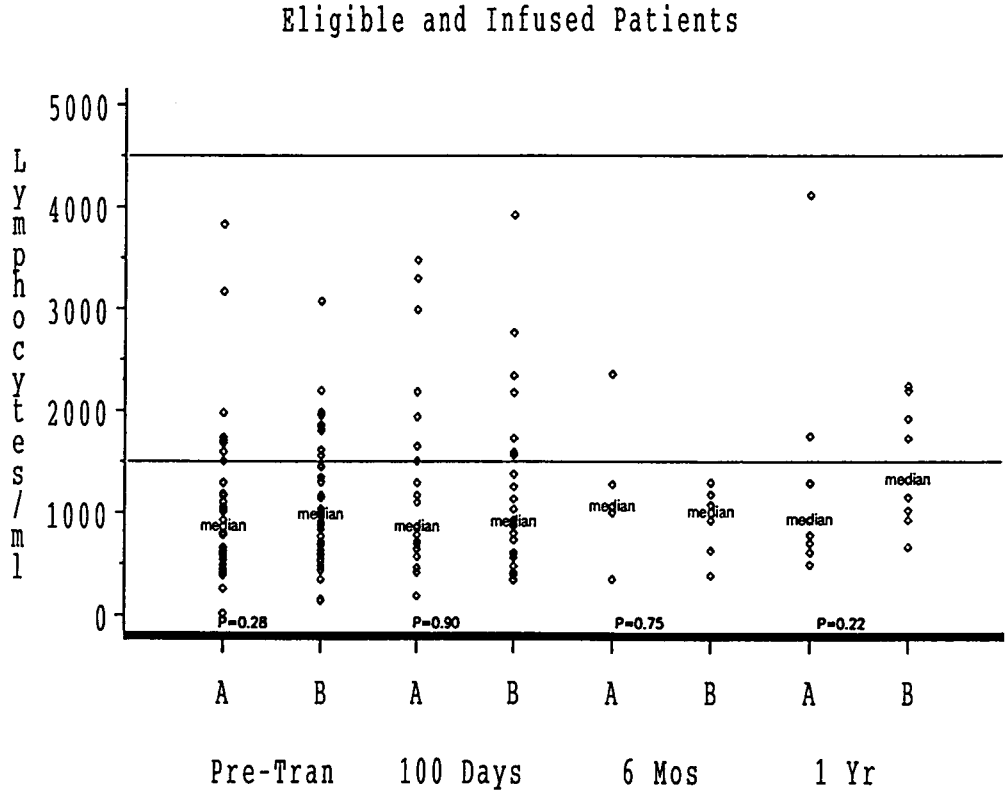

Figure 1. *Absolute lymphocyte counts pre and posttransplant. Horizontal lines represent normal reference range.*

$$\text{Patient SI} = \frac{\text{mean stimulated counts per minute (CPM) patient}}{\text{mean unstimulated CPM patient}}$$

Results for lymphocyte proliferation in response to mitogens are shown by arm in Table 3. Median, minimum and maximum SIs, as well as the number of positive responses/number tested are listed for both arms.

Table 3. General Cell-Mediated Immunity Lymphocyte Proliferation in Response to Mitogens (Current Status as of February 1994)

	Arm A		Arm B		
Mitogen	Median SI (Min-Max)	# Positive # Tested	Median SI (Min-Max)	# Positive # Tested	p-Value[3] (# Positive)
PHA[1]	493 (111-3498)	100% (13/13)	500 (89-1739)	100% (15/15)	NA[4]
ConA[2]	156 (30-924)	77% (10/13)	187 (7-659)	87% (13/15)	0.64
PWM[1]	43 (11-152)	38% (5/13)	69 (5-129)	60% (9/15)	0.45

[1] A positive response to PHA or PWM is defined as an SI >50
[2] A positive response to ConA is defined as an SI >75
[3] Exact test (two-tailed)
[4] Not applicable

Lymphocytes from all 28 patients tested in both arms responded to PHA. Lymphocyte proliferation in response to ConA was observed in most patients including 77% (10/13) of the patients tested in Arm A (CEPRATE) and 87% (13/15) of the patients tested in Arm B (Buffy Coat). Lymphocyte proliferation in response to PWM was observed in some patients including 38% (5/13) of the patients tested in Arm A (CEPRATE) and 60% (9/15) of the patients tested in Arm B (Buffy Coat). No significant difference in lymphocyte proliferation between the arms was observed for any of the mitogen induced proliferation assays.

DISCUSSION

The rate of hematologic recovery in patients who received marrow concentrated with the CEPRATE SC Stem Cell Concentrator was equivalent to the rate of hematologic recovery in patients who received marrow processed by standard buffy coat preparation. In addition, a significant decrease in the hemodynamic side effects of marrow infusion was observed in patients who received marrow concentrated with the

CEPRATE system compared to patients who received standard buffy coat preparation.

The major overall reduction in serious adverse events that was observed with a CD34+ selected marrow infusion compared to a buffy coat infusion in the phase III study should lead to a decrease in the morbidity of patients undergoing ABMT. Additionally, medical interventions especially for serious complications associated with marrow infusion have been markedly reduced in patients receiving CD34+ selected marrow as shown in the phase III study. This has important clinical implications for ABMT patients. Monitoring and treatment for potential cardiovascular, respiratory, and other serious adverse events in an intensive care unit-like setting has been widely used in patients receiving an infusion of buffy coat prepared marrow. This should not be required for patients receiving an infusion of CD34+ cells concentrated using the CEPRATE device. Furthermore, the elimination of life-threatening complications such as acute renal failure, acute respiratory failure, anaphylactoid reactions, and serious cardiovascular complications should result in a decrease in the mortality of patients undergoing ABMT. This will become increasingly important in the near future as mortality from other causes such as infection, bleeding, and chemotherapy-related toxicity continues to be dramatically reduced. As this occurs, reducing the morbidity and mortality associated with autologous marrow infusion will become even more critical to enable the safe application of ABMT in general clinical oncology. By concentrating CD34+ cells using the CEPRATE device, physicians can deliver a safe and effective infusion of autologous marrow to their patients undergoing ABMT.

All marrow transplant recipients have a profound impairment of most immune functions, regardless of the type of graft (autologous or allogeneic), underlying disease, conditioning regimen, or type of preparative regimen.[3,4] Immune reconstitution posttransplant follows a general pattern of development from immature to mature immune functions and is successfully completed in one to two years posttransplant.[3,5-7] After the first year posttransplant, the various components of the immune system of most autologous transplant patients begin to work properly. This pattern of lymphocyte recovery was observed in patients evaluated in the phase III study. In addition, there were no significant differences between the arms in the general pattern of lymphocyte recovery as measured by immunophenotyping at six months or one year posttransplant.

Most transplant studies evaluating recovery of cells have demonstrated that the absolute number of CD4+ cells is lower than normal while the absolute number of CD8+ cells is higher than normal resulting in lower than normal CD4+/CD8+ cell ratios.[3,8-10] This is especially evident in the first six months posttransplant. Although CD4+/CD8+ ratios may normalize after one year posttransplant, absolute numbers may remain abnormal for almost three years posttransplant for CD4+ cells and seven years posttransplant for CD8+ cells.[8] In the phase III study, a reduction in the median CD4+/CD8+ ratio posttransplant was observed in both arms at 100 days, six months and one year posttransplant compared to pre-transplant ratios. There were no significant differences between the arms in the CD4+/CD8+ ratios posttransplant by six months or one year posttransplant.

Mitogens including phytohemagglutinin (PHA), concanavalin A (ConA), and pokeweed mitogen (PWM) were used to evaluate lymphocyte proliferative responses. No significant difference in lymphocyte proliferation between the arms was observed for any of the mitogen induced proliferation assays. Previous studies have shown that *in vitro* proliferative responses after stimulation with mitogens measured by ^{3}H-thymidine uptake are low during the first 100 days posttransplant.[3] Responses to mitogens return to normal or near normal values by six months to one year posttransplant.

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MOBILIZATION OF PERIPHERAL BLOOD PROGENITOR CELLS (PBP) USING TAXOL (T) IN COMBINATION WITH CYCLOPHOSPHAMIDE (C) AND G-CSF, QUANTITATION AND CORRELATION OF CD34+ CELL COUNT, COLONY-FORMING-UNIT-GRANULOCYTE-MACROPHAGE (CFU-GM), AND MONONUCLEAR CELL (MNC) COUNT

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ABSTRACT

We have demonstrated that cyclophosphamide (C)/G-CSF mobilized PBP permit administration of rapidly sequenced courses of high dose chemotherapy. To further assess the role of taxol (T) in PBP mobilization we commenced a phase I study of escalating doses of T, Level (L) I=150, II=200, III=250, IV=300 (mg/M^2) plus high dose C (3g/M^2) with PBP leukaphareses (LP) followed by 4 cycles of high dose carboplatin (CBDCA)/C rescued with PBPs. LP collections were analyzed for CD34+, CFU-GM, and MNC counts. To assess the reliability of our CD34 assay the correlation between CD34 and CFU-GM was analyzed. **Results**: 62 LP collections have been analyzed using flow cytometry for CD33/34 cell surface marker positivity and assessed for CFU-GM/BFU-E (colony formation with SCF+IL-3+IL-6+EPO+G-CSF). Median (range) CD34+cells/kg at level (L) I=14.2 x 10E6 (.2-4.0 x 10E7), L II=6.8 x 10E6 (2.8-14.8 x 10E6), L III=14.2 x 10E6 (.36-2.8 x 10E7), L IV=4.6 x 10E6 (2.0-18.8 x 10E6). The median (range) CFU-GM/kg at L I=2.47 x 10E6 (.1-16.2 x 10E6), L II=5.64 x 10E6 (1.96-11.4 x 10E6), L III=3.32 x 10E6 (.7-11.4 x 10E6) and L IV=4.4 x 10E6 (.14-1.76 x 10E7). The median (range) MNC/kg at level I=5.8 x 10E8 (2.32-12.0 x 10E8), level II=13.6 x 10E8 (7.4-21.3 x 10E8), level III=16.3 x 10E8 (2.48-30.0 x 10E8) and level IV=14.96 x 10E8 (5.64-34.1 x 10E8). Of 54 cycles of high-dose CBDCA/C, a median (range) intertreatment interval of 17 (14-25) days was achieved. Following PBP infusion, median (range) days to ANC >500 was 8 (5-12) and platelets >20K was 11 (8-15). The correlation coefficient for CD34 and CFU-GM for the first 10 patients studied was r=0.83. **Conclusions**: 1) Addition of taxol does not compromise PBP yield following C/G-CSF; 2) Hematologic recovery with PBP mobilized in

this fashion is similar to that achieved with CTX/G-CSF; 3) No dose-response relationship exists in terms of taxol and PBP mobilization.

INTRODUCTION

Advanced ovarian carcinoma remains a disease with a poor prognosis, with long-term disease-free survival reported in approximately 20% of patients. Twelve thousand women will die annually from the disease.[1]

Due to the high response rates and occasional cures seen with this disease, investigators have attempted to improve on the results currently seen through the use of dose intensive therapy. At Memorial Sloan-Kettering Cancer Center we have pursued an aggressive approach to the treatment of advanced ovarian cancer. We previously demonstrated the feasibility of administering rapidly sequenced courses of high-dose chemotherapy in patients by exploiting peripheral blood progenitor cell technology.[2] With the emergence of taxol as an active agent in refractory ovarian cancer we were anxious to explore its potential as a component of a front-line high-dose chemotherapy regimen in the treatment of advanced ovarian cancer. Clinical experience with taxol has shown it to be devoid of cumulative myelosuppression, with thrombocytopenia being uncommon at taxol doses commonly employed. These factors suggest that taxol causes limited toxicity to early hematopoietic progenitor cells and thus would effectively mobilize these cells.

We designed a phase I study of escalating doses of taxol with high-dose cyclophosphamide and G-CSF. The main objective of the study was to determine the maximum tolerated dose of taxol that could be administered with high-dose cyclophosphamide. Additionally, we planned to study it's potential for hematopoietic progenitor cell mobilization, with a view to the design of future studies of high-dose chemotherapy regimens for the treatment of this and other diseases. We studied four dose levels of taxol given with cyclophosphamide and G-CSF. Patients received two cycles of cyclophosphamide/taxol therapy, the first cycle being followed by four leukaphereses. Thereafter, patients received four cycles of carboplatin with cyclophosphamide rescued with taxol/cyclophosphamide and G-CSF primed peripheral blood progenitor cells (Figure 1).

Figure 1. Treatment Plan

Cycle #1:		
Day 1	IV hydration, premedication Cyclophosphamide 3.0 gm/M^2 Taxol*	(inpatient)
Day 10-14	Leukapheresis	(outpatient)
Cycle #2:		
Day 14	IV hydration, premedication Cyclophosphamide 3.0 gm/M^2 Taxol	(inpatient)
Cycle #3:		
Day 28	IV hydration, premedication CBDCA@/CPA#	(inpatient)
Day 31	PBP reinfusion	(day hospital)
Cycle #4:		
Day 14	IV hydration, premedication CBDCA/CPA	(inpatient)
Day 43	PBP reinfusion	(day hospital)
Cycle #5:		
Day 56	IV hydration, premedication CBDCA/CPA	(inpatient)
Day 59	PBP reinfusion	(day hospital)
Cycle #6:		
Day 70	IV hydration, premedication CBDCA/CPA	(inpatient)
Day 73	PBP reinfusion	(day hospital)

* *Taxol escalating dose (150-300 mg/M^2)*
@ *Carboplatin 1000 mg/M^2*
\# *Cyclophosphamide 1.5 gm/M^2*
All chemotherapy courses followed by G-CSF

OBJECTIVES

The objectives of this study were to determine the maximum tolerated dose of taxol that could be administered with high-dose (3.0 gm/M^2) cyclophosphamide. Additionally, we studied the mobilization of hematopoietic progenitors into the peripheral blood in patients treated with G-CSF during the recovery from high-dose cyclophosphamide/taxol, and the ability of these progenitor cells to rescue repeated courses of high-dose carboplatin and cyclophosphamide. The study was approved by the Institutional Review Board of the Memorial Sloan-Kettering Cancer Center, and all patients signed informed consent indicating full understanding of the risks and potential benefits of the research prior to protocol entry.

PATIENTS AND METHODS

Sixteen patients were enrolled on this phase I study. Patient characteristics are detailed in Table 1. Of the 16 patients entered on study, 10 were sub-optimally debulked at initial surgery (>1cm residual disease). There were 11 patients with stage IIIC, 4 patients with stage IV, and 1 patient with stage IIC disease.

Table 1. Patient Characteristics

Number of Patients	16
Median Age	46
Age range	22-63
Histology	
Adenocarcinoma - Papillary-serous	10
- Endometrioid	3
- Undifferentiated	2
- Clear Cell	1
Histologic Grade	
2	6
3	9
Unknown	1
Debulking	
Optimal	6
Sub-optimal	10
Stage	
IIc	1
IIIc	11
IV	4

Eligibility Criteria

To be eligible for this study, patients were required to have histologically proven FIGO stage IIC-IV ovarian cancer. Patients could have received no prior chemotherapy or radiotherapy. Hematologic eligibility criteria included a normal peripheral blood count with an absolute neutrophil count >1.5 x 10^9/L and a platelet count >150 x 10^9/L, normal prothrombin time and normal activated partial thromboplastin time. Patients with heart disease or other active serious medical or psychiatric disease were excluded. The following biochemical parameters were also required: bilirubin <1.5 x upper limit of normal and normal serum creatinine.

Treatment Plan

All patients had double-lumen leukapheresis-grade vascular access catheters placed on entry to the protocol. Prior to chemotherapy, patients were pre-medicated with ondansetron at a fixed dose of 32 mg. They also received decadron 20 mg with lorazepam 2 mg intravenously. Cyclophosphamide 3 g/M^2 was administered as a one-hour infusion followed six hours later by taxol as a 24-hour infusion. Taxol doses were escalated in cohorts of three patients, with no intra-patient dose escalation. Patients received; level I - taxol 150 mg/M^2, level II - 200 mg/M^2, level III - 250 mg/M^2 and level IV - taxol 300 mg/M^2. The treatment was repeated on a planned two-weekly schedule. Patients commenced G-CSF at 5 μg/kg subcutaneously commencing 24 hours following chemotherapy. Upon recovery of absolute neutrophil count to >1.0 patients underwent a series of four daily leukaphereses procedures. Upon recovery following the second cycle of cyclophosphamide and taxol, patients proceeded to cycle 1 of high-dose carboplatin 1000 mg/M^2 with cyclophosphamide 1,500 mg/M^2. Cyclophosphamide was administered as a one-hour infusion followed by carboplatin administered as a 12-hour continuous infusion. Peripheral progenitor cells were reinfused in the day-hospital at 72 hours following completion of the carboplatin. Four sequential cycles of carboplatin/cyclophosphamide were administered.

Progenitor Cell Collection and Reinfusion

Daily leukapheresis procedures were performed using a Fenwall CS 3000 (Baxter, Chicago, Illinois) cell separator. Ten liters were processed per procedure, using continuous flow centrifugation at a flow rate of 70 mls/minute. The procedure usually lasted between 120 to 180

minutes and was not associated with significant complications. Following removal of citrated plasma, the suspension was mixed with an equal volume of dimethyl sulfoxide cryoprotectant solution. The resultant 100 cc product was frozen in marrow freezing bags for storage. For reinfusion, the product was thawed in a 37°C water-bath and reinfused as a bolus solution via the leukapheresis-grade catheter over a three- to five-minute period. Patients received D51/2NS at 200 cc/hr x 2 hours prior to progenitor-cell infusion.

Figure 2. Progenitor Cell Collection

Leukapheresis procedures:
Fenwall CS 3000 (Baxter, Chicago) cell separator.

Ten liters processed per procedure.

Continuous flow centriguation - flow rate of 70 mls/minute.

Following removal of citrated plasma, suspension was mixed with DMSO cryoprotectant solution.

The product was frozen in marrow freezing bags for storage.

Progenitor Cell Reinfusion

Thawed in a 37°C water-bath and reinfused as a bolus solution over a three- to five-minute period.

Patients received D51/2NS at 200 cc/hr x 2 hours prior to progenitor-cell infusion.

Additional premedications included:

Zofran 24 mg IV
Decadron 20 mg IV
Lorazepam 2 mg IV

Patients monitored for evidence of hemoglobinuria for 1 hour prior to discharge.

Analysis of CD34 Antigen Positivity

Aliquots of the daily leukapheresis product were analyzed for presence of circulating hematopoietic progenitors (Figure 3). Cells expressing the surface membrane CD34 and/or CD33 antigens were identified by flow cytometry using direct immunofluorescence analysis. A small aliquot of leukapheresis cell suspension was incubated with a mixture of CD34 FITC-conjugated anti-HPAC-2 antibody (Becton Dickinson) and CD33 phycoerythrin (PE)-conjugated My9 antibody (Coulter). The possibility that non-specific binding of the FIT-conjugated anti-HPCA and PE-conjugated My9 antibodies could also account for CD34+ and CD33+ cells was ruled out using FITC-conjugated isotype-matched IgG2b (Coulter) irrelevant antibodies. After incubation, erythrocytes were lysed with Immunoprep leukocyte preparation system Q PREP (Coulter) and then the entire nulceated cell population was analyzed by flow cytometry using a FACS (Coulter). The frequency of the cells expressing CD34 and/or CD33 antigens was calculated as a percentage of all analyzed cells. The target cell number for leukapheresis for adequate hematologic rescue was 0.5 x 10^6 CD34+ cells per kilogram body weight/cycle of high-dose chemotherapy.

Figure 3. Analysis of CD34 Antigen Positivity

1. Expression of surface membrane CD34 and/or CD33 antigens identified using direct immunofluorescence analysis (flow cytometry).
2. Then incubated with a mixture of CD34 FITC-conjugated anti-HPAC-2 antibody (Becton Dickinson) and CD33 phycoerythrin (PE)-conjugated My9 antibody (Coulter).
3. Non-specific binding of the FITC-conjugated anti-HPCA and PE-conjugated My9 antibodies was prevented by using FITC-conjugated isotype-matched IgG2b (Coulter) irrelevant antibodies.
4. Erythrocytes were lysed with Immunoprep leukocyte preparation system Q PREP (Coulter) and then the entire nucleated cell population was analyzed by flow cytometry using a FACS (Coulter).
5. The frequency of the cells expressing CD34 and/or CD33 antigens was calculated as a percentage of all analyzed cells.
6. The target cell number for leukapheresis for adequate hematologic rescue was 0.5 x 10^6 CD34+ cells/kg body wt/cycle of high-dose chemotherapy.

RESULTS

All patients completed all planned courses of cyclophosphamide/taxol therapy (Table 2). Thirty-two cycles of taxol/cyclophosphamide were administered (level I = 6, level II = 6, level III = 10, level IV = 10).

Table 2. Treatment Intervals, Days to ANC Recovery, Admissions and Documented Positive Blood Cultures at Each Dose Level of Taxol Evaluated

CTX/TXL Level	Cycles	Intervals	Days to ANC >.5 median (range)	Admit	C + S Positive
I	6	14 (13-21)	10 (8-12)	4	0
II	6	13 (13-14)	9 (8-9)	1	0
III	10	14 (12-19)	9 (5-10)	5	1
IV	10	14 (14-17)	10 (7-10)	0	0

Progenitor Cell Mobilization

Sixty-two leukapheresis collections have been analyzed using flow cytometry for CD33/34 cell surface marker positivity. In addition, we evaluated the mobilization of progenitor cells at each taxol dose-level in an effort to identify any possible dose-response effect that may exist. The median number of CD34+ cells/kg/leukapheresis at levels I = 3.56×10^6 (range 5.0×10^5 - 1.0×10^7); level II = 1.72×10^6 (range 7.0×10^5 - 3.7×10^6); level III = 3.56×10^6 (range 9.0×10^5 - 7.0×10^6), and level IV = 1.15×10^6 (range 5.0×10^5 - 4.7×10^6). The median number of CFU-GM at level I = 6.18×10^5 (range 2.55×10^4 - 4.05×10^6), level II = 1.41×10^6 (range 4.90×10^6 - 2.86×10^6), level III = 8.31×10^5 (range 1.75×10^5 - 2.86×10^6) and level IV = 1.10×10^6 (range 3.64×10^4 - 4.41×10^6). The CD34+ cells/CFU-GM/kg/leukapheresis per dose-level is depicted in Table 4 and 5. The CD34+ cells/CFU-GM and MNC yield/Kg/leukapheresis for each patient is outlined in Table 5. To determine the reliability of our CD34 assay we assessed correlation between CD34+ and CFU-GM in our first 10 patients and found a correlation of r = 0.83 (Figure 4).

Fifty-four cycles of carboplatin/cyclophosphamide were given, rescued with taxol/cyclophosphamide/G-CSF primed peripheral blood-derived progenitor cells. Median intertreatment intervals for carboplatin/cyclophosphamide courses when rescued with taxol/cyclophosphamide-primed peripheral progenitor cells was 17 days

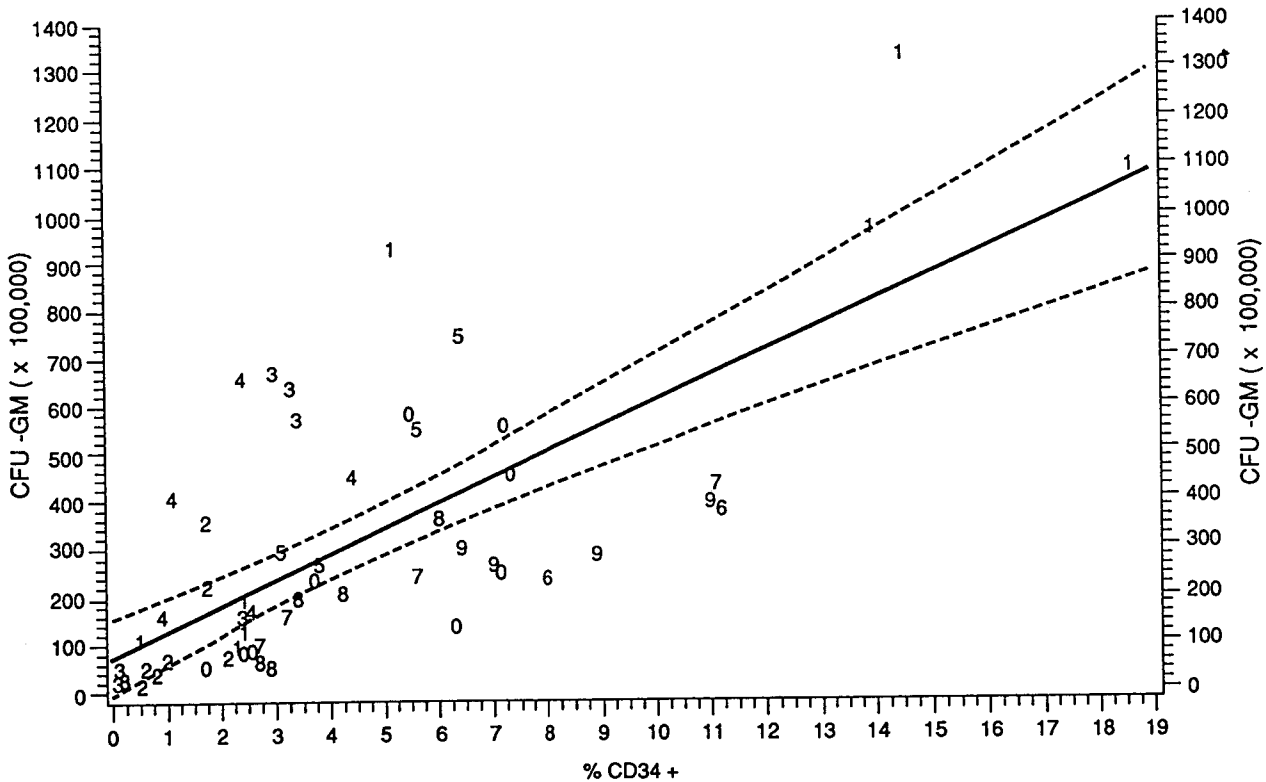

Figure 4. *Leukaphereses Collections: Correlation between CFU-GM and CD34+.*

(range 14-25). The median number of days to absolute neutrophil count >0.5 was 8 (range 5-12) and self-sustaining platelet count >20K was 11 (range 8-15), both measured from the day of progenitor cell infusion (Table 3).

Table 3. Days to Recovery of Absolute Neutrophil and Platelet Counts Measured from the day of Progenitor Cell Infusion

CBDCA/ CPA Cycles	Treatment interval median (range)	Days to ANC >.5 median (range)	Days to Plts >20K median (range)	Admit	C + S Positive
54	17 (14-25)	8 (5-12)	11 (8-15)	10	1

Table 4. Median CD34+ Cell Yield/Kg per Leukapheresis Per Dose-Level

Level	No. Leuk	CD34+/kg (median)	CD34+/kg (range)
I	13	3.56×10^6	5.0×10^5 - 1.0×10^7
II	13	1.72×10^6	7.0×10^5 - 3.7×10^6
III	16	3.56×10^6	9.0×10^5 - 7.0×10^6
IV	20	1.15×10^6	5.0×10^5 - 4.7×10^6

Table 5. Median CFU-GM Yield/Kg Per Leukapheresis Per Dose-Level

Level	No. Leuk	CFU-GM/kg (median)	CFU-GM/kg (range)
I	13	6.18×10^5	2.55×10^4 - 4.05×10^6
II	13	1.41×10^6	4.90×10^5 - 2.86×10^6
III	16	8.31×10^5	1.75×10^5 - 2.86×10^6
IV	20	1.10×10^6	3.64×10^4 - 4.41×10^6

Table 6. Analysis of Mononuclear Cell Count, CD34+ Cell Count and CFU-GM Per Kilogram Per Leukapheresis

Patient	Leuk	MNC/kg	CD34+/kg	CFU-GM/kg
Level I - Taxol 150 mg/M^2				
1	1	1.31×10^8	3.79×10^6	1.89×10^5
	2	2.38×10^8	4.25×10^6	6.18×10^5
	3	2.79×10^8	5.25×10^6	1.58×10^6
	4	2.62×10^8	3.15×10^6	1.54×10^6
	5	1.11×10^8	1.66×10^6	2.72×10^5
2	1	1.18×10^8	1.04×10^7	1.29×10^6
	2	3.04×10^8	1.07×10^7	4.05×10^6
	3	2.94×10^8	9.12×10^6	2.86×10^6
	4	2.10×10^8	3.56×10^6	1.96×10^6
3	1	5.80×10^7	5.07×10^5	2.55×10^4
	2	1.16×10^8	1.64×10^6	1.04×10^5
	3	1.45×10^8	1.20×10^6	3.36×10^5
	4	1.16×10^8	1.26×10^6	4.22×10^5
Level II - Taxol 200 mg/M^2				
4	1	1.86×10^8	1.37×10^6	1.26×10^6
	2	3.22×10^8	1.59×10^6	1.88×10^6
	3	2.03×10^8	1.59×10^6	1.31×10^6
	4	2.88×10^8	1.10×10^6	4.90×10^5
5	1	3.40×10^8	1.72×10^6	1.41×10^6
	2	3.77×10^8	2.40×10^6	1.73×10^6
	3	3.77×10^8	2.23×10^6	2.51×10^6
	4	3.21×10^8	1.96×10^6	5.84×10^5
	5	4.15×10^8	7.00×10^5	7.14×10^5
6	1	2.83×10^8	3.33×10^6	1.58×10^6
	2	5.33×10^8	1.73×10^6	1.45×10^6
	3	5.17×10^8	3.70×10^6	3.87×10^6
	4	3.67×10^8	1.42×10^6	1.12×10^6

Table 6 (cont)

Patient	Leuk	MNC/kg	CD34+/kg	CFU-GM/kg
Level III - Taxol 250 mg/M^2				
7	1	8.90×10^7	6.94×10^6	3.45×10^5
	2	2.35×12^8	3.41×10^6	5.65×10^5
	3	2.75×10^8	9.06×10^5	2.09×10^5
	4	2.65×10^8	1.16×10^6	1.75×10^5
8	1	4.43×10^8	1.46×10^6	7.62×10^5
	2	6.15×10^8	6.15×10^6	2.70×10^6
	3	7.51×10^8	2.63×10^6	1.89×10^6
	4	4.46×10^8	1.11×10^6	5.00×10^5
9	1	7.50×10^7	4.06×10^6	2.79×10^5
	2	6.23×10^8	3.89×10^6	2.86×10^6
	3	5.28×10^8	1.45×10^6	1.10×10^6
	4	6.04×10^8	2.21×10^6	1.30×10^6
10	1	6.20×10^7	7.05×10^6	1.95×10^5
	2	3.11×10^8	6.79×10^6	1.25×10^6
	3	4.08×10^8	3.83×10^6	1.20×10^6
	4	4.08×10^8	3.71×10^6	1.13×10^6
Level IV - Taxol 300 mg/M^2				
11	1	7.71×10^8	1.16×10^6	1.85×10^5
	2	7.82×10^8	5.09×10^5	1.56×10^5
	3	8.18×10^8	9.27×10^5	1.96×10^5
	4	2.60×10^8	9.64×10^5	3.64×10^4
12	1	5.15×10^8	1.03×10^6	1.10×10^6
	2	3.57×10^8	1.02×10^6	2.79×10^5
	3	2.77×10^8	1.15×10^6	2.16×10^5
	4	2.64×10^8	1.11×10^6	1.40×10^6
13	1	2.51×10^8	2.38×10^6	1.00×10^5
	2	1.43×10^8	9.81×10^5	6.19×10^5
	3	2.58×10^8	1.15×10^6	1.29×10^6
	4	4.36×10^8	1.53×10^6	1.43×10^6
14	1	5.34×10^8	4.66×10^6	3.31×10^6
	2	8.53×10^8	3.62×10^6	3.93×10^6
	3	3.05×10^8	2.59×10^6	1.82×10^6
	4	1.41×10^8	2.07×10^6	

Table 6 (cont)

Patient	Leuk	MNC/kg	CD34+/kg	CFU-GM/kg
15	1	5.78×10^8	2.71×10^6	4.41×10^6
	2	4.76×10^8	2.20×10^6	1.15×10^6
	3	3.92×10^8	1.10×10^6	5.95×10^5
	4	2.51×10^8	7.14×10^5	1.11×10^8

Response to Therapy

Fourteen patients have undergone surgical evaluation. Of 13 patients evaluable for response, there were 5 patients with pathological complete responses (38.5%), 6 patients with microscopic residual disease (46%), and 2 patients with pathological partial responses for an overall response rate of 100%. One patient at surgical evaluation was found to have microscopic disease only, but due to an inadequate initial staging procedure was not evaluable for response.

DISCUSSION

The application of high-dose chemotherapy in advanced ovarian cancer remains controversial. A number of investigators have evaluated the role of very high-dose chemotherapy requiring autologous bone marrow support. Consistently high responses have been obtained, however these are frequently of short duration. Agents such as melphalan, cyclophosphamide, and thiotepa are active in ovarian cancer, and can be substantially escalated with the use of autologous bone marrow support.[3,4] Using high-dose melphalan in patients who had failed prior cisplatin therapy, Stoppa et al reported a 75% overall response rate. At a median follow-up of 23 months, 15/35 patients were alive and disease-free. Dauplat et al, utilizing a similar regimen, obtained a 36% 2-year disease-free survival again in patients failing induction cisplatin regimens.[5] Stiff et al, in a survey of autologous bone marrow transplant centers in the United States reported on data collected from eleven centers.[6] The survey identified only 5% of patients transplanted in their first remission. The majority of patients were transplanted with relapsed or refractory disease.

In previous studies at Memorial Sloan-Kettering Cancer Center, we have demonstrated the feasibility of administering rapidly sequenced courses of high-dose chemotherapy in patients with advanced ovarian cancer by exploiting PBP technology.[2] The use of progenitor cells either with or without autologous bone marrow has become an accepted means of abrogating the myelotoxicity of high-dose chemotherapy regimens. Investigators in Adelaide previously reported that the reinfusion of these

"mobilized" PBP, following later high-dose therapy, resulted in rapid hematologic recovery compared to historical controls receiving autologous bone marrow transplantation.[7,8] Socinski et al reported that patients receiving cytokine-mobilized PBP had more rapid hematologic recovery compared to that of historically controlled patients rescued with ABMT alone.[9,10] Siena identified CD34 content of the leukapheresis collection as the best predictor of engraftment[11] although this work has been somewhat controversial. Jannsen et al reported lack of reliability of using the CD34+ cell fraction as a predictor of CFU-GM and subsequent hematologic recovery.[14] Spitzer et al reported that hematological response to cytokines during progenitor cell collection was more predictive of neutrophil recovery than either CD34 cells or CFU-GM.[12-15] In our study, we demonstrated good correlation between CD34+ yield and CFU-GM. A major concern when employing peripheral progenitor cell rescue is that in endeavoring to adequately mobilize progenitor cells there may be a delay in the initiation of effective chemotherapy. In the case of ovarian cancer, the use of cisplatin as initial therapy may hamper subsequent efforts at progenitor cell mobilization, although Shea et al have recently reported on their experience with carboplatin as a progenitor cell mobilizing agent.[16] The possibility of additional antitumor treatment constitutes a theoretical advantage for a chemotherapy-based mobilization. This is of particular relevance in the setting of sequential high-dose chemotherapy for ovarian cancer. Cyclophosphamide has long been recognized as an active agent in the treatment of advanced ovarian cancer. Taxol has obtained responses in both heavily and minimally pretreated ovarian cancer patients (20-37%).[17,18]

The combination of taxol with cyclophosphamide may offer significant antitumor efficacy. A potential added advantage, particularly in relation to the use of high-dose therapy, is that we have demonstrated that the addition of taxol does not compromise mobilization of peripheral blood progenitor cells which may be of value during subsequent high-dose chemotherapy.

The addition of taxol to this regimen does not compromise progenitor cell mobilization and may offer improved antitumor efficacy.

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SESSION X: RADIOLABELED ANTIBODIES

IMPROVED RADIOIMMUNOCONJUGATES FOR CANCER TREATMENT

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ABSTRACT

The potential for selectivity and quantification in radiolabeled immunoglobulin therapy can only be fulfilled by properly designed and quality controlled radioimmunoconjugates. As a first step, monoclonal antibodies, of human or murine origin, recognizing tumor-associated antigens are produced and purified. In the next phase, immunoconjugates are radiolabeled after the development of three essential elements: i) synthetic chemistry of bifunctional chelates, ii) conjugation of chelates to antibodies and radiolabeling, and iii) *in vitro* and *in vivo* preclinical evaluation of the radiolabeled product. Finally, on the basis of the described preclinical results, the best radioimmunoconjugates are selected and prepared for clinical trials.

Indium-111 (^{111}In) and Yttrium-90 (^{90}Y) are considered the most appropriate isotopes for diagnosis and therapy, respectively. Chelation chemistry is required to link radiometals to protein. Derivatives of diethylenetriaminepentaacetic acid (DTPA) chelator are synthesized containing the appropriate number of ligands and carbon backbone substitutions that optimize stability of the metal-chelate complex *in vivo*. DTPA backbone substituted (2B3M) chelate-IgG conjugates (CC49) labeled with In or Y showed higher metal-complex stability, and less retention of radioactivity observed in liver, spleen, and femur when compared to ITCB-DTPA immunoconjugates. ^{111}In and ^{90}Y labeled CC49 conjugates showed similar tumor targeting and pharmacokinetics in nude mice. Prior to *in vivo* administration, radioimmunoconjugates are analyzed for radiochemical purity, immunoreactivity, and *in vitro* serum stability, sterility and pyrogenicity. Preclinical evaluation of radioimmunoconjugates is performed in nude mice bearing human tumor xenografts and in normal beagle dogs.

Different sizes of radioimmunoconjugates are required for different routes of administration (large for i.p. and intratumoral injection; small for IV injection). IgM conjugates are stable after i.p. injection and have a long half-life (100 h) in the peritoneal cavity. Pharmacokinetic patterns of ^{111}In and ^{90}Y labeled DTPA-IgM were similar in nude mice with peritoneal carcinomatosis of a human cancer.

The correlation of clinical and preclinical results is of crucial importance to the development of clinical radiolabeled immunoglobulin therapy because it determines the predictive value of a preclinical analysis. Progress in clinical application will be nearly impossible without good preclinical models.

INTRODUCTION

Optimal selection of radiolabeled antibodies for clinical radiolabeled immunoglobulin therapy (RIT) is based on quantitative evaluation in the laboratory and in animal models. The origin of monoclonal antibodies and design of immunoconjugates play an important role in determining the pharmacokinetic characteristics of RIT reagents. Originally, murine IgG and its fragments were frequently used in the clinic.[1-3] Most patients develop an immune response against the administered murine monoclonal antibody (HAMA - human anti-murine antibody). This precludes the repeated use of the same RIT reagent in HAMA-positive patients. Human monoclonal antibodies (huMoAbs) are potentially non-immunogenic in human patients and are being evaluated.[4] Currently available huMoAbs are IgM, a large pentamer (MW 950.000). Proteolytic digestion of IgG or IgM produces lower molecular weight fragments (Fab or F(ab')$_2$, that can penetrate more efficiently into tumor vasculature when injected intravenously. The radiometals (^{111}In or ^{90}Y) are considered promising radioisotopes for outpatient diagnosis or therapy. Antibodies are conjugated to chelators via a covalent linkage, producing a chelate-immunoconjugate. The function of a chelate is to bind the radiometal (^{111}In or ^{90}Y). Radioimmunoconjugates are evaluated for tumor targeting and pharmacokinetic studies in nude mice bearing human tumors. Beagle dogs are employed for radiotoxicology and pharmacology studies.[5] In this communication we describe some of our newer approaches to the development of radioimmunoconjugates and explore their tumor targeting ability in nude mice.

METHODS AND MATERIALS

Improved Chelate-immunoconjugates

We synthesized new derivatives of the DTPA (diethylenetriaminepentaacetic acid) chelator for ^{111}In and ^{90}Y radioisotopes. These new chelators are characterized by a carbon backbone substitution that induces rigidity and inertness in metal-chelate complex under physiological conditions. In addition to backbone modification, these agents also contain the aminobenzyl group on the carbon framework which is necessary in a linker conjugation procedure. These chelators were synthesized by complex multi-step organic reaction procedures.[6] The final products were purified and analyzed by various spectroscopic techniques. These chelators were converted into isothiocyanatobenzyl (ITCB) derivatives for antibody conjugation.

Murine Monclonal IgG (CC49)

Monoclonal IgG, CC49, was obtained from Dr. Jeffrey Schlom (National Cancer Institute, Bethesda, MD). CC49 has shown to be highly reactive with tumor-associated glycoprotein 72.[7] The 2B3M and ITCB chelators were conjugated to monoclonal antibody (CC49) via a thiourea linkage.

Human Monoclonal IgM (AC6C3)

The human IgM was developed previously by Freedman (Department of Gynecology, MDACC). AC6C3 recognizes a 32 Kd antigen determinant expressed on the cell membrane of various malignancies including ovary, colon, and breast.[8] Human IgM was isolated and purified from cell culture supernatant. Purity of IgM was tested by SDS-PAGE and size-exclusion HPLC. Immunoreactivity was determined by ELISA and fluorescence activated cell sorter (FACS) analysis. Immunoconjugates were prepared by reacting IgM with ITCB-DTPA in a 0.2 M bicarbonate buffer, pH 8.0. Prior to radiolabeling, the IgM conjugates were purified from unconjugated DTPA by centricon-30 filtration.

Radiolabeling of Immunoconjugates

A 20 µl aliquot of $^{111}InCl_3$ (3.7 mCi) was equilibrated with 250 µl of 0.06 M sodium citrate buffer and 0.6 M Sodium acetate buffer, pH 5.5. Two hundred fifty microliters of immunoconjugate (10 mg/ml) in PBS was added to buffered indium, mixed well, and incubated at room temperature

for 45 minutes. This labeling mixture was challenged with a 100-fold excess of free DTPA to sequester free isotope and remove weakly labeled radioisotope from the protein before column chromatography. The labeled immunoconjugates were separated from low molecular weight moieties by size-exclusion chromatography with a Sephadex G50 gel column (1.5 x 20 cm) using 0.05 M PBS as eluant.

A 5 μl aliquot of $^{90}YCl_3$ (6.5 mCi) in 0.1M HC1 was equilibrated with 200 μl of 2.0 M acetate buffer, pH 6.0. An aliquot of 200 μl liters of immunoconjugates solution (5 mg/ml) in PBS was added into buffered yttrium-90, mixed well, and incubated at room temperature for 1 hour. DTPA challenge and size-exclusion chromatography methodology were similar to that described for ^{111}In-labeling except that a Sephadex G100 column (1.5 x 30 cm) was used.

Tumor inoculation

For a subcutaneous tumor model, a human tumor (LS174T or SW620) was xenografted into the right hind leg of nude mice by a subcutaneous injection of tumor cells suspension (2 x 10^6 cells). The human colorectal carcinoma cell line, SW620, was obtained from Janet Price (Cell Biology, MDACC). It was maintained in monolayer culture in ROMI medium containing 10% fetal calf serum in the presence of $C0_2$ at standard cell culture conditions. For intraperitoneal inoculation, cells were washed with 0.05 M PBS and resuspended to a concentration of 2 x 10^6 cells in 0.2 ml RPMI media/mouse. Each mouse was injected through the abdominal wall directly into the abdominal cavity with a 25 gauge needle. Female athymic nude mice (nu/nu, NCR) were housed in a closed colony and provided with sterile food and water. Animal health and tumor growth were monitored daily.

Biodistribution Studies

Biodistribution studies of IgG radioimmunoconjugates were performed in nude mice with subcutaneous human colon carcinoma xenograft (LSI74T). When the size of the tumor was approximately 0.5 to 1 cm in diameter, the mice were injected with purified ^{111}In or ^{90}Y-labeled immunoconjugates (20 μCi, specific activity 2 mCi/mg) via the tail vein. Animals were sacrificed at 1, 2, 4 and 6 days after the injection of the radioimmunoconjugates. Organs were excised, weighed promptly and counted in a gamma counter.

Nude mice with intraperitoneal tumor lumps (SW620) were selected for study. The athymic mice bearing intraperitoneal SW620 human carcinoma lumps were injected with either indium-111 or yttrium-90 labeled IgM conjugate at an activity of 20 μCi (1 mCi/ mg). Using a 25 gauge needle, the radioimmunoconjugates were administered within 2 hours of preparation by a transcutaneous intraperitoneal injection of 0.2 ml total volume per mouse. For biodistribution studies, 4 mice were euthanized with $C0_2$ at each of the 2, 24, 72, 120, and 144 h post-injection time points. Approximately 0.5 ml of blood was removed by cardiac puncture, weighed, and prepared for radioactivity assay. Intraperitoneal tumor and normal tissue samples (whole organs in every case except for the liver and small intestines) were removed, cleaned in saline solution, weighed and assayed for radioactivity. Instruments were cleaned with 70% ethanol between organ samplings to reduce cross-contamination. A gamma counter (Cobra II, Packard Instrument Co.) was used to determine counts per minute per gram of sample.

RESULTS

Improved chelate-immunoconjugates

In vitro serum stability study of ^{111}In and ^{90}Y-labeled immunoconjugates was carried out using analytical chromatography procedures. The data indicate that the substitution of isothiocyanatobenzyl group at C-2 and a methyl group at C-3 produced less leakage of isotope for both indium and yttrium compared to ITCB-DTPA in a DTPA challenge serum stability test.

A comparison of the pharmacokinetics and biodistribution between the 2B3M-DTPA and ITCB-DTPA immunoconjugates labeled with indium-111 is shown in Figure IA. Excellent tumor localization was achieved on day 2 following injection. The two immunoconjugates had similar blood clearance. A significantly lower retention of radioactivity was found in the liver and spleen for the 2B3M-DTPA conjugate when compared to the ITCB-DTPA conjugate. Localization of the 2B3M-DTPA conjugate in other normal organs was very similar to the biodistribution patterns for the ITCB-DTPA conjugate. Bone marrow uptake of indium-labeled immunoconjugates was not observed in any of the mice in this study. Similar biodistribution patterns were obtained with ^{90}Y-radiolabeled 2B3M-DTPA and ITCB-DTPA immunoconjugates when compared in the nude mice tumor model as illustrated in Figure lB. Femur uptake of ^{90}Y for the 2B3M-DTPA conjugate was about 50% less than for the ITCB-DTPA

conjugate for all time points. Lower retention of radioactivity in liver, spleen and femur by yttrium-labeled 2B3M-DTPA conjugate is due to the improved chelation stability. The radioactivity levels in tumors were quite similar for both conjugates labeled with ^{111}In or ^{90}Y.

IgM Conjugates for Compartmental RIT

A DTPA-IgM conjugate was prepared by coupling ITCB-DTPA to IgM and labeled with either indium-111 or yttrium-90. The radioimmunoconjugates were purified by column chromatography. The immunoreactivity of IgM-DTPA conjugate was similar to native IgM as determined by FACS and ELISA. Table 1 shows the quality control analysis of radioimmunoconjugates.

Table 1.

Analysis	^{111}In-DTPA-IgM	^{90}Y-DTPA-IgM
DTPA/IgM	4.0	4.0
Specific Activity	1.0 mCi/mg	1.0 mCi/mg
Radiochemical Purity		
ITLC	98%	94%
TLC	98%	96%
Serum Stability*		
ITLC	93%	91%
Immunoreactivity		
FACS	83%	83%

**after 48 h incubation at 37° C in human serum*

Nude mice bearing multiple intra-abdominal lumps of human colorectal carcinoma (SW620) were used as a model for peritoneal carcinomatosis (Fig. 1). The biodistributions of radioimmunoconjugates were compared in nude mice at 2, 24, 72, 120 and 144 hours after intraperitoneal administration of the In-111 and Y-90 labeled reagents (Fig 2). Both, indium- and yttrium-labeled IgM showed high specific tumor uptake (Fig. 2). The effective tumor half-lives of the immunoconjugates were 39 h and 46 h for indium and yttrium, respectively. The tumor-to-normal organ ratios of tissues surrounding the peritoneal cavity were similar for both reagents. The tumor-to-femur ratio was lower for the yttrium-90-labeled immunoconjugate. A control human monoclonal IgM (CR4E8) reactivity for human squamous cell carcinoma was also labeled with yttrium-90 and studied in the same model. AC6C3 to CR4E8 tumor uptake ratios were 4.5 and 4.6 at 24 and 120 hours, respectively.

IgM Conjugates for Intravenous Administration

The biodistribution of indium-111-labeled IgM was studied after intravenous (i.v.) administration in nude mice bearing a subcutaneous (s.c.) xenograft and compared to intraperitoneal (i.p.) administration in nude mice bearing peritoneal tumor lumps. Intravenously administered IgM cleared rapidly from the blood and was deposited mainly in the liver (50% ID/g), pancreas (20% ID/g), and kidney (10% ID/g) at 24 h (Fig 3). Tumor Deposition was low (≤1.0% ID/g) in the s.c. tumor xenograft. In contrast, high tumor targeting (29% ID/g) was obtained in peritoneal tumor lumps after i.p. administration of ^{111}In-labeled IgM. Long peritoneal residence time and low liver uptake (7% ID/g) were observed after i.p. administration. Blood activity was less than 1% of the injected activity. Tumor-to-normal tissue ratio at 24 h was very high for blood (290), muscle (62), and femur (47). Other organs showed moderate tumor-to-non-tumor ratios, such as spleen (4), kidney (5), liver (4), and pancreas (3). Figure 4 shows the data plotted for the whole body retention of radioactivity in nude mice which was measured over a period of seven days (n=4). The biological half-life for i.p. administration of ^{111}In labeled IgM (AC6C3) was determined by slope of % retention activity over time. The half-life of the radioimmunoglobulin was approximately 67 h for the whole body.

DISCUSSION

Substantial reduction in percent injected dose per gram in liver and spleen were observed with the ^{111}In and ^{90}Y-labeled CC49 antibody using a 2B3M-DTPA chelator. This preliminary data supports the hypothesis that carbon-backbone substituted chelate such as the 2B3M-DTPA conjugate, may provide an improvement in chelation stability over the ITCB-DTPA chelate conjugate. This new chelate conjugate may decrease the normal organ toxicity while maintaining an efficient tumor uptake. The indium-111 labeled DTPA-IgM conjugates results predicted accurately the targeting and specific binding of the same immunoconjugate labeled with yttrium-90 in nude mice. The intraperitoneal route of administration of intact, specific radiolabeled IgM provides rapid and prolonged uptake of radioactivity in tumor and low normal tissue uptake. Again, the distribution of indium- and yttrium-labeled IgM was similar. This compartmental strategy is characterized by early uptake of the labeled immunoconjugate in the tumor, suggesting that the absence of an endothelial barrier removes a major limitation to tumor uptake after i.v. administration. In normal beagle dogs the same long peritoneal residence

time was found for i.p. IgM as for the mice in this study (Vriesendorp, Quadri, Freedman unpublished observations). Intraperitoneal IgM offers a promising new method for the diagnosis and treatment of human patients with ovarian carcinoma and peritoneal carcinomatosis. New studies in human patients with indium-111- and yttrium-90-labeled AC6C3 will be initiated. Two new aspects of preclinical RIT studies were covered in this report (improved chelation chemistry and i.p. IgM). They illustrate the quantitative information that can be obtained on radioimmunoconjugates prior to clinical application. If this information is predictive for the behavior of radioimmunoconjugates in human patients, it will facilitate and accelerate clinical RIT. It also carries the advantage of decreasing the risks of inadvertent side effects in human patients. Continuation of the prescribed preclinical RIT approaches and correlations between preclinical and clinical results are of vital importance to the safe and rapid development of clinical RIT.

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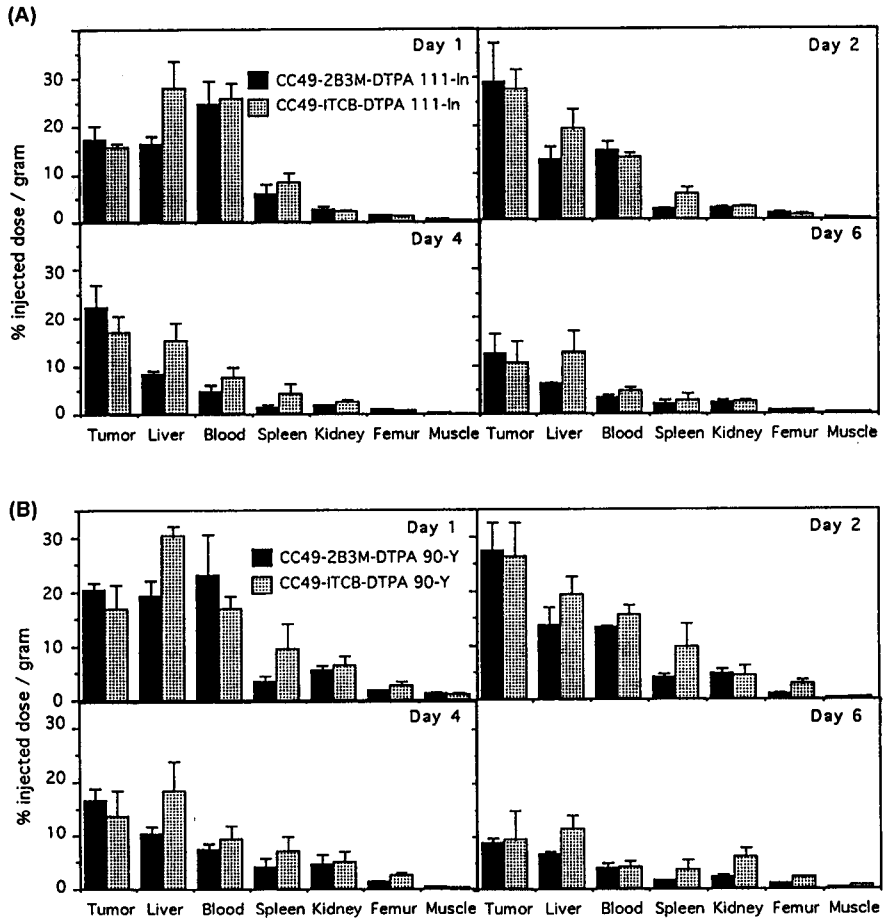

Figure 1. *Biodistribution of (A) 111In (n=4) and (B) 90Y (n=3) labeled CC49-immunoconjugate in nude mice bearing human colon carcinoma xenograft LS174T (n=3)*

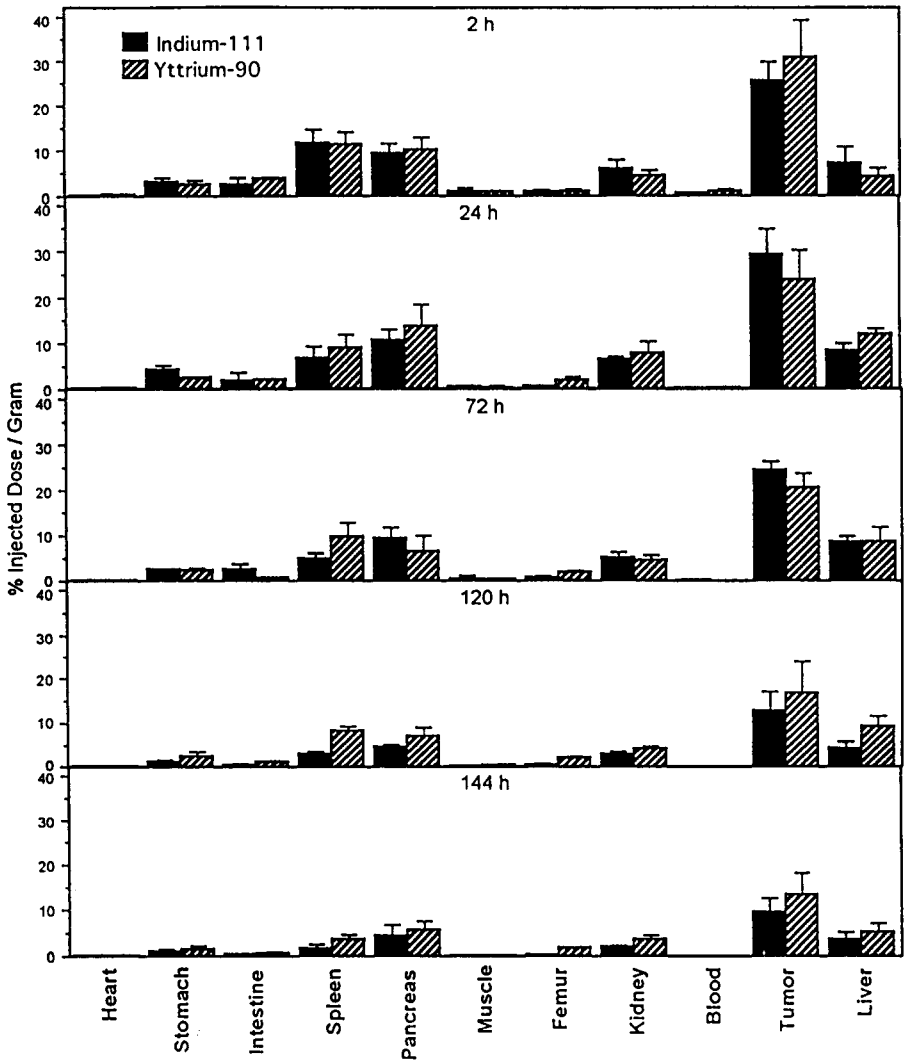

Figure 2. *Biodistribution of ^{111}In- and ^{90}Y-DTPA-IgM conjugates after i.p. administration in nude mice bearing intraperitoneal tumor lumps (SW620).*

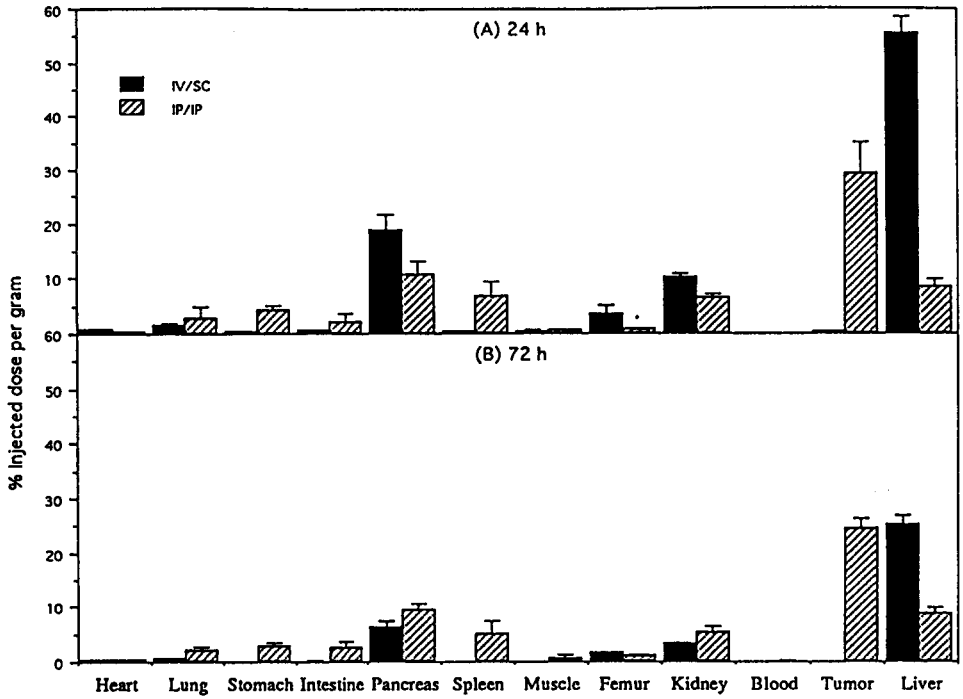

Figure 3. *Biodistribution of IV and i.p. administered In-111 labeled DTPA-IgM conjugates in nude mice bearing human colon carcinoma (SW620)*

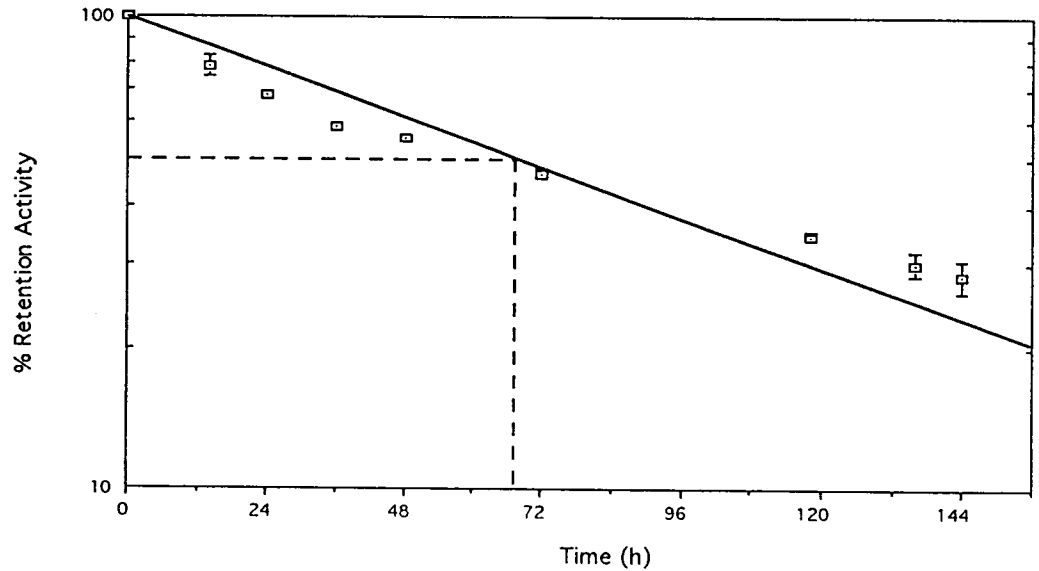

Figure 4. *Whole body retention of intraperitoneal administration of In-111-labeled IgM (AC6C3) in nude mice bearing intraperitoneal human colon tumor lumps (SW620) (n=4)*

HODGKIN'S DISEASE, A PERPETUAL PARADIGM FOR NEW THERAPEUTIC APPROACHES

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INTRODUCTION

Thomas Hodgkin described in 1832 the malignant disease of lymph tissues that now bears his name. More than a century and a half later, the search for the clonogenic tumor cell in Hodgkin's disease (HD) continues with the most advanced histological, immunological and molecular biological techniques. Meanwhile, the biology of HD was found to be unique - a chronic predictable progression from one lymph node region to the next: "contiguous spread." Kaplan formalized the radiotherapy solution for early stage HD by treating patients with large fields, including the involved lymph node regions plus the neighboring clinically uninvolved nodes. He also helped define the radiation dose required for control of HD.[1] Advanced stages of HD were found to be susceptible to multiple-agent chemotherapy regimens in pioneering studies at the NCI.[2] This led to the recommendation of full-dose cyclic chemotherapy, combining agents with different modes of action and nonoverlapping normal tissue toxicity for HD. Later this concept was extrapolated with some success to the treatment of other malignancies. Dicke and coworkers found recurrent HD to be responsive to conditioning regimens of high-dose chemotherapy followed by autologous bone marrow rescue, demonstrating the importance of a dose effect curve for chemotherapy in HD.[3] Most recently, end-stage, treatment-refractory HD appeared to be responsive to radiolabeled immunoglobulin therapy (RIT) in the work of Lenhard and coworkers.[4]

In this communication HD is used once more as a paradigm for the development of a novel cancer treatment i.e., RIT. A review of preclinical and clinical research relating to RIT in HD illustrates that the tradition continues. Some aspects of HD continue to resist explanation notwithstanding intensive research, but therapy trials of RIT in HD appear

extremely helpful in optimizing this new treatment modality for human patients.

MATERIALS AND METHODS

Antigen: Human ferritin, described by Order and coworkers as a tumor-associated antigen in HD[5] was purified using previously reported techniques.[6] Ferritin has a high molecular weight (440 KD), is not a membrane antigen and present in the interstitium and cell cytoplasm of some normal cells in liver, spleen and bone marrow, and tumor cells of different histologies.[6]

Isotopes: Iodine-131, indium-111 and yttrium-90 were utilized for radiolabeling. They were obtained through the courtesy of Hybritech or more recently from New England Nuclear, Boston, Mass. (indium) and from Westinghouse, Richland, Wash. (yttrium).

Radiolabeling methods: Iodine labeling was performed by the indirect lactoperoxidase method using tyrosine residues of the immunoglobulin. Indium/yttrium labeling was performed by a proprietary method of Hybritech, that was isothiocyanatobenzyl (ITCB) DTPA based and uses lysine residues of the immunoglobulin for the connection between chelate and protein. In the second clinical yttrium study a backbone substituted ITCB-DTPA was utilized as described by Quadri and coworkers.[7]

Quality control: Radioimmunoconjugates were tested for immunoreactivity and serum stability *in vitro*. Prior to *in vivo* use they were challenged with a 100-fold excess free DTPA concentration, and purified by column chromatography. Sterility and pyrogenicity tests were performed prior to injection. One percent human serum albumin was added to decrease radiolysis of the radioimmunoconjugate.

Administration to patient: Radioimmunoconjugates were administered intravenously. Polyclonal antiferritin was given in a 2-5 mg total protein dose by rapid bolus infusion. Cold (unlabeled) immunoglobulin was not added. Monoclonal antiferritin was given slowly over fifteen minutes, premixed with 20 mg of cold immunoglobulin. Blood and urine samples were taken at regular intervals after administration of the radioimmunoconjugate for pharmacokinetic analysis. Gamma camera whole body and SPECT scans were performed after iodine- or indium-labeled immunoconjugates only.

Animal models: Nude mice with subcutaneously xenografted human hepatocellular carcinoma cells (HepG2) were used for tumor

targeting studies. HepG2 cells secrete ferritin. Biodistribution, pharmacokinetics, immunoscintigraphy and tumor therapy studies were performed in the same model.[8,9] Beagle dogs were used for biodistribution, pharmacokinetics and radiotoxicology studies of radiolabeled antiferritin.

Patients: End-stage HD patients with measurable disease (n=134) were entered in four different studies. Ages ranged from 10 to 71. Male-to-female ratio was apparently 1.5:1. Most patients were Caucasian. Black and Hispanic minorities were proportionally represented. For full protocol description the reader is advised to consult the original publications.[7,10-13] A summary of the studies is given in Table 1.

RESULTS

Antigen: The great majority of patients (>90%) receiving indium-111-labeled antiferritin (InAF) show tumor targeting, i.e., increased deposition of the radioimmunoconjugate in areas containing HD in comparison to surrounding normal tissues.

The ferritin concentration is higher in tissues containing HD and presumably more accessible than ferritin in normal tissues, due to the leaky capillaries in tumor neovasculature. In three patients that underwent a gallium-67 scan 3 weeks prior to their indium-111 antiferritin study, the gallium scan indicated smaller tumor areas than the indium scan. This might be a reflection of the target for the different isotopes: the transferrin cell membrane receptor for gallium and interstitial ferritin for indium. An interstitial antigen like ferritin might provide a better tumor targeting "margin" for therapy. Targeting was not influenced by serum ferritin levels.[10]

Antibody: Intact mouse monoclonal antiferritin did not target HD due to high deposition in the normal human liver (~60% of injected activity).[6,10] The second trial with a new batch of indium- or yttrium-labeled polyclonal (rabbit) anti-human ferritin produced higher percentages in tumor targeting and response rates than the first batch of polyclonal antiferritins in the first trial (Table 2). Animal studies showed that $F(ab')_2$ stabilized fragments show less normal liver uptake than intact monoclonal antibodies. The chelation chemistry employed (stable versus labile linkers) was another determinant of normal liver uptake.[6,7] Institutional approval has been obtained for new monoclonal antibody studies in HD with indium-111 labeled $F(ab')_2$ stabilized fragments of antiferritin.

Table 1. Studies of radiolabeled antiferritin in patients with end-stage Hodgkin's disease

Study first author (reference)	Antibody (species)	Isotope (mCi administered)	Labeling chemistry	Study specifics	Number of patients
Lenhard (4)	polyclonal[1] (rabbit, pig)	Iodine-131 (50 Mci)	Lacto-peroxidase	2 cycles	37
Vriesendorp (10)	polyclonal[1] (rabbit, pig, baboon), monoclonal QCI	Indium-111 (3-5 mCi) Yttrium-90 (20,30,40,50 mCi)	ITCB/DTPA (Hybritech)	+/- BMT* 1-5 cycles	35
Bierman (11)	polyclonal[1]	Indium-111 (3-5 mCi) Yttrium-90 (20,30 mCi)	ITCB/DTPA (Hybritech)	CBV chemo** + BMT*	14
Herbst (12)	polyclonal[1]	Indium-111 (3-5 mCi) Yttrium-90 (20,30,40,50 mCi)	ITCB/DTPA (Hybritech)	Long-term follow-up	44 (including 35 of ref. 10)
Morton (13)	polyclonal[2] (rabbit)	Indium-111 (5-7 mCi) Yttrium-90 (0.3 mCi/kg) (0.4 mCi/kg) (0.5 mCi/kg)	ITCB/DTPA (Quadri)	No BMT* pharmacokinetics	39

* BMT =- Bone marrow transplantation

** CBV = Cyclophosphamide, BCNU, VP-16

1,2 indicate different polyclonal antiferritins that were produced by different techniques at different times by different investigators.

Table 2. Comparison of trials with indium/yttrium labeled polyclonal antiferritin 1 and 2

Polyclonal antiferritin	Tumor targeting* (indium-111)	Response rates** (yttrium-90)
1	40/45	20/39
2	39/39	12/22

* $p=0.04$

** $p<0.01$

Prescription method: The one hour blood radioactivity levels were compared for yttrium administered intravenously as a total activity, activity per body surface area or activity per kilogram body weight. Only the mCi per kilogram body weight prescription showed a significant positive correlation with one hour blood radioactivity levels ($R=0.44$, $p<0.05$). For later time points (24 and 48 hours after administration) correlations did not reach accepted significance levels for any prescription method due to a pharmacokinetic complication that develops over time. The volume of distribution appears to be increased for some patients with severe B symptoms and some patients that receive an yttrium radioimmunoconjugate with a specific activity over 10 mCi/mg protein.[12,13] For both situations a "capillary leak" is postulated that will increase the delivery of the radioimmunoconjugate to normal interstitial tissues and decrease the secondary (slower) blood half-life of the radioimmunoconjugate.

Toxicity: Hematologic toxicity is the dose-limiting side effect to radiolabeled antiferritin treatment. Platelet levels of 20,000/mm^3 blood occur more frequently after higher administered activities and delay the administration of a new RIT cycle. Serious granulopenia is less common and will respond to treatment with recombinant human G-CSF. Such treatment can delay a new RIT cycle by opening up the blood-marrow barrier for a considerable length of time. A subsequent yttrium-labeled antiferritin administration would lead to increased marrow deposition and bone marrow toxicity for a second cycle. Average cycle length for the first cycle is 10 weeks. Cumulative hematological toxicity has been observed and induces a longer cycle length in subsequent cycles. Most patients can tolerate 3 cycles. One individual was treated 5 times. One patient has developed a myelodysplastic syndrome after completing multiagent chemotherapy, high dose chemotherapy and bone marrow transplantation (approximately 60% of all yttrium-90 treated patients), external beam radiation and finally 3 RIT cycles. Blood count recovery has been slowest

in patients with prior bone marrow transplantation, patients with bone marrow involvement with HD and patients with rapidly progressive HD. Bone marrow transplantation can accelerate hematopoietic recovery after RIT with yttrium-90 labeled antiferritin.[10,11] The timing of the administration of hematopoietic cells after RIT is crucial and should be delayed until the dose-rate in the bone marrow has decreased to 1 cGy per hour or less.[6] Further RIT studies that include bone marrow transplantation can be postponed until the radioimmunoconjugate and its administration has been optimized (e.g. monoclonal AF in a fractionated schedule). Two patients had circulating pre-existing anti-antibodies prior to the initiation of RIT. In only one patient, anti-rabbit antibodies were found after single InAF YAF cycle. Anti-antibody formation occurs in less than 5% of HD patients and facilitates the analysis of cyclic RIT in this patient population. The lack of a strong anti-antibody response in HD patients is due to their disease and treatment-induced immunodeficiency. No toxicity was observed in any organ system outside of the hematopoietic system.

Response of Hodgkin's disease: Response rates for single agent radiolabeled antiferritin studies are shown in Figure 1. The studies are not concurrent, but consecutive. Response rates are higher after Y-90 AF than after I-131 AF. The administration of higher yttrium-90 activity levels per kilogram resulted in significantly higher response rates. Complete response rates show the same differences between trials (Figure 2). The differences between the first and the second yttrium-90 studies are probably due to a better radioimmunoconjugate and a better prescription method in the second study. Response durations are from 2 months to years with an average of 8 months. Survival studies were performed after the first yttrium study.[12] Fifty percent of complete responders, partial responding and progressive disease patients were alive at 2 years, 1 year and 4 months respectively. Survival of patients on the second yttrium study is still under analysis. For the YAF/CBV/BMT study, 4 out of 8 evaluable patients are alive at 2 years, 3 are NED.[11] Most patients have recurred after RIT for HD and succumbed to their disease. One patient developed a fatal gliosarcoma of the brain, while undergoing RIT. Three patients died while NED for HD, two with pre-existing lung fibrosis, one in a car accident. Small numbers of patients are alive 1 to 5 years after RIT in the different studies. Patients receiving InAF and YAF that were not treated with bone marrow transplantation remained outpatients, unless disease progression made inpatient management necessary. Response rates are more common in patients with small volume disease and long

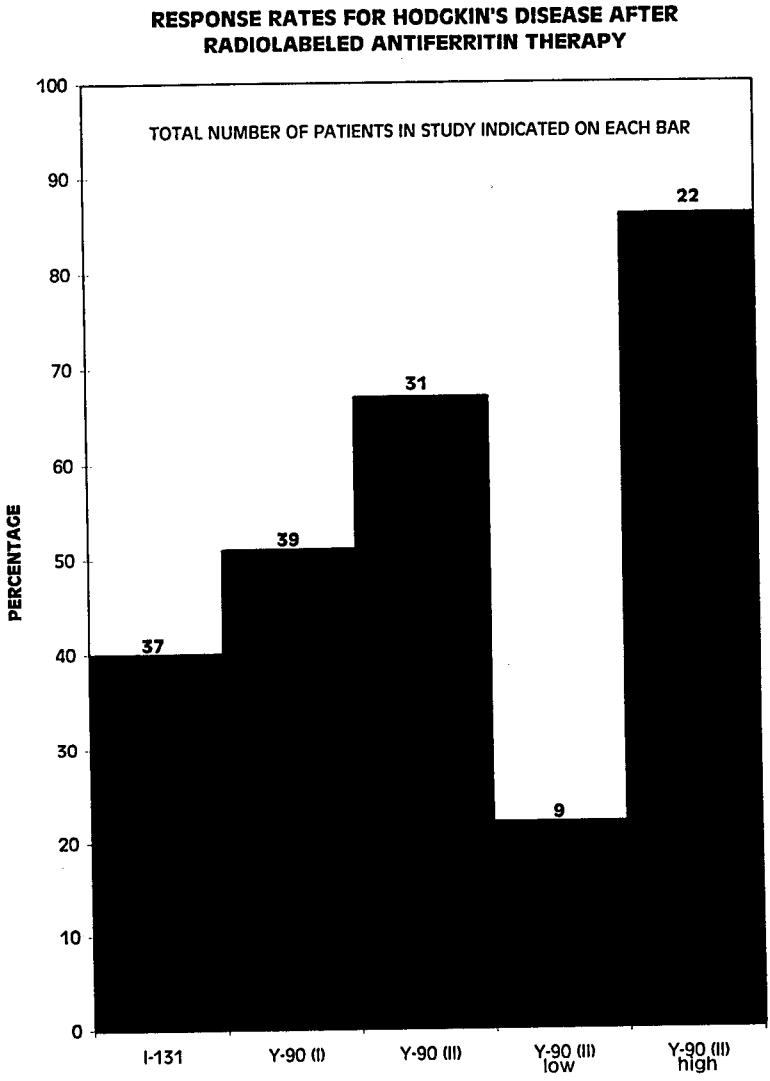

Figure 1. *Response rates for Hodgkin's disease after radiolabeled antiferritin therapy. (Footnote: I-131 is Iodine-131 antiferritin study (ref. 4). Y-90 (I) is first yttrium-90 antiferritin study (ref. 10). Y-90 (II) is second yttrium-90 antiferritin study (ref. 13). Low indicates patients received 0,.3 mCi Y-90/kg, high indicates patients received 0.4 or 0.5 mCi Y-90/kg.)*

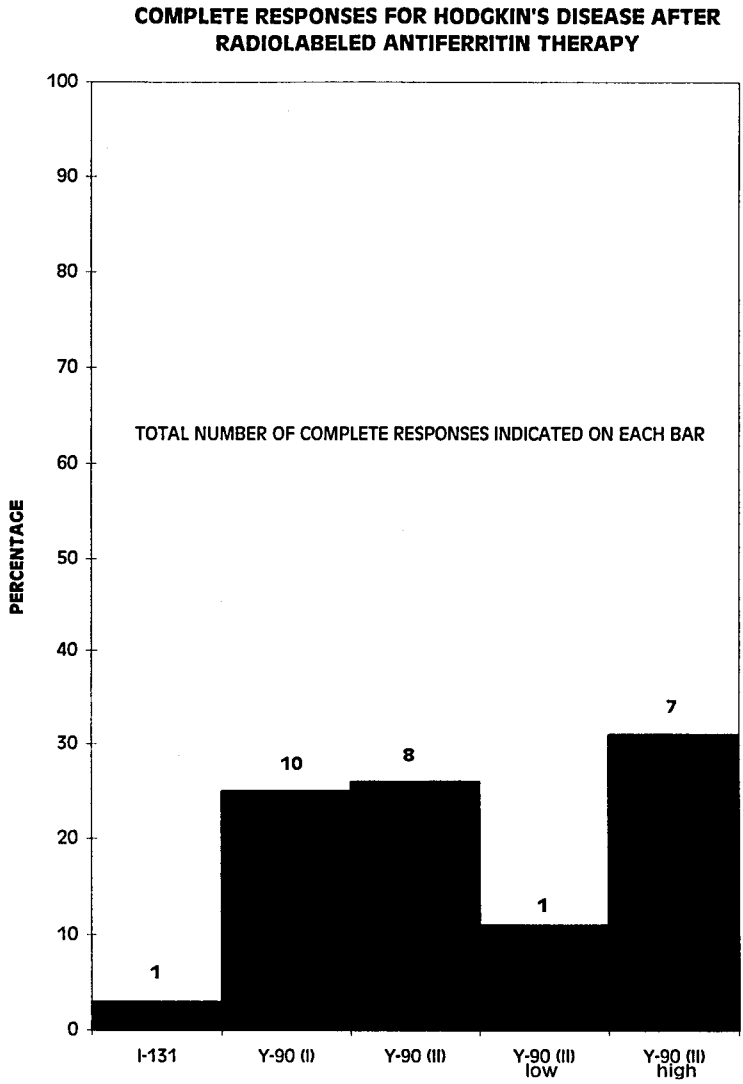

Figure 2. *Complete responses for Hodgkin's disease after radiolabeled antiferritin therapy. (Footnote: For explanations of symbols see footnote Figure 1.)*

disease histories at time of RIT initiation. One-third of the HD recurrences is in a previously uninvolved area, while the response to RIT is maintained in the initial disease sites.[10]

Tumor dosimetry: Techniques for tumor dosimetry depend on quantitative information obtained from whole body and SPECT scans after InAF. This information will only be predictive for YAF if the biodistribution and pharmacokinetics of AF is the same for the indium and the yttrium labeled conjugate. With the use of the proper chelation chemistry and specific activity this appears to be the case. Tumor doses of up to 30 Gy in 1 week can be obtained after polyclonal YAF. A full dosimetric analysis, including correlation of tumor doses to tumor responses is being performed (P. Leichner in preparation). In the first YAF study no correlation was found between tumor dose and subsequent response.[10]

DISCUSSION

In the United States approximately 7500 patients are diagnosed with HD each year. For two out of three patients the administered radiotherapy and/or chemotherapy will be curative: one of the success stories in modern oncology. Approximately 2500 patients will not respond to treatment or recur. Second line treatment (including high-dose chemotherapy/radiation followed by bone marrow transplantation) is expected to be curative for one-third of these patients, leaving less than two thousand patients per year for third-line treatment. In this heavily pretreated patient population, radiolabeled antiferritin appears to provide a high tumor response rate at a low toxicity cost. Yttrium-90 has shown stronger antitumor effects than Iodine-131. The most disappointing experience in the reviewed clinical studies is the failure of intact mouse monoclonal antiferritin to target HD. Further preclinical animal studies are needed and initial returns are promising for $F(ab')_2$ stabilized fragments.

The experience obtained in HD with RIT indicates that a low amount of radiolabeled protein (<5 mg) can induce a tumor response. This argues for a radiation effect and against an immunological effect of the radioimmunoconjugate. In addition, the interstitial location of tumor-associated ferritin will prevent the occurrence of immune-mediated cellular cytotoxicity or complement-dependent cytotoxicity on tumor cells. The range of yttrium-90 beta emissions is on average over 5 mm in tissue and corrects to some degree for uneven deposition of the

radioimmunoconjugate in the tumor and the risks for "cold" spots in the tumor.

The design of clinical RIT protocols has been improved on the basis of the studies in HD: a) prescription method: mCi/kg, b) delay of toxicity/dose escalation studies until the radioimmunoconjugate is optimized (see also 14), c) increased chance for tumor response in early clinical studies, d) low toxicity, low cost, outpatient studies (InAF followed by YAF), e) select patients with measurable disease for single agent studies (i.e. RIT only). The variables in clinical RIT remain overwhelming. A combination of mouse and dog studies has provided information that appeared to be helpful and predictive for subsequent RIT studies in HD.[6] Translational research in preclinical animal models is essential for progress. Hodgkin's disease does not have a counterpart in veterinary oncology. Nude mice with xenografted human hepatoma cells can provide a ferritin-positive target. Normal tissue toxicity is best analyzed in a larger animal model. For beagle dogs, RIT will not be close to total body irradiation and the same normal tissue dose gradients as experienced by human patients can be reproduced. The execution of translational research for RIT is an organizational and financial challenge due to the many talents/specialties required for the proper execution of such research. Fractionation of RIT and monoclonal AF are the most pressing issues for preclinical analysis at this time.

The advantages of RIT over most other cancer therapies are selectivity and quantification (mCi/g in tumor and normal tissues can be determined over time by non-invasive methods). For further development of RIT, selective and quantitative approaches remain necessary to maintain and enhance the already excellent therapeutic ratio of RIT. HD is once more the best paradigm for studies to improve RIT, the next in line of new treatment approaches. The scientific community and the authors of this communication are in debt to the courage, enthusiasm and willingness of HD patients and their caregivers to participate in such studies.

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USE OF ^{131}I-LABELED ANTI-CD33 MONOCLONAL ANTIBODY M195 FOR MYELOID LEUKEMIAS

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INTRODUCTION

M195 is a mouse IgG2a monoclonal antibody reactive with the myeloid surface glycoprotein CD33. This antigen is found on most myeloid and monocytic leukemic blasts and leukemic progenitor cells in addition to committed normal myelomonocytic and erythroid progenitor cells. It may be expressed in low numbers on early hematopoietic stem cells but is not found on non-hematopoietic tissues.[1,2] These characteristics make CD33 a suitable target for the immunotherapy of myeloid malignancies.

In a pilot trial, ten patients with myeloid leukemias were treated with escalating doses of M195. The initial dose was trace-labeled with ^{131}I to allow detailed pharmacokinetic and dosimetric studies by serial sampling of blood and bone marrow and whole body gamma camera imaging. These studies demonstrated that M195 could saturably target leukemia cells within one hour after injection at doses of approximately 5 mg/m^2 and that M195 was rapidly internalized into these cells after binding to its antigenic target. The antibody was retained within the marrow for up to 48 hours. Dosimetric analysis showed that M195 could deliver up to six-fold more radiation to the marrow than to the liver and other normal organs, suggesting that an ^{131}I-M195 conjugate could be useful as a cytoreductive agent.[3]

LEUKEMIC CYTOREDUCTION WITH ^{131}I-M195

In a second pilot trial, 24 patients with relapsed or refractory myeloid leukemias received from 50 to 210 mCi/m^2 of ^{131}I-labeled M195 in divided doses. Areas of leukemic involvement, including bone marrow, liver and spleen, were clearly imaged by gamma camera for up to 72 hours after administration of ^{131}I-M195. Thirty-seven percent of assessable patients developed human antimouse antibodies (HAMA). In two patients

with HAMA who received second courses of treatment with ^{131}I-M195, plasma levels could not be maintained, and no therapeutic effect was observed. Non-hematologic toxicity was limited to hyperbilirubinemia in one patient who had previously undergone allogeneic bone marrow transplantation (BMT). Twenty-three patients demonstrated decreases in peripheral blood blast counts, and 89% of patients demonstrated substantial reductions in the number of bone marrow blasts. Profound marrow suppression was seen at ^{131}I doses of 135 mCi/m^2 or greater, and eight patients had sufficient marrow cytoreduction to proceed to either autologous (n=3) or allogeneic (n=5) BMT. Three of these patients attained marrow remission, with one lasting 9 months and another 28+ months. These data suggested that leukemic cytoreduction could safely be achieved with ^{131}I-M195 and that this agent could have a role as part of a preparative regimen for BMT.

^{131}I-M195 PRIOR TO BONE MARROW TRANSPLANTATION

In an effort to reduce relapse after allogeneic BMT for advanced myeloid malignancies, a conditioning regimen combining escalating doses of ^{131}I-M195 with busulfan (16 mg/kg) and cyclophosphamide (120 mg/kg for first BMT, 90 mg/kg for second) has been studied. Patients received cyclosporine and corticosteroids as graft-versus-host disease (GvHD) prophylaxis. Nineteen patients (median age, 38 years) were treated. Fifteen patients underwent first BMT for relapsed (n=2) or refractory (n=8) acute myelogenous leukemia (AML), or accelerated (n=2) and myeloblastic (n=3) chronic myelogenous leukemia (CML). Four patients received a second BMT for either relapsed, chronic phase (n=1) or accelerated (n=3) CML. Patients were treated with 120 to 160 mCi/m^2 of ^{131}I-M195 given in 2 to 4 divided doses. Extramedullary toxicity attributable to ^{131}I-M195 was limited to urticaria in one patient. All patients engrafted with median time to an absolute neutrophil count over 500/μL of 14 days (range, 10 to 23) and median time to a platelet count over 20,000/μL of 27 days (range, 12 to 85). Seven patients developed acute GvHD (2 with grade I, 3 with grade II and 2 with grade IV); two developed chronic GvHD. HAMA was seen in 6 of 16 patients evaluated. Among the 15 patients who received first BMT, there were 3 peri-transplant deaths due to infection (n=2) and GvHD (n=1). Eight of these patients remain alive with a median follow-up of 8 months. Seven are in remission at 2, 3, 4, 9, 17, 20 and 28 months post-BMT. Four patients relapsed 3, 5, 6 and 16 months following transplant. The four patients

who underwent a second BMT all achieved remission but died of transplant-related complications 1 to 8 months after BMT. Although the impact of this preparative regimen on disease-free survival will require longer follow-up, this approach potentially enables intensification of antileukemic therapy prior to BMT without increased toxicity or impaired engraftment.[5]

^{131}I-M195 FOR MINIMAL RESIDUAL DISEASE

The effects of ^{131}I-M195 on minimal residual disease have been studied in patients with relapsed acute promyelocytic leukemia (APL). APL provides an ideal setting since most patients with relapsed disease can be salvaged with all-*trans* retinoic acid (RA), and minimal residual disease can be monitored using a reverse transcription and polymerase chain reaction (RT-PCR) assay that detects the PML/RAR-α fusion mRNA associated with t(15;17). Seven patients with relapsed APL in second remission after all-*trans* RA induction were treated with either 70 (n=2) or 50 (n=5) mCi/m^2 of ^{131}I-M195. There was no immediate toxicity. Late toxicity was limited to myelosuppression, but no episodes of febrile neutropenia were seen. The maximum tolerated dose of ^{131}I-M195 in this post-remission setting resulting in neutropenic periods of less than 14 days approximated 50 mCi/m^2. Six patients had detectable PML/RAR-α mRNA after all-*trans* RA therapy; two had transiently negative RT-PCR determinations following ^{131}I-M195. Median disease-free survival of the seven patients was 8 months (range, 3 to 14.5). Four patients with median follow-up of 26 months remain alive, and median overall survival exceeds 22+ months (range, 5.5 to 34+). Four patients have had longer disease-free intervals than the maximum disease-free survival lasting 6 months of 11 patients with relapsed APL induced into remission and maintained with all-*trans* RA therapy on the study immediately preceding this trial. Five of the 7 patients achieved longer survival duration than 10 of the 11 patients in the prior trial. Although the number of patients in these series is small and these trials are not randomized, patients' outcomes in the present study compare favorably with those of patients treated on this earlier study as well as with chemotherapy-based regimens and BMT. These data support further study of antibody-based therapy for minimal residual disease in acute leukemia.

HUMANIZED M195

Because of the lack of intrinsic cytotoxicity of murine M195 and its immunogenicity which limits repeated dosing, a complementarity determining region (CDR)-grafted human IgG1 version of M195 (HuM195) was developed. HuM195 displayed up to a 9-fold increase in binding avidity over the murine antibody and has the capability to mediate antibody-dependent cellular cytotoxicity against leukemic target cells *in vitro*.[7] Thirteen patients were treated on a twice weekly schedule for 3 weeks at 4 dose levels ranging from 0.5 to 10 mg/m^2. Two patients were retreated for a total of 12 doses. The first dose of HuM195 was trace-labeled with ^{131}I in order to facilitate detailed pharmacokinetic and biodistribution studies by serial sampling of blood, radioimmunoassay of cells, and whole-body gamma camera imaging. Reversible fever and rigors were seen at the highest dose levels. Pharmacokinetics were similar to the parental murine form. The entire bone marrow was clearly imaged within hours after infusion with optimal biodistribution occurring after the administration of a 3 mg/m^2 dose. Adsorption of HuM195 onto targets *in vivo* was demonstrated by flow cytometry with near saturation at the 3 mg/m^2 dose level. ^{131}I-HuM195 was rapidly internalized into target cells with re-expression of the target antigen occurring approximately 72 hours after the infusion. HAHA responses were not seen. A decrease in the number of marrow blasts was seen in one patient with refractory erythroleukemia at the highest dose level. These data indicate that HuM195 would be a suitable agent to deliver radionuclides to leukemic cells, that HuM195 may have intrinsic activity in the setting of minimal residual disease where normal effector cells are present, and that repeated administration is possible due to its apparent lack of immunogenicity.[8]

FUTURE DIRECTIONS

Investigation of HuM195 constructs for myeloablation prior to BMT and for minimal residual disease are underway. The trials previously discussed indicate that these two applications of monoclonal antibody-based therapy require different approaches. As part of a BMT preparative regimen, the lack of immunogenicity and increased binding avidity of HuM195 offer advantages over use of the murine antibody. A trial using ^{131}I-labeled HuM195 for myeloablation has begun. The use of ß particle-emitting radiometals such as ^{90}Y which may be better retained within the marrow could further improve delivery of isotope to leukemic cells. In the

setting of minimal residual disease, one wishes to avoid the nonspecific cytotoxic effects of ^{131}I. Currently, several trials have been initiated to study the effects of unconjugated HuM195 on minimal residual disease given as adjuvant therapy where normal effector cell populations are present. *In vitro* studies have shown that interleukin-2 (IL-2) can potentiate the antileukemic activity of HuM195, and clinical trials combining these two agents are planned. Finally, isotopes which emit shorter-ranged α-particles such as ^{212}Bi and ^{213}Bi, or Auger electrons, such a ^{123}I and ^{125}I, may provide more specific cytotoxicity. A ^{213}Bi-HuM195 construct has been generated,[9] and clinical trials are planned.

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INTENSIVE RADIOLABELED ANTI-BREAST MONOCLONAL ANTIBODY ^{90}Y-BrE-3 WITH AUTOLOGOUS HEMATOPOIETIC CELL SUPPORT PRELIMINARY RESULTS

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INTRODUCTION

Intensive monoclonal antibody therapy offers a new option for effective cancer therapy. One group of high molecular weight mucin antigens are often found on malignant human epithelial cells, and are referred to as the human milk fat globulin (HMFG) or human mammary epithelial (HME) antigens.[1] Monoclonal antibodies to these antigens have been developed by several investigators and have been reported to react with high percentages of human epithelial cancers, especially those of breast, lung, ovarian and colon origin.[2] Ceriani, et al developed an IgG-1 monoclonal antibody to an epitope of HMFG named BrE-3.[3] This MoAb was used for our studies described below.

SELECTION OF ANTIBODY, ISOTOPE AND CHELATE

Combining antibody targeting capability and radioisotope technology, multiple radiolabeled monoclonal antibodies have demonstrated potential both as imaging agents for cancer detection and as therapeutic modalities. Successful tumor localization has depended on antibody-antigen specificity, tumor antigen density, dose of infused antibody, tumor vascular supply, size of tumors, and radiochemical properties of the isotope and stability of the radiolabel-antibody conjugate.[4] Successful tumor therapy requires optimization of the above characteristics, and close attention to development of dose-limiting normal organ toxicity. For example, myelosuppression has been the major toxicity in therapeutic radiolabeled antibody trials utilizing ^{131}I or ^{90}Y as the isotope.[5]

Recently, 19 patients with multiply-relapsed lymphoma were treated with a single high dose (234 to 777 mCi) of ^{131}I-labeled (MB-1)

anti-pan B-cell antibody at the University of Washington. Sixteen of 19 patients experienced a CR, and 9 remain in continuous complete remission 3-53 months later.[6] Patients received autologous marrow support, and two incidents of possible cardiopulmonary toxicity were described. These investigators also observed that favorable antibody distribution usually occurred in patients with smaller tumor volume. Kaminski, et al treated 9 lymphoma patients with an ^{131}I-anti-B1 antibody in doses varying from 35-70 mCi. Six of 9 patients experienced objective tumor responses at doses which produced a maximum of Grade 2 myelosuppression.[7] These two studies, taken together, illustrate exciting therapeutic promise for this modality. It is now important to test radioimmunotherapy in more common tumor types.

The choice of isotope for use in therapeutic targeting depends on multiple factors, including the type of radiation emission and effective range, isotope half-life, tumor dose exposure, radiochemical linking technology[8], and radiation safety issues. For therapeutic application, investigators have utilized gamma or beta-irradiating isotopes such as ^{131}I or ^{90}Y or 186Re, or alpha-emitting particles such as 212Bi. ^{90}Y has the advantage in sharing chemical chelation properties of the well characterized ^{111}In and a relative short, 60 hr half-life. ^{90}Y emits high-energy beta-articles which possess effective range length of approximately 0.3 cm.[9] Thus, ^{111}In-labeled MoAb is the gamma-emitting isotope often used for tumor imaging, and ^{90}Y-labeled MoAb is the beta-emitting isotope used for therapeutic purposes. It must be kept in mind, however, that chelate stability differs between Y and In, and that radionuclide deposition will thus be somewhat different, particularly in bone. Tissue biopsies are important to allow accurate dosimetric calculations. Zimmer, et al have reported that a ^{111}In B72.3 radioimmunoconjugate is reasonably predictive of ^{90}Y-B72.3 binding to tumor, though plasma PK of the two compounds suggested more rapid release of ^{90}Y than ^{111}In from the chelate.[10] ^{131}I is less desirable for high-dose therapeutic purposes though its gamma emission allows scanning. It is eliminated in expired air and sweat as well as urine and stools; its energetic gamma emission additionally makes for considerable biohazard problems for the health care staff.

Potential normal organ toxicity also varies with the isotope and strength of chemical binding to antibody. In a nude mouse colon tumor xenograft model, bone marrow hypoplasia and splenic fibrosis were progessively greater at doses from 40 to 160 uCi of ^{90}Y-labeled anti-CEA antibody and all doses were nonlethal.[11] When used for human

radioimmunotherapy in a patient with B-cell lymphoma, 10 mCi of ^{90}Y-labeled anti-idiotype monoclonal antibody resulted in partial regression of disease.[12] Furthermore, there was minimal toxicity with resultant mild thrombocytopenia (platelets 83,000/ul), which resolved by 8 weeks following therapy. With the 60 hr half-life of ^{90}Y, more than 90% of any isotope localized to bone or bone marrow will have decayed within 15 days, even ignoring important elimination of ^{90}Y by all other mechanisms (mostly renal, biliary, or GI excretion). Thus, administering cyopreserved marrow followed by G-CSF 15 days after dosing should afford reliable reengraftment even at high isotope doses.

SCID mice which were either tumor or nontumor bearing were treated at Colorado with ^{131}I-BrE-3. The clearance of radioactivity was comparable in tumor and nontumor bearing mice. Since immune complexation with circulating antigen can mediate rapid MoAb clearance, this data is consistent with a low circulating antigen level under the conditions of these experiments.

NORMAL AND NEOPLASTIC HUMAN TISSUE DISTRIBUTION OF BrE-3 BY HISTOPATHOLOGY AND IMAGING

Studies were carried out in formaldehyde fixed, paraffin-embedded tissue blocks. Binding of BrE-3 to normal human tissues was less extensive than MoAbs Mc5, HMFG-2, and DF3. BrE-3 bound weakly (as compared to breast tumors) to the alveolar lining of lung, some kidney tubules, pancreas and stomach. Normal human breast tissue was negative.

To date, we have evaluated tumor biopsy specimens of 41 patients for BrE-3 staining preparatory to entering the clinical trial. All specimens stained positively with BrE-3 using an alkaline phosphatase-antialkaline phosphatase technique. Within individual specimens, 10-50% of the tumor cells stained, with an intensity of individual cell staining varying from 1-3+.

Circulating BrE-3 Antigen

Thirty-one patients' plasma has been assayed for circulating BrE-3 levels. To date, the highest measured level has been 60 ng/ml, well below the 5 ug/ml cutoff for eligibility for this trial, indicating that <1% of BrE-3 is likely to be bound in the circulation.

Preclinical Radioimmunotherapy (RIT) with BrE-3 Conjugates

^{131}I-BrE-3 binding was tested by flow cytometry in an immunodeficient mouse model grafted with MX-1 tumor that has an intermediate to low content of BrE-3 Ag. After a dose of 500 uCi (considered the maximal tolerated dose) of ^{131}I-BrE-3 was given to mice carrying MX-1 tumors, a >1 month growth delay with a 90% tumor growth inhibition at 28 days was noted. No deaths were recorded in the experimental group.

When a 250 uCi ^{90}Y-conjugate of BrE-3 was used in the same model, extensive tumor necrosis was noted within one week. Tumors in 4 out of 4 animals were eradicated after 20 days. The ^{90}Y-BrE-3 was injected when the tumors were 100 mm^3 and the control, untreated tumors grew to an average 1162 mm^3 volume 40 days after placebo injection. No deaths were recorded in the experimental group.

In summary, antibody BrE-3 has a number of positive characteristics which accentuate its potential clinical utility with ^{90}Y, including: 1) the widespread expression of BrE-3 antigen on human breast cancer with acceptable normal tissue reactivity, 2) relatively low serum antigen levels in patients with advanced breast cancers, 3) successful localization of ^{111}In-BrE-3 to antigen positive human carcinoma nude mouse xenogafts, 4) successful localization in humans with advanced breast cancers, and 5) development of low levels of serum human anti-mouse antibodies, as evaluated clinically in a series of studies at the University of Colorado. While the data for clinical activity of ^{90}Y is limited, it is clear that the dose-limiting toxicity of this treatment without AHCS is marrow toxicity.

CLINICAL TRIAL DESIGN

Protocol Schema

Days	-14	-13	-12	-11	0	+1
G-CSF					→	
Marrow reinfusion					•	
^{90}Yt-BrE-3 dose	•					
Scan	•					
^{111}In-BrE-3 dose	•					

PRELIMINARY CLINICAL TRIAL DATA

To date, 9 patients have been treated with ^{90}Y-BrE-3. The only toxicity seen to date is hematologic, with 5/8 evaluable patients experiencing Gr 3-4 granulocyte toxicity and 5/8 patients experiencing Gr. 3-4 platelet toxicity. No infections have been noted. Cohort 1 (15 mCi/M^2) was treated without incident. One of the first three patients in Cohort 2 (20 mCi/M^2) experienced delayed platelet engraftment. She expired from tumor progression on day +57 while still receiving platelet transfusions; the ANC recovered on day +17. Three additional patients were treated at the 20 mCi/M^2 dose without difficulty, and dosing at the 34 mCi/M^2 level will proceed with future patients. Demographic data for the 9 patients is shown.

^{90}Y-BrE-3 Protocol
Demographic Data (N=9)

• Age, median	48
• Stage 4 breast cancer, female	9
• No. prior chemo regimens, median	2
• Adequate cardiac, renal, pulmonary and hepatic function	
• Failure of conventional chemotherapy	
• Ineligible for Phase 2 Studies	
• Low levels of circulating HAMA, BrE-3	

In our historical series of marrow-supported chemotherapy, between 5-10% of patients have delayed platelet recovery similar to that described above. In view of the timely granulocyte recovery, it seems unlikely that irradiation of the reinfused marrow caused the platelet delay.

Hematologic Toxicity (N=9)

Cell Type	# Gr. 4 Toxicity	Duration (median days)	Days to nadir (median)
Platelets	5	20 (5->30)	27 (D+11)
WBC	2	[3,9]	25 (D+9)
	G-CSF used		

All patients recovered granulocyte counts to >500/ul by day +20. Importantly, no extra-hematologic toxicities have been noted. Of eight patients evaluable for response, four demonstrated partial responses. The

one patient no evaluable for response had tumor limited to bone. There was subjective improvement in bone pain following treatment.

Dosimetry and Scanning

Successful imaging of tumor was achieved in all patients. Most striking was the ability to image brain metastases in the two patients where they were present.

Limited data is available about biodistribution and elimination of radioactivity. Urinary excretion of radioactivity varied from 7.8 to 10.8% over 96-hr following dosing. The effective total body half-life of ^{90}Y is 50 $\pm$ 15 hr, indicating important excretion in addition to physical decay. Preliminary dosimetry estimates of total body radiation vary between 80-120 rads for the first six patients treated. Scanning and volumetric data are being collated to allow organ or tissue-specific dosimetry estimates. It is probable from scanning and biopsy data that liver is a major site of nonspecific tissue binding. In view of the radiosensitivity of this organ, liver chemistry tests will be watched closely as the trial proceeds.

Serial bone marrow biopsies performed in two patients demonstrate an actual increase in specific activity between one and two days after dosing, in spite of the radioactive decay of ^{90}Y. The specific activity ratios of 1.70 and 1.72 (day 2/day 1) contrast with ratios of 0.79 and 0.18 for ^{111}In. This data strongly suggests that chelate decomposition results in rapid uptake of ^{90}Y by bone, where physical decay largely governs disappearance. Additionally, it emphasizes the unreliability of ^{111}In scanning as a measure of ^{90}Y bone uptake acutely. One patient had marrow sampling performed one and six days after treatment and showed a specific activity ratio of 0.18 (day 6/day 1) versus 0.25 which would be predicted on the basis of physical decay. Though the data are obviously limited, it suggests that marrow reinfusion 15 days after treatment is safe.

One patient had serial liver biopsies performed one and two days after dosing. The ^{90}Y specific activity ratio (day 2/day 1) was 0.85 compared to 0.80 predicted by physical decay, and the specific activity on day 1 was 2.3 uCi/gm tissue compared to 2.3 and 2.2 uCi/gm tissue for bone marrow in two other patients whose dose of radionuclide was identical. Assuming uniform distribution of isotope throughout the body, an approximate specific activity of 0.35 uCi/gm tissue would be expected, indicating a 6-7-fold concentration of ^{90}Y in these tissues. This result emphasizes the value of a marrow-supported strategy with this program and the importance of monitoring liver function carefully during the trial. It will be critical to obtain tumor biopsies for comparison purposes in

future patients. Appelbaum[13] has demonstrated that the marrow stroma can be irradiated to >15,000 cGy and recover, thus making it unlikely that stromal injury will be dose-limiting in this study.

SUMMARY AND CONCLUSIONS

Early data from this trial suggest that an encouraging response rate is seen in patients with heavily treated metastatic breast cancer. The absence of extra-hematologic toxicity will clearly permit further dose escalation using autologous hematopoietic progenitor cell support. The goal of this ongoing Phase I study is to define the maximally tolerated dose of ^{90}Y-BrE-3 as defined by extra-hematologic toxicity. Subsequently, we will perform a Phase II study in patients with breast cancer at the MTD, and ultimately attempt to add this program to multiagent chemotherapy to treat patients with metastatic breast cancer.

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PROSPECTS FOR MORE RAPID AND SAFER DEVELOPMENT OF CLINICAL RADIOLABELED IMMUNOGLOBULIN THERAPY

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HISTORY

The German bacteriologist, Paul Ehrlich, received a Nobel prize in 1908 for his work in the development of selective chemical compounds for the eradication of infectious diseases. He introduced the metaphor "magic bullet" for selective therapeutic chemicals. Early investigators of radiolabeled immunoglobulin therapy (RIT) have emphasized the promise of RIT by repetitive allusions to the magic bullet metaphor. Ironically this concept is diametrically opposed to the unique advantageous characteristic of properly designed and executed RIT.

A bullet is a nondiscriminating brutal vehicle of force and destruction. Magic has the connotation of elegantly entertaining (and fooling) a gullible audience. In contrast, RIT is **selective**. After administration the radioimmunoconjugate follows the normal preexisting delivery pathways in the body and deposits itself in small volumes in the body, predetermined by the specificity of the immunoglobulin. Usually the first radioisotope connected to the immunoglobulin is selected for diagnostic purposes. After *in vivo* administration it will generate a diagnostic picture on an external scanning device (gamma camera) with gamma emissions in the range of 100-300 KeV; the second isotope is selected to give localized radiation around the deposited immunoglobulin by its high energy beta emissions. There is **no** magic to RIT; it is visible and before and after *in vivo* administration it is accountable in location and activity. Current technical limitations of gamma cameras and biological limitations on tumor deposition of radioimmunoconjugates provide the possibility to detect tumor volumes larger than 0.5-1 cm in diameter and estimate radioactivity in such volumes in mCi/g with an accuracy of $\pm$ 25-50%.[1,2] The most promising clinical results with RIT have been obtained

in patients with lymphoma and Hodgkin's disease. Hematological toxicity has been the only major side effect noted.

The difficult responsibility and task for current RIT investigators is to leave the magic bullet metaphor behind and develop new investigative, quantitative, techniques that will demonstrate unequivocally the merits of RIT in patients with nonhematological malignancies. The experience obtained in the development of RIT for end-stage Hodgkin's disease can serve as a paradigm.[3]

RIT Strategy

A priority appears logical to develop RIT applications in human patients by following as guidelines the attractive RIT properties of **selectivity** and **accountability**. Patients should have measurable cancer involvement (larger than 1 cm in diameter) and radiosensitive disease. RIT studies in patients with melanoma or colon cancer have been unrewarding.[4,5] Low tumor response rates and shallow dose-effect curves make it difficult to optimize the all important therapeutic ratio of RIT. Expectations are that after RIT has been optimized in patients with radiosensitive tumors, RIT deserves to be tested again in more radioresistant tumors.

The potential advantages of RIT can be enhanced by selection of the most appropriate radioisostopes. For diagnostic purposes this will be a gamma emitter in the range of 100-300 KeV. Radioisotopes with short half-lives (<10 hours) can be attached to low molecular weight (<50,000) targeting molecules. Larger targeting molecules require radioisotopes with longer half-lives to accommodate the delay in tumor uptake of such radioimmunoconjugates. Tc-99m and In-111 are the isotopes of choice (Table 1).

For therapeutic purposes the isotope selection is controlled by tumor size. Higher tumor doses and more homogeneous tumor doses are delivered by higher energy beta emissions in cancer patients with measurable disease. In small tumors (single cells or single cell sheets), lower energy beta emissions have an advantage because less radiation is wasted in normal tissues surrounding tumor cells.[1,6] In Table 1, Y-90 and Cu-67 are given as the radioisotopes of choice for large and small tumors, respectively. Alpha emitters are still being considered for low tumor volume applications. The number of alpha emitters with properties advantageous to RIT is limited.[7,8] The high LET of alpha particles is of interest as a most effective way to kill tumor cells over a short range, but it

can also destroy the chemical bonds necessary for binding the isotope to the immunoglobulin (radiolysis).

Table 1. Selection of Radioisotopes for Immunoconjugates*

	Isotope	Emissions	Energy (KeV)	T 1/2 (hrs)
A. Radiolabeled immunoglobulin Scintigraphy (RIS)				
Low MW immunoconjugates	Tc 99M	γ	140	6
High MW immunoconjugates	In 111	γ	173 + 247	67
B. Radiolabeled immunoglobulin Therapy (RIT)				
Measurable Disease (>1 cm)	Y 90	β	2270	64
Small tumor masses (leukemia, leptomeningeal disease)	Cu 67	β γ	570 92 + 184	59

*RIS should always precede RIT in patients

By definition, the chemical nature of the daughter product is different from the mother isotope after alpha emission and this can lead to additional radioimmunoconjugate instability. The chelation chemistry required for a stable, kinetically inert radioimmunoconjugate differs per isotope. Unstable radioimmunoconjugates are of limited therapeutic value and carry considerable risks for undesirable normal tissue side effects. Radioisotopes with beta emissions only can be adapted to outpatient use for RIT.

Less than 5% of the beta emissions will be converted to low energy photons (Bremsstrahlung). Mixed emission radioisotopes (gamma and beta) will require inpatient management for activity escalation studies due to the radiation safety measures required. A double injection approach to RIT, i.e. first low activity gamma emitter immunoconjugate for diagnosis and quality control, and second high activity beta emitter immunoconjugate for therapy has important advantages to the patient: low toxicity, low cost, outpatient. This approach requires that the gamma emitting immunoconjugate is predictive for the beta emitting isotope and that the two immunoconjugates have similar biodistributions and *in vivo* pharmacokinetics.[10,12] This can be accomplished with the application of

appropriate chelation chemistry methods. The double injection RIT strategy is summarized in Table 2 for intravenous administration of radiolabeled immunoconjugates.

Table 2. RIT Strategy*

1. Administration of low activity (5-7 mCi) gamma emitting (In-111) radioimmunoconjugate
2. Determine *in vivo* stability radioimmunoconjugate (serial gamma camera, blood radioactivity, urine radioactivity measurements)
3. Determine tumor targeting and normal tissue uptake
4. Determine tumor dosimetry from gamma signal for beta emitting radioimmunoconjugates
5. Only if 2, 3, 4 favorable, administer beta emitting (Y-90) radioimmunoconjugate in 0.3 mCi/kg or higher activity

*This strategy applies to single agent studies in patients with radiosensitive malignancies, and IV RIT. Lower radioactivity levels are required for intracompartmental or intralesional RIT or RIT reagents that target bone marrow or have a blood half-life over 50 hours.

Preclinical Analysis

The primary prerequisite for successful RIT is the design and synthesis of an optimal radioimmunoconjugate. Novel chelation structures have been developed that after conjugation to an immunoglobulin preserve immunoreactivity of the antibody. *In vitro* stability of these radioimmunoconjugates in serum is excellent for indium and yttrium.

Preclinical *in vivo* studies are controversial at this time. Previously the unsupported contention was that the specificity of the radioimmunoconjugate (reactive with human tumor associated antigens) would only be expressed in human patients. Animal testing would not be appropriate. The variables involved in clinical RIT are numerous.[3,8] Most variables cannot be explored in human patients for ethical or financial reasons. Therefore, preclinical animal studies are essential to progress in clinical RIT and need to be adjusted to provide optimal, quantitative information that is predictive for clinical RIT. Table 3 lists the animal models available for preclinical RIT research. The mouse and dog models have made important contributions to the development of RIT for Hodgkin's disease.[3,8,14] Confirmation of the usefulness of such models in the development of RIT for other human malignancies is needed.

Table 3. Animal Models for RIT*

Species	Modification	Application
Nude mice	Human tumor xenografts s/c or i/p	tumor targeting biodistribution pharmacokinetics tumor therapy
Beagle dogs	No malignancy; size closer to human patient	biodistribution pharmacokinetics radiotoxicology
Rhesus monkeys	No malignancy; normal tissue antigens similar to human antigens: crossreactivity	biodistribution pharmacokinetics radiotoxicology

*An artificial "preclinical" therapeutic ratio can be obtained by comparing tumor dosimetry developed in mice with normal tissue dosimetry obtained in dogs or rhesus monkeys.

The research mission is clear: 1) increase uptake of radioactivity in malignant tissues, 2) minimize normal tissue damage, 3) minimize anti-antibody formation. The number of permutations in combinations of different experimental variables is high. In Figure 1 the decision tree for an individual RIT reagent is provided. Usually the initial RIT product was made with a specific patient population in mind (histological type and stage). If this is not the case, such a population needs to be identified to facilitate the selection of variables. The shaded boxes in the diagram indicate the areas of greatest current interest: 1) chelation chemistry, 2) origin of Ig, 3) route of administration (intravenous, intraperitoneal, intralesional), 4) size of Ig, 5) fractionation of RIT, 6) correlation of animal and patient studies. The variables are interdependent, e.g. large Ig's and i.p. administration; fractionation and human Ig for RIT. This interdependence can confine the preclinical analysis to a more manageable, smaller project. In the upper part of the diagram, the Ig will be permutated into several modifications. Subsequently, the number of permutations will be narrowed down in animal models to one or two with the most promising characteristics for applications in human patients. The difference with the "old" application of RIT in human patients is that most

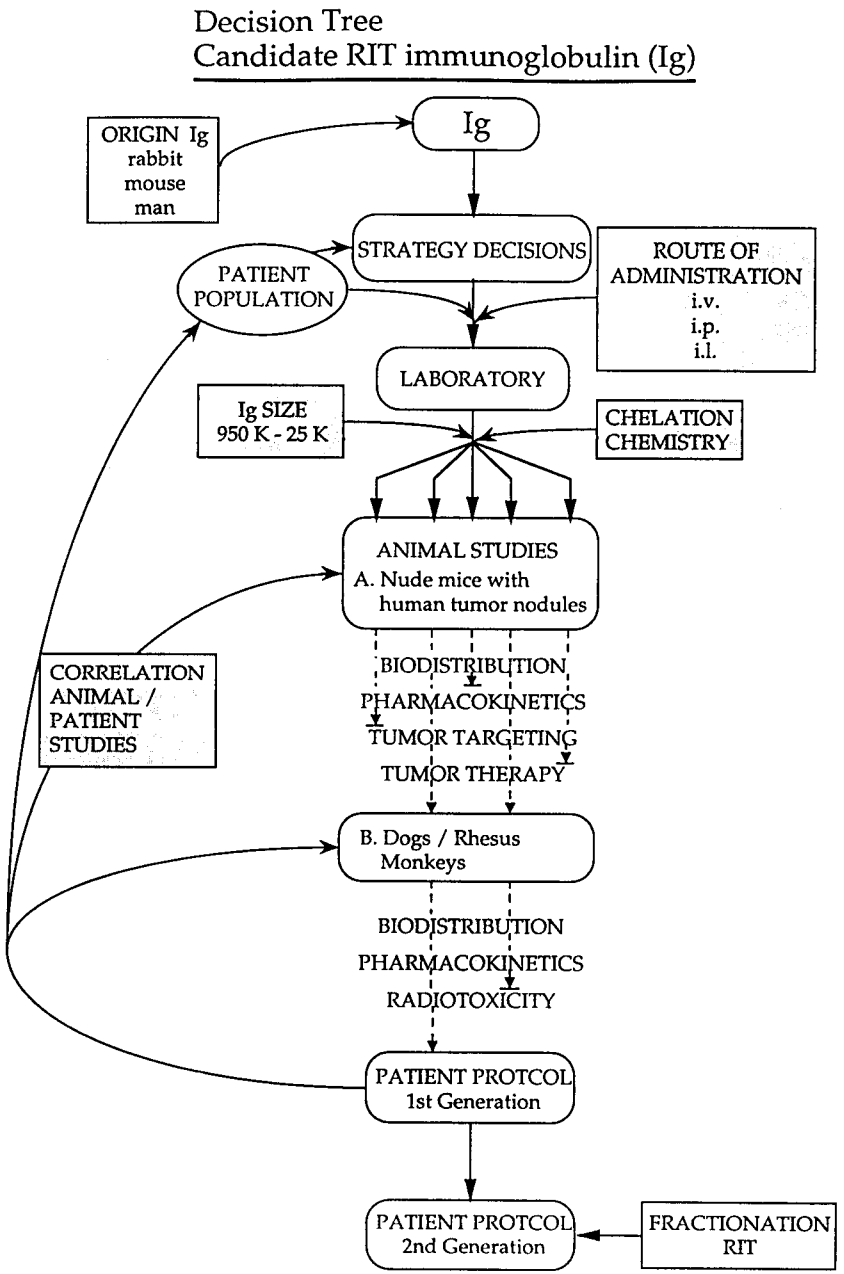
Decision Tree
Candidate RIT immunoglobulin (Ig)
ORIGIN Ig
rabbit
mouse
man
Ig
STRATEGY DECISIONS
PATIENT POPULATION
ROUTE OF ADMINISTRATION
i.v.
i.p.
i.l.
LABORATORY
Ig SIZE
950 K - 25 K
CHELATION CHEMISTRY
ANIMAL STUDIES
A. Nude mice with human tumor nodules
CORRELATION ANIMAL / PATIENT STUDIES
BIODISTRIBUTION
PHARMACOKINETICS
TUMOR TARGETING
TUMOR THERAPY
B. Dogs / Rhesus Monkeys
BIODISTRIBUTION
PHARMACOKINETICS
RADIOTOXICITY
PATIENT PROTCOL
1st Generation
PATIENT PROTCOL
2nd Generation
FRACTIONATION
RIT

Figure 1.

permutations of an Ig are not investigated in patients but screened in preclinical animal models. The crucial test to the new approach illustrated in Figure 1 is the comparison of the animal data to the results obtained in human patients. This will show whether the animal models utilized are indeed predictive or need to be adjusted. It is evident that the absence of accepted animal models for preclinical RIT testing slowed down the clinical development of RIT. Recommendations for optimal adjustment of the relatively simple variables in the diagram are not available yet and await the development of predictive animal models.

Clinical RIT

A new treatment modality such as RIT needs to be tested first as a single agent. It is tempting to develop RIT along the same lines as its competition: cancer chemotherapy. Previously we have argued that classical sequency of Phase 1 (toxicology), Phase 2 (activity) and Phase 3 (randomized trial) is not applicable or potentially dangerous for RIT reagents.[13] The RIT strategy outlined in Table 2 allows for the early elimination of RIT reagents that do not target tumor or have unfavorable pharmacokinetics. Obviously such reagents should not be utilized for toxicology studies. If the preclinical analysis as outlined in the previous section is applied, most clinical RIT reagents will target tumors and show the predicted pharmacokinetics. The first human studies with an RIT reagent can show considerable antitumor activity (in contrast to Phase 1 chemotherapy studies). This will enhance patient accrual and morale in the RIT team. A limitation to patient accrual is that patients must have measurable disease, otherwise tumor targeting (an important issue in the RIT strategy, see Table 2) cannot be evaluated. The prescription method for a radioimmunoconjugate is in mCi per kg, not mCi per body surface area or a total number of mCi per patient.[14] A safe starting activity for a mouse or rabbit RIT reagent that does not show bone marrow targeting is 0.3 mCi of yttrium-90 labeled Ig per kilogram.[2] Human reagents with a long (>50 hrs) blood half-life or RIT reagents that target bone marrow require a lower prescription activity per kilogram. The recommended protein dose is low ($\leq$ 5 mg/patient). This dose will not cause significant activation of acute phase reactants and the accompanying early adverse effects. It will also limit the immunological effects of the Ig on the tumor and permit a cleaner analysis of the radiation effects of the radioimmunoconjugate. Fractionation effects of RIT reagents need to be studied in much greater detail before specific recommendations can be made other than single shot RIT. In general, fractionation is expected to

decrease side effects in normal tissues (late side effects more than acute side effects) and not to decrease antitumor effects. In later studies RIT agents can be dose (activity) escalated and be incorporated in multimodality studies such as high dose chemotherapy regimens with bone marrow rescue.[15] The discovery of optimal RIT principles in patients with radiosensitive tumors (leukemia, lymphoma, small cell lung cancer, ovary and possibly breast) can lead to the application of those principles in patients with more radio-resistant malignancies. The purpose of the preclinical RIT analysis and the new methodology for clinical RIT studies is the same: decrease adverse effects in patients and enhance the chance for a tumor response. The interest in RIT can only be sustained if patients can receive effective, low toxicity, outpatient treatment.

CONCLUSIONS

The future of clinical RIT is a major challenge that requires the participation of many different specialists. Methodologies in the chemistry laboratory and the animal laboratory have been developed that allow for the introduction and testing of a translational approach (laboratory-animal-patient) to clinical RIT. Without a successful translational approach, the prospects for clinical RIT are dim. The initial results of RIT in patients with Hodgkin's disease are encouraging. A sixty-to-eighty percent response rate was obtained in an unfavorable end-stage patient population. Toxicity and costs were limited. Outpatient management was feasible and highly appreciated by the patients and their caregivers. A cautious, step-by-step analysis might provide similar results in patients with tumors more common than Hodgkin's disease and should include a preselection for the more promising radioimmunoconjugate permutations in predictive preclinical models. The design of curative clinical RIT protocols might require several investigational cycles in the decision tree of Figure 1.

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SUMMARIES

SUMMARY OF AML AND ALL SESSIONS

K.A. Dicke

The meeting started with the assessment of the role of auto-BMT in first remission of acute myelogenous leukemia. Data from 5 randomized trials were presented. The MRC trial chaired by Dr. Burnet was still ongoing so that definite conclusions were not presented. Of the 4 other trials, it became clear that the total number of patients randomized in each arm was disappointingly low; no more than 20% of patients entered in the studies were actually treated by one of the two arms: chemotherapy or auto-BMT. The EORTC trial demonstrated a significantly longer DFS rate in favor of auto-BMT, whereas in the other 3 trials, no significant difference was demonstrated. However, in 2 of these 3 trials auto-BMT was slightly superior. Meta-analysis would be necessary to look at these 5 trials all together and may give a more conclusive answer whether auto-BMT plays a significantly positive role.

The role of tumor cell contamination was addressed by Dr. Gorin who presented the EMBTR data. This registry, already in effect for the last 10 years, provided data about the role of purging. Ex vivo treatment of marrow had a positive effect on DFS after auto-BMT in patients whose marrow was collected within 3 months after onset of CR and transplanted within 6 months after onset of CR. Most likely at the time of harvest, the tumor cell contamination was high. In the majority of these patients, marrow was collected before intensification of remission. These results may indicate that tumor cell contamination of the graft has a biologically relevant role after transplantation confirming the elegant studies of Brenner et al which showed the participation of transplanted leukemic cells in relapse after bone marrow transplantation It is not known whether infused leukemic cells initiate relapse or if a minimal number of infused leukemic cells is necessary for participation in relapse. In other words, there may be a threshold of leukemic cells in the graft above which this population becomes biologically relevant.

Analysis of the data of the Autologous Blood and Marrow Transplant Registry, North America (NABMTR), demonstrated no difference in DFS rate between auto and allo-BMT. Relapse rate was higher after auto-BMT but that was offset by the lower treatment related mortality. We are looking forward to the analysis of auto-BMT versus chemotherapy by the NABMTR and hopefully results will be presented at the next meeting.

Dr. Ball presented promising data with antibody purged marrow cells in second remission AML. He reported a 50% inversion rate i.e. transplant remissions are longer than first remission. These data are seemingly superior to the 4-HC data of the Baltimore group. Differences in patient population may account for this. Randomized studies to test the superiority of purging methods are difficult to organize due to the relatively small number of patients available. In addition, the relatively small difference in results may be another major stumbling block of organizing such trials. In this situation, the two registries may be of great value. Data might be combined and hopefully an answer to these major issues can be obtained. It is important to include in such an analysis also the newer approaches of in vitro purge: 4HC/VP-16 and the in vitro incubation of marrow cells prior grafting.

An important issue is the role of posttransplant treatment. The Johns Hopkins team presented data of induction of graft versus host disease and its effect on prevention of relapse. The relapse rate does not seem to differ from that in the historical control group of patients without cyclosporin treatment. Another approach is the immune modulation advocated by Nilsson et al with Linomide. This study, as well as the interleukin-2 studies, are ongoing and hopefully in the next meeting more cumulative data are available.

An "old", but important, issue is that of the "best" or optimal regimen. This was again addressed in this symposium. In AML there is no difference in results between CY+TBI and the busulphan/cyclophosphamide regimen, which is in contrast with ALL in which TBI containing regimens seem to be superior according to the EBMTR analysis. The Rome group presented the updated BAVC results in second remission AML and the 52% 5-year CCR rate is one of the best results available. In this patient group, no marrow purging was used. These results are equivalent to Ball's results in second CR after Bu-Cy; however, in that study marrow was purged with a monoclonal antibody cocktail. The influence of patient selection on these results is not known. Randomization or a multi-institutional study will be necessary to address such issues.

In ALL the role of auto-BMT in CR1 is less obvious. In the randomized study organized by the French; Professor Fiere presented no difference between chemotherapy and auto-BMT. The Minnesota results indicate inferior results after auto-BMT to allogeneic BMT but better than chemotherapy. The future may bring gene-markers to monitor relapse as well as efficiency of purging, improvement of conditioning regimens by

improving the TBI regimens and by introduction of other modalities such as RIT (radiolabeled immune therapy). Post transplantation therapy with either immunotoxins or low dose chemotherapy are important areas of development, which may bring improvement of results in the future. Time will tell!

AUTOTRANSPLANTS FOR LYMPHOMA

SUMMARY

Armand Keating, M.D.

The field of intensive therapy and autotransplantation for lymphoma appears to have entered a period of consolidation. Two prospective trials comparing conventional dose salvage therapy versus ABMT for patients with relapsed or refractory Hodgkin's disease and for chemotherapy-sensitive relapsed non-Hodgkin's lymphoma (NHL) have been completed. The BLNI study in patients with Hodgkin's disease (HD), although having accrued small numbers of subjects, nonetheless showed superior disease-free survival after ABMT. The Parma study for NHL has been analyzed and while full details are pending, the results apparently also favor the autotransplant arm.

The definitive role of ABMT in low grade NHL remains to be established. A randomized trial of autotransplants versus conventional dose therapy for this indication is most worthwhile and should be supported strongly. A. Porcellini presented the European CUP trial protocol that will compare chemotherapy versus autotransplant with purged or unpurged marrow in patients with poor risk relapsed follicular NHL.

In the meantime, the Dana Farber data reported by A. Freedman at this Symposium continues to show superior DFS in patients whose autografts were rendered t(14;18) negative as detected by PCR. Longer follow-up is required to determine if survival is prolonged.

Timing of autotransplants for the lymphomas continues to be investigated. For example, M. Prince on behalf of the Toronto Program reported that disease status at transplant is the single most important prognostic factor determining DFS and objective survival after transplant for patients with intermediate grade NHL. This may not apply to low grade disease however, since the Dana Farber group showed a similar outcome for patients transplanted in CR or PR.

For HD, D. Reece (Vancouver) described promising results in patients transplanted at first relapse. In a series of 58 patients followed for a median of 2.3 years, actuarial progression free survival was 61% at 5 years. In contrast, for patients receiving so-called standard dose CBV (and with more heterogeneous disease- and patient-related characteristics) with a minimum follow-up of 4 years and median follow-up of 77 months, DFS

at 4 years was 25% as reported by P. Bierman on behalf of the M.D. Anderson Hospital and the University of Nebraska.

The Nebraska group also reported long-term complications after transplant for HD, including late relapses at a frequency of approximately 10 percent and the development of myelodysplastic syndrome.

Other issues that will be followed with interest will include the advantages of blood cell transplants versus marrow autografts, the role of immunotherapy including monoclonal antibody-radionuclide combinations for management of localized disease and transplants for patients with high risk disease in first remission.

HIGHLIGHTS ON AUTOTRANSPLANTS IN MULTIPLE MYELOMA

SUMMARY OF THE MYELOMA SESSION

Philippe Henon

Institut de Recherché en Hématolgie et Transfusion, Mulhouse, France

Within the past five years, high-dose therapy followed by autologous blood cell transplantation (ABCT) has been extensively used as front-line or salvage therapy in patients with stage II or III multiple myeloma (MM). When compared with allogeneic transplantation, ABCT-related mortality dramatically dropped below 10% versus about 40%. Consequently, this procedure can be offered safely up to age 70 in contrast to allo-transplant trials that have an age limit of 55 years and encompass less than 10% of MM patients. Furthermore, while complete remission (CR) rates (up to 50%) and overall survival (4-5 years) are comparable after both procedures so far, quality of life and cost effectiveness seem to be significantly better in the case of ABCT.

However, several crucial issues still remain unresolved. First, failure of blood cell (BC) mobilization occurs more frequently in MM than in other diseases. So, collecting adequate numbers of BC for further transplantation may be problematical in 20-30% of MM patients. In an attempt to offset such a failure, Henon et al reported a short pilot study evaluating the effect of a mobilizing regimen combining high-dose melphalan (HDM) 100 mg/m^2 in a single IV bolus and sequential administration of rh IL-3 (early acting cytokine) and of rh G-CSF (late acting cytokine). Two different schedules for cytokine administration were tested. Schedule A consisted of IL-3 (5 μg/kg/day) administered subcutaneously from day 7 to day 11 after HDM, followed by G-CSF (5 μg/kg/day) from day 12 to day 20; two patients (one non-pretreated, one having prior standard chemotherapy for one year) underwent this schedule. Under schedule B, HDM was followed by IL-3 alone at the same dosage from day 1 to day 3, IL-3 + G-CSF (overlap) from day 4 to day 7 and G-CSF alone from day 8 until completion of leukaphereses. Three patients underwent this second schedule; one non-pretreated, two having received prior standard chemotherapy for 6 and 12 months, respectively. It resulted from this pilot study that only schedule B,

comprising the IL-3/G-CSF overlap, was capable of substantially enhancing BC mobilization (a 25 to 165-fold increase in CD34+ cells from baseline), more evident in non-pretreated than in pretreated patients, and also of significantly reducing the post-HDM aplasia (3 to 9 days). Once reinfused, mobilized BC permitted a hastened posttransplant granulocytic (9 days) and platelet (10-12 days) recovery. Additionally, schedule B also noticeably reduced the occurrence of stomatitis and diarrhea usually related to HDM.

A second unresolved issue is the absence of total eradication of the disease. Even if post-ABCT overall survival is successfully prolonged when comparing with standard therapies, the median event-free survival at 3 years still does not exceed 40-60%. Moreover, the absence of a plateau in the survival curves suggest that most of the patients, if not all, will relapse. Disease recurrence may occur either from persistence of clonogenic cells in the autograft product, or from "sanctuary sites" in the patient.

Several groups have reported their attempt to improve the event-free survival. Vescio et al, having previously shown the lack of CD34 antigen expressing malignant cells in MM, have chosen to purge BC autografts by CD34 positive selection using the CellPro system (CellPro, Inc., Bothell, WA). This technique, at least, allows a 1.5-2.5 (and, in some cases, a 4-5) log reduction in tumor cell infusion, as demonstrated using PCR. Thirty-one patients with MM have been transplanted with purified blood CD34+ cells to date, after a conditioning regimen combining Busulfan and cyclophosphamide, 500 μg/day of GM-CSF were added posttransplant to improve hematopoietic recovery, and maintenance therapy with Intron A and dexamethasone was initiated at day 100. There were two treatment-related deaths. All patients have had partial or complete response to therapy, except one who died of disease progression.

Barlogie et al have preferred to explore another way to improve event-free survival rates, still strengthening high-dose therapy in an attempt at *in vivo* purging. One hundred seventy-six patients initially underwent 3 courses of standard therapy (VAD regimen) followed by one BC mobilizing course combining high-dose cytoxan and GM-CSF before cell collection. Fifty percent presented with a stage III MM at diagnosis, 51% were over 50 years old. The first transplant was preceded by a conditioning regimen combining HDM 200 mg/m^2 and GM-CSF. Before the second one, patients received either the same conditioning regimen in case of good response to the first one, or else HDM 140 mg/m^2 + TBI.

About 2/3 of patients underwent both transplants, which were followed by a maintenance therapy with interferon 3 M.U./m^2 until eventual relapse. Ninety percent of patients were responders, 50% achieving CR. Overall survival and event-free survival were 60% and 40% respectively at 4 years. Patients were hospitalized 14 days for each transplant.

Last, but not least, R. Champlin interestingly reported the first attempt of targeted radiotherapy on bone marrow using Holmium 166. This bone-seeking radioisotope shows a higher affinity for trabecular bone than for cortical bone, thus secondarily radiating adjacent marrow. Nine MM patients received 6 to 12 mCi/kg in 3-4 IV infusions. Blood radioactivity rapidly decreased, proportionally to the increase of bone radiolabelling. Total doses infused ranged from 519 to 264 l/mCi. Bone retention ranged from 26 to 33% of the total dose infused, 67 to 74% being excreted in urine. Pancytopenia with hypocellular marrow was observed in all patients, normal neutrophil count recovering after 5 to 17 days. Extra-medullary toxicity was tolerable, not exceeding WHO grade II. Such an approach, which seems to be less aggressive than total body irradiation, might be interestingly developed for pretransplant conditioning. However, in MM, two major problems are still limiting its use: first, a certain microheterogeneity of dose distribution within marrow seems to be frequent; second, this radiation technique might not be used in case of extra-medullary MM.

To substantially prolong life in high-grade MM is a major step toward achieving durable disease control. This may eventually lead to another step, that of a definitive cure for MM. However, it will still require more effort and additional therapeutic strategies. As a whole, the communications presented during this session proved to worthwhile.

SUMMARY OF BREAST CANCER SESSION

Giovanni Rosti

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In the field of solid tumors as well as for hematologic disorders, high-dose chemotherapy is a policy which is increasing world-wide. In Europe in 1993, more than 7,000 patients received BMT for their diseases: four hundred of them had breast cancer, 168 germ cell tumors and nearly 100 Ewing's sarcoma, and even more have been transplanted in the U.S.

The reason for the increase in the number of transplants for breast carcinoma is the poor outcome for advanced metastatic disease despite several "active" combinations, as well as, the dismal prognosis of patients with high risk ($\geq$4 or $\geq$10 positive axillary nodes). Moreover, good results are emerging in selected cases of metastatic patients, particularly those grafted while in complete remission after upfront chemotherapy as shown in the data presented by Dr. G. Spitzer, Dr. G. Rosti, Dr. S. Williams and several others. An interesting option was presented by Dr. R. Jones from Denver who treats patients in what they call the "Stage IV NED" breast cancer; these patients are rendered free of disease with radiation or surgery or have micrometastatic disease in the bone marrow as single site. The magnitude of high doses in terms of dose size may play an additional role, as clearly shown by Dr. K. Fields and Dr. G. Elfenbein from Tampa. With the ICE regimen developed at their institution, patients receiving higher doses have a better disease-free outcome compared with the ones who received "smaller" high doses. And that may be an important point for future investigations.

Two registries have been set up, one in Europe on behalf of the EBMT Group Solid Tumor Working Party chaired by Prof. Philip from Lyon, and one in the U.S. and Canada by the NAABMTR (North American Autologous Bone Marrow Transplantation Registry). In the latter, which enrolls several hundred patients, a clear tendency to shift toward high-dose treatment in adjuvant poor risk cases is emerging. But the message is that patients with metastatic disease still need to be investigated with ABMT/PBSCT supported high-dose therapies, especially if they have shown a sensitivity to the upfront chemotherapy (so-called "sensitive relapses"). The 100 days mortality is decreasing in the U.S. and in Europe due to better supportive therapies as well as to the use of peripheral blood progenitor cells (still to be definitively proven). Adjuvant

cases are grafted in Europe with four or more positive axillary nodes (Amsterdam, Milan) while the two major US randomized trials require at least ten positive axillary nodes as an entry criterion. In the near future, clear data are expected from the US (SWOG, ECOG) and Europe (Milan, Dutch trial, etc.) to answer the question, "is more better?" in adjuvant breast cancer patients.

During the symposium many questions regarding treatment with transplants arose and are summarized below.

Questions to be answered in the near future

1. Which is the "best" high-dose policy?
   - Single shot - Elfenbein, Williams, Antman
   - Double shot - Rosti, Spitzer
   - High-dose sequential chemotherapy - Gianni
   - Multiple repeated high-dose courses - Repets
   - Others

2. Are PBSC clearly superior to ABMT?
   - Which is the exact role of positive selection or *in vivo* expansion?

High-dose chemotherapy in solid tumors, including breast cancer, was a desperate option in the early 80's and it is rapidly becoming a common policy of treatment which is considered experimental even in centers with appropriate know-how. For some tumors like breast cancer, the time for good randomized phase III trials has definitively come for adjuvant patients, and for metastatic disease or inflammatory breast carcinoma new strategies are warranted (Phase I trials included). We hope that at the Eighth International Symposium, we will hear many answers to questions posed during the Seventh.

SUMMARY OF HIGH-DOSE CHEMOTHERAPY FOR SOLID TUMORS

Craig R. Nichols

Indiana University

There is increasing activity in the area of high-dose chemotherapy for a variety of solid tumors. For example, presentation of the summed data from the North American Autologous Bone Marrow Transplant Registry demonstrates that almost 3000 patients with locally advanced or metastatic breast cancer were registered in the data base since 1989. Overall, the two-year survival for those who received high-dose treatments as adjuvant therapy is 77% and for metastatic disease is 38%. The therapy-related mortality has dropped over this time frame from 22% to 6%. To date, the bulk of the investigations in most solid tumors have revolved around Phase I and II studies that have resulted in high-dose regimens that can be delivered safely and are associated with substantial anti-tumor effect. In these meetings, a variety of such studies were presented focusing on more intensive chemotherapy regimens, repetitive application of high-dose treatments and new protocols in breast cancer, ovarian cancer, germ cell tumors, brain tumors and pediatric sarcomas. It is clear that these novel high-dose regimens have resulted in durable responses in patients with relapsed and refractory solid tumors.

It is also clear that the initial development phase of high-dose regimens has entered a plateau period. It is unlikely that substantially more effective regimens can be developed in light of the limits of nonhematologic toxicity that are currently constraining further dose escalation. There are few new drugs that are amenable to dose escalation that could find a niche in the current regimens. Thus, it appears that it is time for rigorous evaluation of the true impact of these intensive and expensive approaches to treatment of advanced solid tumors. At this symposium, we heard the beginnings of such plans in several areas. Ongoing trials in Europe and the USA for breast cancer comparing high-dose chemotherapy to standard therapy are critically important and represent the highest priority trials for patients with high risk or metastatic breast cancer. In germ cell tumors, a randomized trial of standard dose chemotherapy compared to high-dose chemotherapy in untreated patients with poor risk features has begun as an intergroup trial in the USA. In Europe, cooperative investigations are comparing VIP X 4 cycles to VIP X

3 plus high-dose chemotherapy consolidation in patients with recurrent germ cell tumors. In ovarian cancer, the NCI is sponsoring a trial comparing high-dose chemotherapy to continued paclitaxil and carboplatin in patients with low-bulk disease at second look surgery after 4 initial cycles of cisplatin and paclitaxil. In other disease sites such as melanoma and small cell lung cancer, the data are not sufficiently compelling to encourage phase III investigations of high-dose chemotherapy. It is these comparative trials to which we should commit our patients so that we may measure the true impact of these promising new treatments.

SUMMARY OF CML SESSION

Angelo M. Carella

Genoa, Italy

Over the last decade, the main developments in the management of chronic myelogenous leukemia (CML) have been the introduction of interferon-α (IFN-α), which may prolong life in patients achieving a cytogenetic response and the confirmation of the role of allografting in younger patients. For the majority of patients with CML, alternative treatment strategies are needed in the absence of a compatible related donor (or of a matched or partially matched unrelated donor through organized registries), or for cases refractory to IFN-α. Autografting has been recently explored as an option for this group especially in view of the clear demonstration of the presence of normal hematopoietic stem cells in the bone marrow of CML patients.

The crucial point is how best to assess response or clinical benefit. If one considers that after many years of experience with IFN-α in newly diagnosed patients, it is still not entirely clear to what extent and in which patients IFN-α prolongs life, it may be a little optimistic to think that the precise contribution of autografting will become clear after 3-4 years from now. Nevertheless, the ability to achieve and maintain Ph-negative hemopoiesis for one or more years may lead to a survival benefit.

In the 7th International Symposium on Autologous Bone Marrow Transplantation held in Arlington, Texas, a session was dedicated to CML. In particular the *in vivo* and *in vitro* methodology favoring Ph-negativity were discussed.

Dr. Simonsson reported the results of an intensive and complex approach, employing IFN-α and chemotherapy, with the principal aim to eliminate or minimize the Ph-positive clone. The conclusions suggest that this approach is effective in reducing Ph-positive cells and may prolong survival in CML. Another promising approach is with the immunomodulator roquinimex (Linomide). This drug is known to enhance T-cell, NK cell and macrophage activity and it is now studied in Europe and USA in AML following autografting. J. Rowe and colleagues employed this drug in Ph-chromosome positive CML post-ABMT. The preliminary data suggest that roquinimex may have significant activity even though further follow-up and more patients are needed.

J. Reiffers updated the results of the European experience with autografting using different conditioning regimens. Some patients were in earlier or late chronic phase and received different stem cell rescue (blood or marrow). In any event, the results were similar in terms of survival to those recently presented by P. McGlave and colleagues in "Lancet" with a longer survival for patients who achieved a complete hematologic remission or major cytogenetic remission after autografting versus those patients who did not. C. Carlo-Stella presented an update of the Parma studies on the selection of Ph-negative progenitors. This study demonstrates that many stroma-adherent, mafosfamide-resistant progenitors were Ph-negative. These progenitors are detected with CD34+ fraction and, last but not least, stroma-adherence was no longer necessary to improve selection of Ph-negative clones when the CD34+ cells were incubated with mafosfamide. Clearly, further confirmation of the clinical usefulness of this procedure, will permit important progress in the CML autografting to be made.

Finally, our team reported the experience with mobilization of Ph-negative progenitor cells. From our first experience in blastic phase CML, we were able to demonstrate that the *in vivo* approach results in a higher collection of Ph-negative progenitors in the blood if the cells are collected in the early phase of recovery after aplasia induced by chemotherapy. These results have been recently confirmed in patients with CML at diagnosis. In conclusion, the data briefly presented here open new perspectives for patients without HLA-identical donors or for patients who do not respond to IFN-α.

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