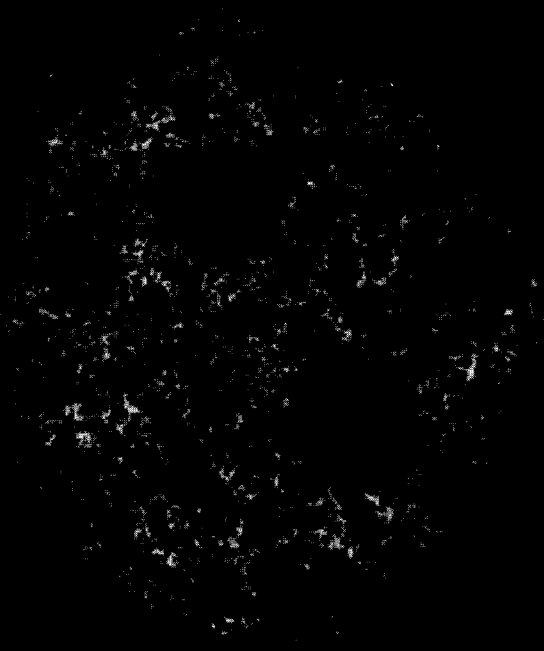
AUTOLOGOUS MARROW AND BLOOD TRANSPLANTATION

Proceedings of the Eighth International Symposium Arlington, Texas



Edited by

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PREFACE

The Eighth International Symposium on Autologous Marrow and Blood Transplantation held in Arlington, Texas served once again as a venue for over 90 leading investigators to present and discuss current and prospective studies in the field. The Symposium presentations underscored the considerable energy, impetus and advances in this area of clinical practice. High-dose therapy and autologous transplantation has matured over the last five years, as demonstrated by the presentation of numerous prospective randomized trials for a number of diseases including leukemia, lymphoma, and breast cancer. However, innovative new approaches continue to be supported in the transplant community as highlighted by developments in mobilization of blood cells, ex vivo expansion of hematopoietic progenitors, and post-transplant immune modulation and eradication of minimal residual disease. Indications for intensive therapy and autologous transplants continue to be refined, but also extended, possibly to include severe auto-immune connective tissue disorders.

A highlight of the Symposium was the presentation of the CMOMC Award. CMOMC is an acronym for "cell meeting our morphological criteria," the candidate hematopoietic stem cell described back in 1971 by van Bekkum and his colleagues. The award is in the form of a CMOMC molded in gold. Drs. Derek van Bekkum and Malcolm A.S. Moore were the first recipients for their outstanding contributions to bone marrow transplantation and experimental hematology.

In the future, this award will be named the "van Bekkum Stem Cell Award" in tribute to Derek van Bekkum for his pioneering work in bone marrow transplantation and as founder and director of the Radiobiological Institute in Rijswijk, The Netherlands, which was for so many years an international center for the study of experimental hematology. His visionary leadership is an inspiration to so many of us and his impact on the careers of numerous laboratory and clinical researchers has been considerable.

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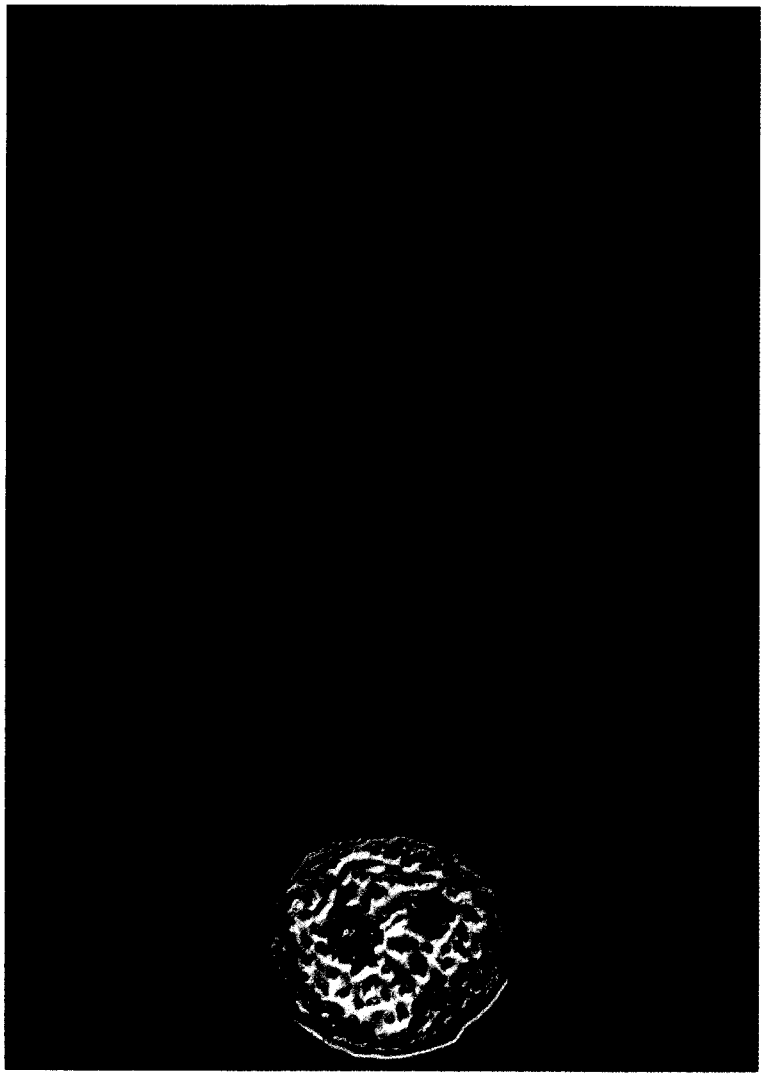

THE VAN BEKKUM STEM CELL AWARD

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ABSTRACT

The AML10 protocol is the first study to prospectively compare daunorubicin, mitoxantrone and idarubicin in remission induction and consolidation therapy of previously untreated acute myeloid leukemia (AML). All patients subsequently undergo allogeneic or autologous stem cell transplantation (alloBMT and autoBMT, respectively). Main end-points of the study are remission rate, overall survival, disease-free survival (DFS) and toxicity. Patients aged 15–60 years with previously untreated AML are randomized to receive remission induction therapy with either of the following three anthracyclines: idarubicin 10 mg/m^2/day, mitoxantrone 12 mg/m^2/day or daunorubicin 50 mg/m^2/day IV days 1, 3 and 5 plus etoposide 100 mg/m^2/day IV days 1–5 and Ara-C 25 mg/m^2 IV as a bolus injection on day 1, followed by Ara-C 100 mg/m^2/day IV as a continuous infusion on days 1–10. Consolidation therapy consists of intermediate dose Ara-C (500 mg/m^2 IV q12 hours for 6 days) plus the same randomized anthracycline on days 4–6 (6+3

regimen), and as postconsolidation therapy a stem cell transplantation in all patients using bone marrow stem cells.

Between December 1993 and June 1996 a total of 1009 patients have been included in the study. Overall results of remission induction are available in 859 patients. Six hundred and thirteen patients (71.4%) entered a complete remission (CR), 567 (66%) after one course. Five hundred and eleven (83.4%) received consolidation treatment. The main known reasons patients in CR did not receive consolidation treatment were toxicity (38 patients) and early relapse (11 patients). AlloBMT was performed in 69% of 146 patients who received the consolidation course and had a family donor. An autograft was performed in at least 144 of 358 patients without a donor; in 41 patients the information was not yet available. Main known reasons an autograft was not performed were toxicity (72 patients) and relapse (56). Delayed marrow recovery after consolidation and poor quality of the bone marrow stem cell harvest were major reasons for delaying or canceling the planned autograft.

In conclusion: In comparison with the earlier EORTC-LCG/GIMEMA AML8A study in which a 3+7 regimen was used, the CR rate in the present 3+5+10 regimen is higher. More often one course suffices to achieve CR. Compliance with alloBMT is better than with autoBMT. The protocol is now amended to mobilize and harvest peripheral stem cells (PSC) in all patients without a donor and to randomly assign them to autologous transplantation with bone marrow (autoBMT) or peripheral stem cells (PSCT). In patients allocated to autoBMT, cryopreserved PSC may be used for rescue in case of bone marrow failure or for transplantation in CR2.

INTRODUCTION

In the previous AML8A study of the EORTC and the GIMEMA (1986–1993) for untreated acute myeloid leukemia (AML) in adults, remission induction consisted of daunorubicin and conventional dose cytarabine (Ara-C) in a 3+7 regimen. The first consolidation course was composed of intermediate dose Ara-C and amsacrine (6+3). Patients who had an HLA-identical family donor were assigned to an allogeneic stem cell transplantation (alloBMT), those without donor were randomly assigned to autologous bone marrow stem cell transplantation (autoBMT) or a second course of intensive chemotherapy with high dose Ara-C and daunorubicin (4+3). Only patients younger than 45–55 years were eligible for transplantation. A complete remission (CR) was achieved in 66% (in 54% after one course).[1] The number of courses required to achieve CR was an important prognostic factor for the long-term outcome of alloBMT.[2] AutoBMT resulted in longer disease-free survival (DFS) than intensive chemotherapy.[1] At 5 years follow-up the DFS in patients in the alloBMT, autoBMT and second intensive chemotherapy arms are 55, 42 and 30%, respectively (unpublished data).

Therefore, in the present AML10 study an alloBMT or autoBMT was planned for all eligible patients as post-consolidation therapy.

Bishop et al.[3] conducted a prospective randomized trial in patients with previously untreated AML, comparing Ara-C, daunorubicin and 7 days of etoposide 75 mg/m^2/day with Ara-C and daunorubicin alone. The CR rate was not different in the two groups, but remission duration was longer with etoposide than without. The AML10 study of the Medical Research Council (MRC) combined in one arm 3 days of daunorubicin (days 1, 3 and 5), 5 days of etoposide (days 1–5) and 10 days of Ara-C (days 1–10) and has an overall CR rate of 82% in younger adults.[4] It was therefore decided to add etoposide to the EORTC-LCG/GIMEMA AML10 regimen.

An additional way to improve the CR rate in AML could be to replace daunorubicin by one of the more recently developed cytostatic drugs, idarubicin (4-demethoxydaunorubicin) or mitoxantrone. A randomized study in adult patients with AML, comparing idarubicin and daunorubicin, each in combination with Ara-C, demonstrated a higher overall CR rate and a higher % CR after one course in the idarubicin arm.[5] In a group of 21 patients who had failed remission induction in the above indicated AML8 protocol and who thus represented a poor prognosis group, 11 patients entered CR after one (8) or two (3) courses of Ara-C 500 mg/m^2, q12 h for 7 days and idarubicin 12 mg/m^2/d on days 1, 3 and 5.[6] Carella et al.[7] reported a CR rate of 83.6% in 67 untreated AML patients using idarubicin, etoposide and Ara-C in a 3+3+5 regimen.

Mitoxantrone might be less cardiotoxic than daunorubicin, and more effective for remission induction.[8,9] Mitoxantrone 12 mg/m^2/d in combination with intermediate dose Ara-C for 3 days, followed by a chemotherapy-free interval of 4 days, and 3 days of etoposide 200 mg/m^2/d in combination with Ara-C, in patients with secondary, relapsed or refractory AML rendered a complete remission percentage of 61%.[10] An EORTC-HOVON study in elderly patients with newly diagnosed AML (the AML9 study) confirmed that the combination of mitoxantrone and Ara-C results in a higher CR rate than daunorubicin and Ara-C, in the context of equivalent toxicity (Löwenberg et al., to be published).

In the present AML10 study the Leukemia Cooperative Group of the European Organization for Research in the Treatment of Cancer (EORTC-LCG) and the Gruppo Italiano Malattie Ematologiche Maligne dell' Adulto (GIMEMA) compare the three anthracycline/anthracene derivatives. Patients receive the same randomly assigned anthracycline/anthracene during remission induction and consolidation. Finally each patient in first CR, also in the age group of 46–60 years, is planned for autologous stem cell transplantation. The study is ongoing and the present report represents an interim analysis of 859 of 1009 patients, who were randomized between December 1993 and March 1996 and who were evaluable for response to induction. Follow-up was until June 1996.

METHODS

Patients

Patients aged 15–60 years with previously untreated AML are eligible. Diagnosis and response cytology are being reviewed by an independent cytology committee. AML M3, as classified according to the French American British (FAB) criteria,[11] is excluded. Also patients with AML secondary to myelodysplastic syndrome (MDS) of more than 6 months' duration and patients with myeloproliferative diseases are excluded. Informed consent is obtained according to the rules of each participating institution. At diagnosis and complete remission bone marrow samples are evaluated for the presence of cytogenetic abnormalities. The abnormalities are grouped according to risk, as described by Keating.[12]

Treatment protocol

All eligible patients receive remission induction treatment with cytarabine (Ara-C) 25 mg/m^2 IV as a bolus injection on day 1, followed immediately by Ara-C 100 mg/m^2/d IV as a continuous infusion on days 1–10, etoposide 100 mg/m^2/d in a 1-hour IV infusion on days 1–5, and randomly assigned one of the following three anthracyclines: idarubicin 10 mg/m^2/d (ICE), mitoxantrone 12 mg/m^2/d (MICE) or daunorubicin 50 mg/m^2/d (DCE) via a short IV infusion on days 1, 3 and 5. If a partial remission is obtained, the same course is repeated. In the case of resistant disease salvage treatment is recommended. Patients in CR undergo consolidation treatment consisting of intermediate dose Ara-C, 500 mg/m^2 via a 2-hour IV infusion every 12 hours on days 1–6, and the same randomly assigned anthracycline, idarubicin 10 mg/m^2/d (IDIA), mitoxantrone 12 mg/m^2/d (NOVIA) or daunorubicin 50 mg/m^2/d (DIA), via a short IV infusion on days 4–6. Patients in confirmed complete remission who have an HLA-identical family donor are assigned to alloBMT. Patients in CR who do not have a related HLA-identical donor are assigned to undergo a bone marrow stem cell harvest. After confirming the CR status of the patient and checking quantity and quality of the harvest, an autoBMT is performed. Because of a delayed and sometimes poor harvest of bone marrow stem cells, after a pilot phase the protocol was amended to include peripheral stem cell mobilization in patients without a donor and a second randomization between autologous peripheral stem cell transplantation and autologous bone marrow stem cell transplantation.

Definitions and statistics

Complete response (CR) and partial response (PR) are defined according to the criteria of the NCI sponsored workshop.[13] Complete remission requires the presence of peripheral blood neutrophils of $>1.5\times10^9$/L, platelets of $>100\times10^9$/L and normal cellularity of the bone marrow with less than 5% blast cells. Auer rods

should not be present. Extramedullary leukemia, such as central neural system involvement or leukemic infiltration of the skin or other sites, should not be present. A partial remission is characterized by all criteria of CR except the presence of 5–20% blasts in the bone marrow or <5% blasts with Auer rods. An untreated CR lasting shorter than one month is only a PR. Failures are resistant disease or death during the induction phase. Resistant disease is considered as patients who survive for at least 4 weeks after the beginning of the first remission induction course without having reached PR or CR, and those surviving after two induction courses without CR. Early death is defined as occurring during days 1–10 of the remission induction course; death occurring after completion of the remission induction course and before repopulation is called hypoplastic death. Relapse following CR is defined as the recurrence of blasts in the blood and/or the finding of more than 5% blasts in the bone marrow after documentation of CR.

Disease-free interval is defined as the time from first CR to relapse; DFS, as the time from CR to the first relapse or to death. Duration of survival was calculated from the time of diagnosis. The probability of survival was calculated according to the Kaplan-Meier method.[14] Tests of statistical significance for difference in survival curves were performed using the two-tailed logrank test.[15]

RESULTS

Characteristics of the patients and their AML

The median age of the 859 randomized evaluable patients was 43 (15–60) years. The most frequent FAB types were M2 (34%), M5 (21%), M4 (20%) and M1 (17%). FAB types M0 (4%), M6 (3%) and M7(1%) were rare. Fourteen percent of 316 patients of whom the cytogenetics have been reviewed had good prognostic karyotypes (t(8;21) or inversion 16), 48% had a normal karyotype in all examined cells or -Y and were considered intermediate risk patients. Eight percent had 11q abnormalities or trisomy 8, and 9% had monosomy or partial deletion of chromosomes 5 or 7 or complex abnormalities. Twenty-one percent had other single karyotypic abnormalities. Thus 38% were considered to be in the poor prognosis cytogenetic category.

Response

In June 1996 a total of 859 randomized patients were evaluable for response to induction. The results are indicated in Table 1. A complete remission was achieved in 613 patients (71.4%) and in 567 patients (66%) after a single course. Two hundred and fifty-two patients were treated in centers that had also participated in the pilot study before initiation of the AML10 study and 607 were treated in nonpilot centers. The CR rates in the two groups were 75 and 70%, respectively (NS).

Table 1. Results of induction

Response	*n* (%)*
CR (after 1 or 2 courses)	613 (71.4)
resistant	131 (15.2)
death	115 (13.4)

**n= number of patients*

Resistance occurred in 131 patients. One hundred and fifteen patients (13.4%) died during the induction period: 2.7% died early (during the ten days of induction therapy) and 10.7% died during the hypoplastic phase.

Of the 613 patients who entered a CR, 511 (83.4%) received the consolidation course. The main reasons for not receiving the consolidation course after achievement of CR, excluding induction deaths, were toxicity in 38, early relapse in 11, refusal in 3 and other in 6 patients. In 44 patients the reasons were not yet known. Twenty-seven (5%) patients died within two months after day one of the consolidation course. Of 511 patients who received the consolidation course, 146 had an HLA-identical sibling and 358 did not. In 7 patients the availability of a donor was not yet known. AlloBMT was performed in 101 patients (69%) with a donor. Reasons for not performing the alloBMT were mainly relapse (20 patients), toxicity (10) and refusal (6). Among patients without a donor, stem cells were harvested in 162 patients (bone marrow in 132, peripheral stem cells in 19 and both bone marrow and peripheral stem cells in 11 patients). An autologous transplantation was performed in 144 patients, using bone marrow stem cells (125 patients), peripheral stem cells (14) or both bone marrow and peripheral stem cells (5). The main reasons for not performing the autograft were toxicity (72 patients), relapse (56) and refusal (31). For 41 patients the results were not yet known. The present estimated rate of performing an autograft therefore is between 40 and 45%.

With a median follow-up of 15 months the overall survival at 1.5 years was 40%. Survival in patients younger than 46 was longer than in patients 46–60 years old ($p=0.04$). There was a significant difference in survival between patients treated in pilot and nonpilot centers ($p=0.003$). Among the most frequently occurring FAB types (1, 2, 4 and 5) survival was longest in patients with types M4 and M2 and shortest with type M5.

The probability of DFS from the time of CR at 15 months was 42%, the risk of relapse was 45%. Transplant-related mortality was approximately 20%: 30% in the alloBMT and 13% in the autoBMT group. DFS was longest in patients with good risk cytogenetics (70% at 14 months) and shortest in patients with monosomy 5, 5q-, monosomy 7, 7q-, 11q23 abnormalities, trisomy 8 or complex cytogenetic abnormalities. DFS was longest for patients with FAB types AML M2 and shortest for AML M5.

Table 2. Types and incidence of grade 3 and 4 toxicity in the remission induction and consolidation phase

Toxicities grade 3–4	*Remission induction (%)*	*Consolidation (%)*
infection	41	23
diarrhea	23	5
oral	16	3
hemorrhage	14	3
nausea	14	10
liver	11	3
pain	10	5
pulmonary	6	2
renal	1	1
central neural system	2	2
allergy	1	1
cutaneous	2	3
constipation	3	<1
heart	3	6

Toxicity

The types and incidence of grade 3 and 4 toxicity according to the WHO criteria in the remission induction and consolidation phase are indicated in Table 2. Grade 3 and 4 extramedullary toxicity was more frequent in the induction than in the consolidation phase. Infection was the most frequently observed type of toxicity in both phases. Severe gastrointestinal toxicity was more often associated with the remission induction than with the consolidation course.

The time to bone marrow recovery after the remission induction course was acceptable as is shown in Table 3. In a few patients recovery was extremely slow.

The time to recovery of hypoplasia after the consolidation course is demonstrated in Table 4. After the consolidation course, recovery of the bone marrow was slow in more patients than after remission induction.

Autologous bone marrow stem cells were harvested at a median of 10 weeks following day 1 of the consolidation course. Twenty-three percent of the stem cell harvests were delayed until 3–6 months following day 1 of the consolidation course. Reasons for delaying the bone marrow harvest were, among others: insufficient in vitro growth of the myeloid progenitor cells (CFU-GM) and hypoplasia of the bone marrow. Twelve percent of evaluable harvests (both early and delayed) were insufficient as determined by the amount of CFU-GM per kg body weight. In those cases autoBMT could not take place. Eighty-two percent of patients harvested within the first 3 months following day 1 of the consolidation course completed the full protocol, versus 61% of those harvested later than 3 months.

Table 3. Recovery of blood platelets and polymorphonuclear cells (PMN) from day 1 of remission induction treatment

	Median/range (weeks)	*No recovery at 2 months (%)*
platelets $>20\times10^9$/L	3.5 (2–30)	4
platelets $>100\times10^9$/L	4 (3–30)	7
PMN $>0.5\times10^9$/L	4 (2–9)	10
PMN $>1.5\times10^9$/L	4 (2–12)	10

DISCUSSION

In the ongoing AML10 study of the EORTC-LCG and the GIMEMA, 1009 patients were enrolled in 2.5 years. This interim analysis shows an overall CR rate of 71.4%. More patients than in the previous AML8A study required only one course to enter CR, instead of two. It is too early to see the implications of this with regard to the DFS after completion of the protocol.

The percentage of completion of the full protocol was higher in alloBMT patients than in those who were assigned to autoBMT. The consolidation course was associated with an acceptable fatality rate but a relatively prolonged time to marrow recovery. This implied a delayed or insufficient bone marrow harvest in a significant number of patients who were otherwise eligible for autoBMT. A delayed harvest was more often associated with failure to complete the full protocol because of relapse or toxicity.

Also, after autoBMT hematologic recovery was sometimes slow (data not shown). AutoBMT was associated with a considerable transplant-related mortality (13%).

Table 4. Recovery of blood platelets and polymorphonuclear cells (PMN) from day 1 of consolidation treatment

	*Median/range (weeks)**	*No recovery at 2 months (%)**
platelets$>20\times10^9$/L	3.5 (2–18)	11
platelets$>100\times10^9$/L	4 (2–30)	28
PMN$>0.5\times10^9$/L	4 (2–13)	13
PMN$>1.5\times10^9$/L	4.5 (3–20)	18

**A few patients received recombinant human glycosylated granulocyte colony stimulating factor (rHuG-CSF) from day 20 on, to mobilize peripheral stem cells, in the context of the pilot study before the amendment of the protocol.*

Recovery of neutrophils after autoBMT in AML was reported to be faster when peripheral stem cells were used.[16,17] Recovery of platelets was more variable. Preliminary clinical data from nonrandomized studies indicate a similar relapse rate and DFS after autoBMT and peripheral stem cell transplantation (PSCT) in AML.[16,17]

Therefore after a pilot phase, the AML10 protocol was amended in June 1996 to include peripheral stem cell collection in the recovery phase of the consolidation course in all patients without a donor. Recombinant human glycosylated granulocyte colony stimulating factor (rHuG-CSF) is being administered (150 $\mu g/m^2/d$, s.c.) from day 20 after the beginning of the consolidation course to mobilize stem cells. Irrespective of the harvest, patients without a donor are then randomized between autoBMT and PSCT. Bone marrow is harvested and autoBMT performed only in patients randomized to undergo autoBMT. The peripheral stem cells of these patients remain stored in liquid nitrogen and can be used for rescue only if the neutrophil count has not reached $0.2\times10^9/L$ on day 28 after the day of bone marrow infusion or if the patient is still platelet dependent on day 90 after marrow infusion. If the collection at recovery from the consolidation course is not sufficient, a second or even third attempt to mobilize can be undertaken. Since the meaning of the delayed recovery after the consolidation treatment in terms of the chance of relapse is not yet known, it was decided to abstain from further treatment if the harvest of bone marrow in patients in the autoBMT arm or peripheral stem cells in the PSCT arm is insufficient.

The amendment of the protocol is expected to improve the percentage of completion of autologous transplantation, to decrease the mortality associated with autologous transplantation and to resolve whether recovery after PSCT in AML is faster and DFS indeed comparable to autoBMT.

APPENDIX

The following centers and investigators from the EORTC Leukemia Cooperative Group participated in this study: Austria: Innsbruck, Innsbruck Universitätsklinik (G.Gastl); Belgium: Antwerpen, Algemeen Ziekenhuis Middelheim (R. De Bock) and University Hospital of Antwerpen (Z. Berneman); Brugge, Academisch Ziekenhuis St. Jan (D. Selleslag); Brussels, Hôpital Universitaire Erasme (W. Feremans), Hôpital Universitaire St. Pierre (A. Efira) and Institut Jules Bordet (L. Debusscher); Liège, Centre Hospitalier Regional de la Citadelle (F. Andrien) and CHU Sart-Tilman (G. Fillet); Verviers, Centre Hospitalier Peltzer-La Tourelle (J. Andrien); Croatia: Zagreb, Clinical Hospital Rebro (B. Labar) and O Novosel Medical School - University of Zagreb (B. Jaksic); Czech Republic: Olomouc, University Hospital (K. Indrak); Prague, Institute of Hematology and Blood Transfusion (J. Cermak); France: Lyon, Hôpital

Edouard Herriot (E. Archimbaud); Nice, Centre Antoine Lacassagne (A. Thyss); Paris, Hôpital Cochin (F. Dreyfus), Hôtel-Dieu de Paris (R. Zittoun) and Hôpital Necker (P. Bourquelot); Suresnes, Centre Medico-Chirurgical Foch (E. Baumelou); Villejuif, Institut Gustave Roussy (J. Bourhis); Germany: München, Klinikum Grosshadern, Ludwig-Maximilians Univ. (U. Jehn); The Netherlands: Amsterdam, Onze Lieve Vrouw Gasthuis (K. Roozendaal); Eindhoven, Catharina Ziekenhuis (W. Peters); Enschede, Medisch Spectrum Twente (M. Schaafsma); s'Hertogenbosch, Groot Ziekengasthuis (H. Sinnige); Leiden, Academisch Ziekenhuis Leiden (R. Willemze); Nijmegen, St. Radboud University Hospital (T. de Witte, P. Muus); Veldhoven, St. Josef Ziekenhuis (G. Vreugdenhil); Portugal: Coimbra, Hospitais da Universidade de Coimbra (I. Sousa); Porto, Hospital Escolar San Joao (M. Ribeiro); Turkey: Ankara, Ibni Sina Hospital (M. Beksac).

The following centers and investigators from the GIMEMA group participated in this study: Italy: Alessandria, Ospedale Civile (A. Lewis); Ancona, Ospedale Torrete University Ancona (P. Leoni); Avellino, Ospedale Civile Avellino (E. Volpe); Aviano, Centro di Riferimento Oncologico (S. Monfardini); Bari, Università degli Studi di Bari - Policlinico (V. Liso); Brindisi, Ospedale Regionale A. di Summa (G. Quarta); Cagliari, Ospedale Oncologico A. Businco (G. Broccia); Catania, Ospedale Ferrarotto (E. Cacciola); Catanzaro, Ospedale Regionale A. Pugliese (A. Alberti); Cremona, Centro Trapianto Midollo Osseo (A. Porcellini); Cuneo, Ospedale Santa Croce (E. Gallo); Firenze, Università degli Studi di Firenze-Policlin. di Careggi (P. Rossi Ferrini); Foggia, Ospedali Riuniti Foggia (M. Monaco); Gallarate-Varese, Ospedale S. Antonio Abate (R. Mozzana); Genova, Ospedale San Martino (A. Bacigalupo, A. Carella, E. Damasio) and Università degli Studi (M. Gobbi); Latina, Ospedale Gen. Provinciale Santa Maria Goretti (L. Deriu); Milano, Istituto Scientifico H. S. Raffaele (C. Bordignon) and Ospedale Maggiore ca Granda (E. Morra); Napoli, Ospedale A. Cardarelli (R. Cimino), Ospedale Nuovo Pelligrini (R. De Biasi), Ospedale S. Gennaro (G. Buonanno) and University Federico II Medical School (B. Rotoli); Nuoro, Ospedale San Francesco (A. Gabbas); Palermo, Ospedale Cervello (F. Caronia) and Policlinico P. Giaccone - Università di Palermo (A. Cajozzo); Parma, Ospedali Riuniti di Parma (V. Rizzoli); Perugia, Policlinico Monteluce (M. Martelli); Pesaro, Ospedale San Salvatore (G. Lucarelli); Pescara, Ospedale Civile Pescara (G. Fioritoni); Potenza, Ospedale San Carlo (F. Ricciuti); Reggio Calabria, Azienda Ospedaliera Bianchi-Melacrino-Morelli (F. Nobile); Reggio Emilia, Arcispedale di S. Maria Nuova (I.Iori); Roma, Ospedale San Eugenio (S. Amadori), Ospedale S. Camillo (A. De Laurenzi), Policlinico A. Gemelli - Universita del Sacro Cuore (G. Leone) and Policlinico Umberto Primo - University La Sapienza (F. Mandelli); San Giovanni Rotondo, Ospedale Casa Sollievo Della Sofferenza (M. Carotenuto); Sondalo, Azienda Ospedaliera - Ospedale E. Morelli (G. Camerone); Taranto, Ospedale SS Annunziata (P. Mazza); Torino, Ospedale Molinette (L. Resegotti) and S. Luigi Gonzaga Ospedale - University of Torino (U. Mazza).

REFERENCES

1. Zittoun RA, Mandelli F, Willemze R et al.: Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. *New Engl J Med* 332:217–223, 1995.
2. Keating S, Suciu S, De Witte T et al.: Prognostic factors of patients with acute myeloid leukemia in first complete remission: An analysis of the EORTC-GIMEMA AML8A trial. *Bone Marrow Transplant* 17:993–1001, 1996.
3. Bishop JF, Lowenthal RM, Joshua D et al.: Etoposide in acute nonlymphoid leukemia. *Blood* 75:27–32, 1990.
4. Wheatley K, Burnett AK, Goldstone AH et al.: Factors relating to the achievement of complete remission in younger patients with acute myeloid leukemia in the United Kingdom Medical Research Council (MRC) AML10 trial. *Blood* 88(suppl 1):214a, 1996.
5. Berman E, Heller G, Santorsa et al.: Results of a randomized trial comparing idarubicin and cytosine arabinoside with daunorubicin and cytosine arabinoside in adult patients with newly diagnosed acute myelogenous leukemia. *Blood* 77:1666–1674, 1991.
6. De Witte T, Suciu S, Selleslag D et al.: Salvage treatment for primary resistant acute myelogenous leukemia consisting of intermediate-dose cytosine arabinoside and interspaced continuous infusions of idarubicin: A phase-II study (no.06901) of the EORTC Leukemia Cooperative Group. *Ann Hematol* 72:119–124, 1996.
7. Carella AM, Gaozza E, Piatti G et al.: A new combination of idarubicin, etoposide and cytarabine in untreated acute non-lymphoblastic leukemia. *Leukemia Lymphoma* 2:317–322, 1990.
8. Estey EH, Keating MJ, McCredie KB et al.: Phase II trial of mitoxantrone in refractory acute leukemia. *Cancer Treat Rep* 67:389–390, 1983.
9. Arlin Z, Case DC Jr, Moore J et al.: Randomized multicenter trial of cytosine arabinoside with mitoxantrone or daunorubicin in previously untreated adult patients with acute nonlymphocytic leukemia. *Leukemia* 4:177–183, 1990.
10. Archimbaud E, Leblond V, Michallet M et al.: Intensive sequential chemotherapy with mitoxantrone and continuous infusion etoposide and cytarabine for previously treated acute myelogenous leukemia. *Blood* 77:1894–1900, 1991.
11. Bennett JM, Catovsky D, Daniel MT et al.: Proposed revised criteria for the classification of acute myeloid leukemia: A report of the French-American-British cooperative group. *Ann Intern Med* 103:620–625, 1985.
12. Keating MJ, Smith TL, Kantarjian H et al.: Cytogenetic pattern of acute myelogenous leukemia: a major reproducible determinant of outcome. *Leukemia* 2:403–412, 1988.
13. Cheson BD, Cassuleth PA, Head DR et al.: Report of the National Cancer Institute Sponsored Workshop on definitions of diagnosis and response in acute myeloid leukemia. *J Clin Oncol* 8:813–819, 1991.
14. Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 53:457–481, 1958.
15. Buyse ME, Staquet MJ, Sylvester RJ (eds) Cancer Clinical Trials: Methods and Practice. Oxford Medical Publications: Oxford, England, Oxford University Press, 337–406, 1984.
16. Körbling M, Fliedner TM, Holle R et al.: Autologous blood stem cell versus purged bone marrow transplantation in standard risk AML: Influence of source and cell composition

of the autograft on hematopoietic reconstitution and disease-free survival. *Bone Marrow Transplant* 7:343–349, 1991.

17. Henon PR et al.: Clinical aspects of autologous bone marrow transplantation. In: Wunder EW, Henon PR (Eds) Peripheral Blood Stem Cell Autografts. Springer Verlag (ISBN 3-450-52612-9) 1993, pp 209–240.

COMPARISON OF AUTOLOGOUS BONE MARROW TRANSPLANTATION AND INTENSIVE CHEMOTHERAPY AS POST-REMISSION THERAPY IN ADULT ACUTE MYELOBLASTIC LEUKEMIA: FINAL ANALYSIS OF THE GOELAM 1 STUDY

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INTRODUCTION

Post-remission therapy remains a critical issue in acute myeloblastic leukemia (AML).[1,2] During the past ten years, aggressive postremission strategies have been proposed for younger patients in complete remission (CR) after induction treatment. These options include allogeneic bone marrow transplantation (alloBMT), autologous hematopoietic stem cell support and intensive consolidation chemotherapy (ICC). Because of selection bias in published pilot series, large randomized studies comparing these strategies were mandatory. The first large randomized trial comparing alloBMT, autologous bone marrow transplantation (autoBMT) and ICC was recently published by the EORTC and GIMEMA groups.[3] In this study, alloBMT and autoBMT resulted in significantly better disease-free survival (DFS) than ICC with high dose cytarabine (HD Ara-C). We herein report the final analysis of a multicenter randomized study conducted by the GOELAM group also comparing autoBMT and ICC. In this study no significant difference between the two approaches could be demonstrated.

PATIENTS AND METHODS

Inclusion criteria

Patients aged 15 to 55 with previously untreated AML were eligible for entry into the trial. The diagnosis was made by a bone marrow aspirate showing at least 30% blast cells or, in case of myelofibrosis, by a bone marrow biopsy. Each case was classified according to the French-American-British (FAB)

system. Patients with a previously diagnosed myelodysplasia for more than 3 months were excluded, but patients with an antecedent of unexplained cytopenia could be included. Also excluded were patients with a myeloproliferative disorder in blast crisis, patients who had previously received cytotoxic chemotherapy or radiotherapy (or both), patients with clinical or electrocardiographic signs of heart failure or coronary disease and patients with hepatic or renal disturbances (hepatic enzymes levels over four times the normal values, creatinine level over 130 micromoles per liter).

Karyotypic abnormalities were classified as favorable (t[8;21], t[15;17] or inv[16]), unfavorable (-5, 5q-, -7 or multiple abnormalities) or intermediate (all other abnormalities).

Study design

The induction treatment consisted of a continuous infusion of Ara-C (200 mg/m^2/day) on days 1 through 7 with either Idarubicin (IDR) given intravenously on days 1 through 5 at a daily dose of 8 mg/m^2 or Rubidazone (RBZ) given intravenously on days 1 through 4 at a daily dose of 200 mg/m^2.

A bone marrow aspiration was performed on day 17. If the marrow was hypoplastic and nonblastic, no further treatment was administered. If the marrow was hypoplastic and contained less than 50% blasts, a second induction course was administered with a combination of three days of Ara-C and two days of the anthracycline initially allocated. If the marrow was normocellular and blastic or contained more than 50% blasts, the treatment was considered a failure and the patient was withdrawn from the study and treated with a salvage regimen. All patients in CR after one or two courses of induction treatment were scheduled to receive intensive consolidation therapy. An alloBMT was offered to all patients aged 40 and younger and with an HLA-identical sibling. The chemotherapy between CR achievement and BMT, the conditioning regimen, the prophylaxis and treatment of graft-versus-host disease were administered according to the protocols of each transplant center.

All other patients aged 50 and younger were assigned to receive a first course of ICC consisting of HD Ara-C (3 g/m^2) given as a 3 hour infusion every 12 hours on days 1 through 4 (total dose 24 g/m^2), combined with either IDR given intravenously on days 5 and 6 at a daily dose of 10 mg/m^2 or RBZ given intravenously on days 5 and 6 at a daily dose of 200 mg/m^2.

The first course of ICC was to be given as soon as possible after CR achievement and no later than 75 days after initiation of induction treatment. Bone marrow was harvested after the first course of ICC without any in vitro manipulation. All patients still in CR were randomly assigned to receive a second course of ICC or an autoBMT if the collected marrow contained at least 2.10^8 mononucleated cells/kg. The second course of ICC consisted of m-Amsa given as a one

hour infusion on days 1 through 5 at a daily dose of 150 mg/m^2 and etoposide given as a two hour infusion on days 1 through 5 at a daily dose of 100 mg/m^2.

The conditioning regimen consisted of busulfan given orally at a daily dose of 4mg/kg for 4 consecutive days and cyclophosphamide given intravenously at a daily dose of 50mg/kg for 4 consecutive days.

Response criteria and statistical analysis

CR was defined as a normocellular bone marrow containing less than 5% blasts and more than 1×10^9 granulocytes/L and more than 100×10^9 platelets/L.

Comparison between the different treatment groups was performed with chi-square test for binary variables and Kruskal-Wallis for continuous variables. DFS was calculated from the date of CR until the date of first relapse or the date of death from any cause. Overall survival was defined as the time from first randomization to the time of death. Patients who did not receive the induction treatment as indicated by the protocol were excluded for the calculation of DFS but data were collected on these patients for the analysis of overall survival. Actuarial curves were plotted following the Kaplan-Meier method and differences between the curves were analyzed with the logrank test.

RESULTS

Between November 1987 and May 1994, 535 patients were included into the study by 16 institutions. Eighteen patients were considered ineligible. The median age of the 517 eligible patients (256 male, 261 female) was 36 years. The FAB classification was M_0 31, M_1 111, M_2 154, M_3 44, M_4 69, M_5 88, M_6 9, M_7 6, undetermined 9. A karyotype was performed in 337 cases and was evaluable in 309 cases. Karyotype abnormalities were found in 176 cases and were classified as favorable (47), intermediate (93) or unfavorable (36). Fourteen patients were unevaluable for the induction treatment: four because they died before receiving the first day of treatment, five because of a major protocol violation, four because of a wrong randomization and one because of missing data. There was no significant difference between the two groups (IDR or RBZ) regarding the initial characteristics.

Induction treatment

Of the 503 patients evaluable for induction treatment, 366 (73%) achieved a CR, including 338 patients treated with only one course of chemotherapy (92% of the CR). There were nine early deaths, 22 deaths during aplasia (4%) and 106 (21%) patients with persisting leukemia. The outcome was not significantly different between the two induction treatment arms (IDR or RBZ).

Table 1. Protocol GOELAM 1 (patients aged 15 to 50). Reasons for not completing the protocol as scheduled

	Total	*After complete remission achievement n=366*	*Patients assigned to alloBMT n=87*	*Patients assigned to first ICC n=251*	*After first ICC n=228*	*After randomization n=164*	
						ICC2 n=78	*autoBMT n=86*
relapse	22	2	5	–	7	3	5
infections complications	19	11	1	–	7	–	–
poor hematologic reconstitution	21	3	–	–	18	–	–
extrahematologic toxicity	27	9	5	–	12	–	1
toxic death	7	1	–	–	6	–	–
refusal	24	2	4	–	14	–	4
protocol violation	26	–	–	22*	–	4	–
lost to follow–up or other reasons	2	–	–	1	–	–	1
total	148	28	15	23	64	7	11

**Including 14 alloBMT instead of first course of ICC.*

Feasibility of intensive postremission therapy

Of the 366 patients in CR, 28 could not undergo any kind of intensive postremission therapy (alloBMT or ICC). Fourteen patients underwent alloBMT but should have received ICC. Eighty-seven patients under the age of 41 who had an HLA-identical sibling were scheduled to receive alloBMT but 15 could not actually be transplanted in first CR. Thus, 72 patients underwent an alloBMT according to the protocol.

Two hundred and thirty-seven patients underwent the first ICC, but only 228 patients are fully evaluable for this step of the procedure. The median duration of neutropenia (less than 0.5×10^9/L) after the first course of intensification was 18 days (range 9–59 days). The median duration of thrombocytopenia (less than 30×10^9/L) was 16 days (range 5–48 days). The median length of hospitalization was 30 days in both arms. Six toxic deaths were recorded (2.5%).

Of the 228 evaluable patients, 64 could not be randomized between autoBMT and a second course of ICC, and only 164 patients were randomized according to the protocol (86 autoBMT, 78 ICC). Eleven of the 86 patients randomly assigned to autoBMT and 7 of the 78 patients randomly assigned to ICC did not actually receive the scheduled treatment. Therefore, 218 patients actually received the intensive postremission treatment as scheduled in the protocol (59% of the patients achieving CR and 40% of the total number of included patients): 72 alloBMT, 75 autoBMT, 71 ICC. The reasons for not completing the protocol at each step are shown in Table 1.

Overall results of postremission therapy

With a median follow-up of 4 years, the 4-year actuarial DFS of the 366 patients who achieved CR was 39% (±3%), with no significant difference between the two induction treatment arms.

The median overall survival of the 517 eligible patients was 21 months and the 4-year actuarial survival was 39% (±2%), with no significant difference between the two induction treatment arms.

AlloBMT

The median interval between CR achievement and alloBMT was 68 days. Of the 72 patients who underwent the scheduled alloBMT, 20 have relapsed, 31 patients have died: 16 from procedure-related toxicity and 15 after relapse. Four-year actuarial DFS was 47% (±6%) and the 4-year actuarial overall survival was 54% (±6%). The actuarial risk of relapse at 4 years was 37% (±7%).

We compared the outcome of the 87 patients for whom the intention was to perform an alloBMT with the 134 patients up to the age of 40 without an HLA-identical sibling for whom the intention was to perform another type of intensive consolidation therapy. There was no significant difference in the distribution of initial clinical characteristics between the two groups, except for age and karyotype

Table 2. Characteristics of the patients according to the assigned postremission treatment

	*alloBMT n=87**	*First course of intensive consolidation chemotherapy n=134***	*p value*	*Randomized autoBMT n=86*	*Second course of intensive consolidation chemotherapy n=78*	*p value*
age	15–40 (30)	15–40 (30)	.93	16–50 (38)	15–50 (37)	0.58
white blood cell count	0.5–172 (9.9)	0.6–350 (14.4)	0.29	0.6–219 (10.6)	0.4–350 (17.5)	0.23
FAB classification						
M2-M3 (%)	39 (45)	51 (38)	0.14	37 (43)	29 (37)	0.52
other subtypes (%)	48 (55)	83 (62)		49 (57)	49 (63)	
cytogenetic group*** (%)						
normal	58	42.5		35.5	41	
favorable	25	15.5		17	14	
intermediate	13.5 } 17	35 } 47	0.001	40 } 47.5	29 } 47	0.34
unfavorable	3.5 }	12 }		7.5 }	16 }	

**Patients aged 15–40 with an HLA-identical sibling.*

***Patients aged 15–40 without an HLA-identical sibling.*

****The karyotype could be analyzed in 60 patients assigned to alloBMT, in 191 patients assigned to ICC1, in 65 patients assigned to randomized autoBMT and in 56 patients assigned to randomized ICC2.*

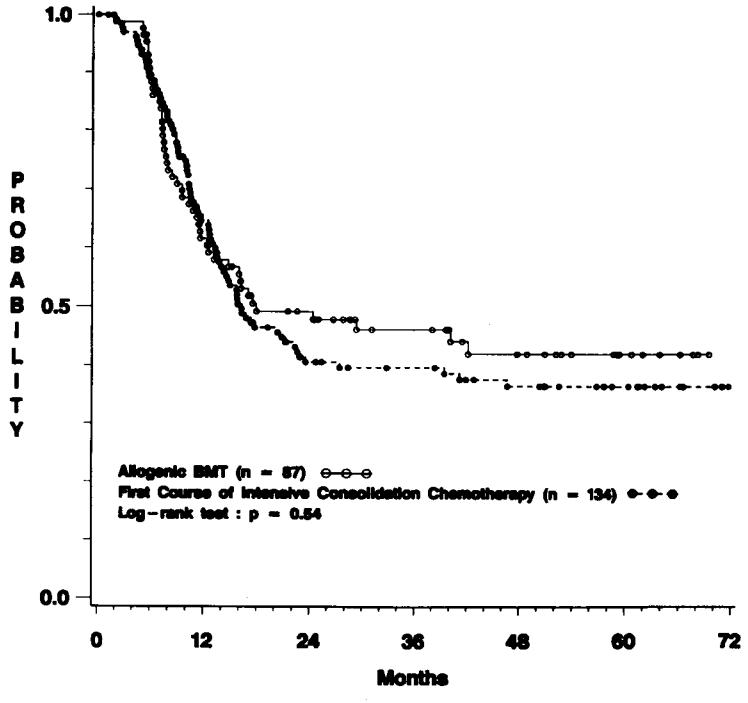

Figure 1. *Comparison (on an intention to treat basis) of 87 patients assigned to undergo alloBMT with 134 patients in the same age category without an HLA-identical sibling assigned to receive the first course of intensive consolidation chemotherapy (DFS).*

analysis (Table 2). The 4-year DFS was 42% (±6%) for alloBMT and 40% (±3%) for intensive consolidation (p=0.54) (Figure 1).

The 4-year overall survival was 51% (±6%) for alloBMT and 52.5% (±3.5%) for intensive consolidation (p=0.63).

Comparison of intensive consolidation chemotherapy and autoBMT

Of the 74 patients who actually underwent autoBMT according to the protocol, 31 have relapsed. Five patients (6.7%) have died from procedure-related toxicity and 24 after relapse. The 4-year DFS and overall survival were 46.5% (±6%) and 51.5% (±7%), respectively. Of the 71 patients who underwent the second course of ICC as assigned, 37 have relapsed. Two patients (2.8%) have died from procedure-related toxicity and 23 after relapse. The 4-year DFS and overall survival were 40% (±6%) and 58.5 (±6.5%), respectively.

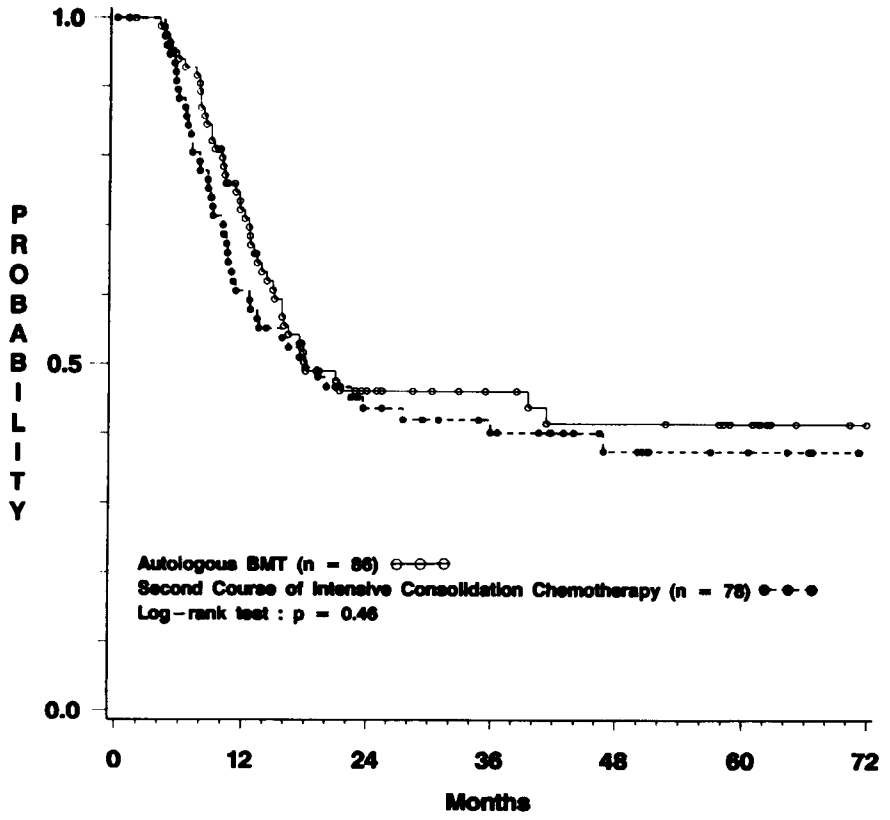

Figure 2. *Comparison (on an intention to treat basis) of 86 patients randomly assigned to undergo autoBMT with 78 patients randomly assigned to receive a second course of intensive consolidation chemotherapy (DFS).*

The comparison between the two groups was performed according to the intention to treat principle. There was no significant difference between the 86 patients randomly assigned to autoBMT and the 78 patients randomly assigned to ICC in regard to initial clinical characteristics (Table 2). The median interval between the achievement of CR and the completion of the last intensive postremission therapy as assigned by the randomization was 85 days for autoBMT and 72 days for ICC (p=0.0001).

The 4-year actuarial DFS was 42% (±6%) for autoBMT and 38% (±6%) for ICC (p=0.46) (Figure 2). The 4-year actuarial survival was 48% (±6%) for autoBMT and 56.5% (±6%) for ICC (p=0.51). The median duration of neutropenia (less than 0.5×10^9/L) was 25 days after autoBMT and 24 days after ICC (p=0.48). The median duration of thrombocytopenia (less than 30×10^9/L) was 109.5 days after autoBMT (range 10–2000+ days) and 18.5

days after the second course of ICC (range 1–50 days) (p=0.0001). The median duration of hospitalization was 39 days after autoBMT (15–99) and 32 days after the second course of ICC (22–52) (p=0.006).

DISCUSSION

The main objective of the GOELAM1 study was to compare different strategies of intensive postremission therapy. In our hands, alloBMT, autoBMT and ICC yielded comparable 4-year DFS rates. Unpurged autoBMT and ICC were randomly assigned and administered after a first course of HD Ara-C containing ICC. With this approach, we failed to demonstrate any superiority of autoBMT since, when analyzed on an intention to treat basis, the 4-year DFS and overall survival were, respectively, 42% and 48% for autoBMT; 38% and 56.5% for ICC. Although these results are comparable with those achieved in a pediatric study,[4] they differ from other randomized studies showing longer DFS for adult patients randomized to autoBMT.[3,5–7] However, in two of these studies autoBMT was compared with conventional chemotherapy.[5,6] The large trial conducted by the CALGB demonstrated that for patients up to the age of 60, the 4-year DFS is significantly longer with high doses of Ara-C than with low or intermediate doses.[8] The British MRC also conducted a large randomized trial testing the impact of autoBMT; however, in this study, autoBMT was performed later and was compared with a no treatment arm.[7]

The design of our study was quite comparable with that of the EORTC/GIMEMA trial, which showed a better 4-year DFS for autoBMT (48%) compared with ICC (30%).[3] Our results differ in that the 4-year DFS is slightly lower after autoBMT (42%) and slightly higher after ICC (38%), making the difference not significant. The differences in outcome after autoBMT are not explained by a higher toxic death rate (6.7% death rate in our study versus 10.4% in the EORTC/GIMEMA trial). The difference may be related to the preparative regimen since all our patients were prepared by a combination of busulfan-cyclophosphamide while 45% of the patients in the EORTC/GIMEMA trial were prepared with total body irradiation. However, the superiority of total body irradiation over the Baltimore regimen has never been demonstrated. The apparently better results of our ICC may be explained by the higher doses of Ara-C given during the first cycle of ICC and by the use of m-Amsa and VP-16 in the second course. Our results with 2 courses of ICC are similar to those published by the CALGB with 4 courses of HD Ara-C.[8] We can therefore assume that the ICC arm of the EORTC/GIMEMA was not optimal.

Since overall survival is better after ICC than after autoBMT due to better salvage of relapsed patients,[3] and because of longer thrombocytopenia after autoBMT, we believe that in future trials ICC should be the standard treatment that is compared with innovative approaches.

REFERENCES

1. Mayer RJ: Allogeneic transplantation versus intensive chemotherapy in first remission acute leukemia: Is there a "best choice"? *J Clin Oncol* 6:1532–1536, 1988.
2. Löwenberg B: Post remission treatment of acute myelogenous leukemia. *N Engl J Med* 332:260–262, 1993.
3. Zittoun RA, Mandelli F, Willemze R et al.: Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. *N Engl J Med* 322:217–23, 1995.
4. Ravindranath Y, Yeager A, Chang M et al.: Autologous bone marrow transplantation versus intensive consolidation chemotherapy for acute myeloid leukemia in childhood. *N Engl J Med* 334:1428–1434, 1996.
5. Reiffers J, Gaspar MH, Maraninchi D et al.: Comparison of allogeneic or autologous bone marrow transplantation and chemotherapy in patients with acute myeloid leukaemia in first remission: A prospective controlled trial. *Br J Haematol* 72:57–63, 1989.
6. Reiffers J, Stoppa AM, Attal M et al.: Autologous stem cell transplantation versus chemotherapy for adult patients with acute myeloid leukemia in first remission: The BGMT experience. *Nouv Rev Fr Hematol* 35:17–9, 1993.
7. Burnett AK, Goldstone AH, Stevens RF et al.: The role of BMT in addition to intensive chemotherapy in AML in first CR. Results of the MRC AML-10 trial. *Blood* 84(10):252a, 1994.
8. Mayer RJ, Davis RB, Schiffer CA et al.: Intensive postremission chemotherapy in adults with acute myeloid leukemia. *N Engl J Med* 331:896–903, 1994.

THE ROLE OF STEM CELL TRANSPLANTATION IN ACUTE MYELOID LEUKEMIA IN FIRST REMISSION: THE BGMT GROUP EXPERIENCE

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INTRODUCTION

In patients with acute myelogenous leukemia (AML), most induction chemotherapy regimens include a combination of cytosine arabinoside (Ara-C) and an anthracycline such as daunorubicin (DNR) or, more recently, idarubicin or mitoxantrone.[1] These regimens bring about complete remission (CR) rates ranging from 50 to 80%, depending on the age of patients or the degree of cytogenetic abnormalities.[1] Once CR is achieved, the treatment to be administered to maintain CR (or prevent leukemic relapse) is still controversial. Three therapeutic strategies can be proposed: allogeneic (alloBMT) or autologous stem cell transplantation (autoSCT) and chemotherapy. In most prospective randomized studies, alloBMT seems to be superior to other forms of treatment (although not significantly in some of these studies); autoSCT has been reported to be superior to chemotherapy in the EORTC study,[2] but this has not been confirmed in some others.[3,4]

Since 1984, the BGMT Group has conducted three consecutive studies (BGM 84, BGMT 87, BGMT 91) in the younger AML patient to compare prospectively, these postremission treatments. The main results are summarized herein.

MATERIALS AND METHODS

Inclusion and exclusion criteria

All patients with a diagnosis of AML according to the FAB criteria were included when their age was between 15 and 55 years (50 years in the BGM 84 study) and there was no evidence of preleukemic phase.

Induction and consolidation chemotherapy regimens

In the three studies, all patients were given induction chemotherapy consisting of Ara-C (100 mg/m^2/day, continuous infusion, 10 days) and DNR (60 mg/m^2/day, bolus, 3 days). The patients who were not in CR after this chemotherapy received a second course of induction chemotherapy (at the same doses). The patients who were not in CR after this second course were excluded. The CR patients (after one or two cycles) were given consolidation with DNR (2 days) and Ara-C (7 days) at the same daily dose as induction.

AlloBMT

The patients who were still in CR after consolidation were planned to undergo alloBMT when they fulfilled the following criteria: age less than 46 years, presence of an HLA-identical sibling donor, no contraindication for alloBMT. For alloBMT, the conditioning regimen consisted of the standard combination of cyclophosphamide and total body irradiation (TBI) (10–13 Grays) although some patients of the BGMT 87 study received a combination of busulfan and cyclophosphamide as part of the GEGMO study published by Blaise et al.[5] Most patients received cyclosporine and methotrexate as graft-versus-host disease prophylaxis.

BGM 84 study[4]

The CR patients who did not fulfill the inclusion criteria for alloBMT were randomized to receive either autoSCT or intensive sequential chemotherapy (ISC). autoSCT consisted of a double transplant of unpurged marrow with high dose melphalan (HDM) (140 mg/m^2) as myeloablative treatment before each transplant. ISC consisted of four monthly regimens of chemotherapy with rotating drugs.

BGMT 87 study[6]

The patients who were not assigned to receive alloBMT were given intensive chemotherapy consisting of high dose Ara-C (3 g/m^2/12h$\times$8 doses) and DNR (45 mg/m^2/day, 3 days) before being randomized to receive either autoSCT or maintenance chemotherapy. Before autoSCT, the patients were given busulfan (16 mg/kg) and HDM as preparative regimen. Unmodified bone marrow or peripheral blood progenitor cells (PBPC) collected after high dose Ara-C and DNR were used to reconstitute hematopoiesis. Maintenance chemotherapy consisted of five cycles of DNR + Ara-C with oral administration of 6-mercaptopurine and methotrexate between the cycles then subsequently for a total period of two years after CR.

BGMT 91 study

The patients excluded from alloBMT underwent a bone marrow harvest before receiving an intermediate dose of Ara-C (500 mg/m^2/12h$\times$8 doses) and DNR (45

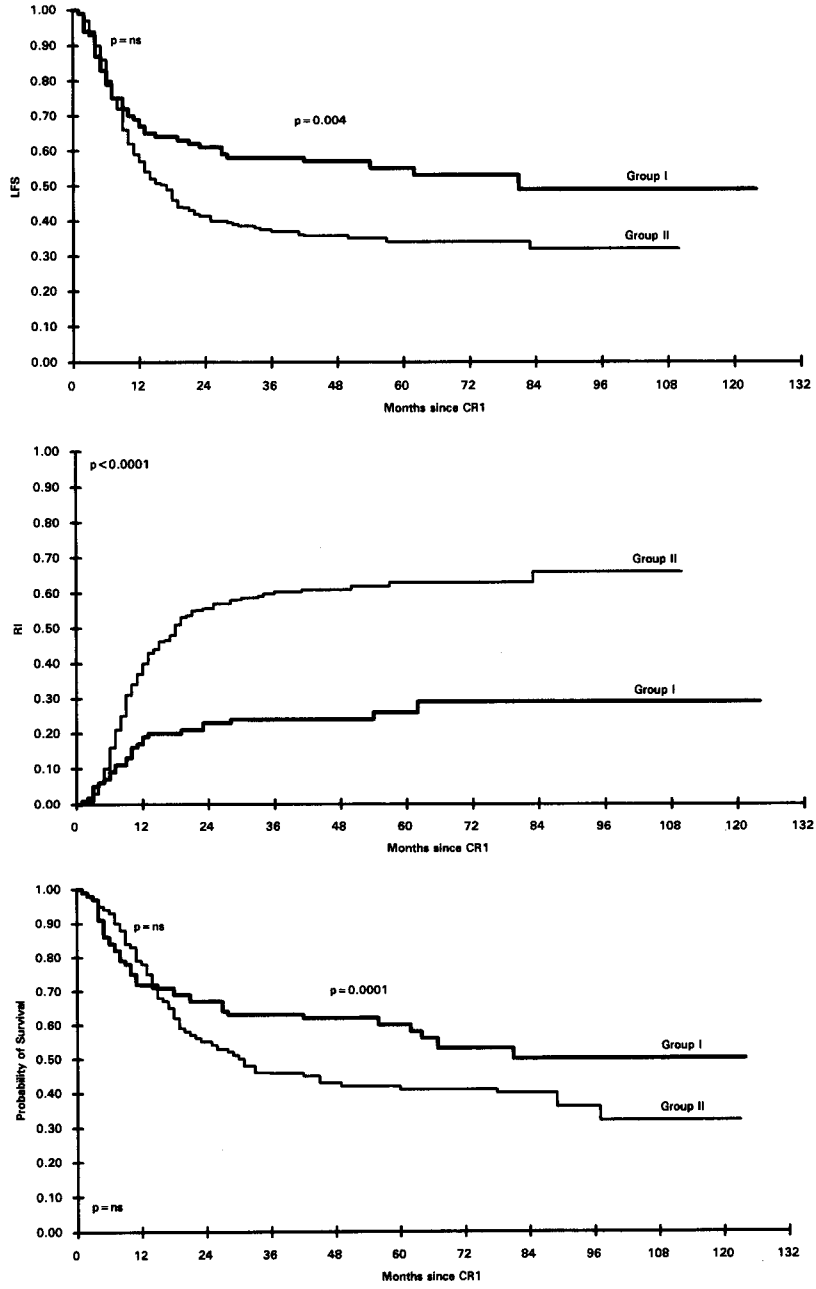

Figure 1. *Leukemia-free survival (LFS), relapse incidence (RI) and survival of patients enrolled in the BGMT studies according to the type of treatment they were intended to receive (Group I: allogeneic bone marrow transplantation; Group II : autologous stem cell transplantation or chemotherapy).*

Table 1.

	BGM 84 *n=85*	*BGMT 87* *n=204*	*BGMT 91* *n=281*	*Total* *n=570*
age (years)*	35.3 (16–55)	39.8 (15–55)	40.3 (15–55)	38.9 (15–55)
sex (male/female)	40/45	113/91	159/122	312/258
FAB morphology (M1 M2 M3/Others)	49/36	116/88	142/139	307/263
initial leukocytosis (giga/L)	15.8 (0.6–340)	15.7 (0.5–309)	16.6 (0.5–720)	16 (0.5–720)
cytogenetics (low/intermediate/high risk/not done)**	10/4/4/67	32/74/24/74	51/121/46/63	93/199/74/204

*$p<0.006$

**$p<0.03$

mg/m^2/day, 3 days). Thirty to 45 days after completion of chemotherapy, the patients underwent autoSCT after the standard cyclophosphamide + TBI conditioning regimen. When hematopoietic recovery was achieved, the patients who were still in CR were randomly assigned to receive or not to receive interleukin-2 (IL-2) (12 million U/m^2/day, continuous infusion) for five cycles given every other week (first cycle = 5 days, cycles 2⇒5 = 2 days) (BGMT 91 Study). When IL-2 became unavailable, the patients received autoSCT according to the same modalities as in the BGMT 87 Study. However, G-CSF was given after high dose Ara-C and DNR to reduce the toxicity of chemotherapy and increase the yield of PBPC to be collected (BGMT 91 b Study).

Statistical evaluation

The Kaplan-Meier method was used to calculate the leukemia-free survival (LFS), relapse incidence (RI) and survival of patients from CR to death ± relapse. The curves were compared using the logrank test.[7] The comparisons were done according to the intention to treat.[8]

RESULTS

Induction and consolidation chemotherapy

A total of 570 patients were given induction chemotherapy (BGM 84=85; BGMT 87=204; BGMT 91=281). The main characteristics are summarized in Table 1. The overall CR rate was 77%; ($^{441}/_{570}$) and similar in each of the three studies. Three hundred ninety of these CR patients (study population) were still in CR after consolidation treatment approximately 60 days after diagnosis.

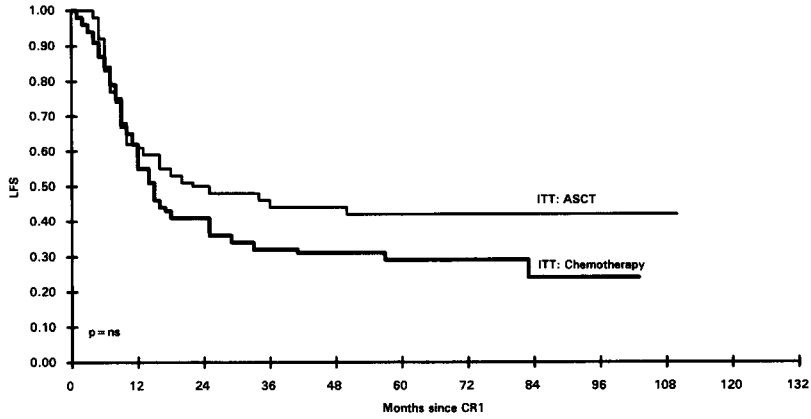

Figure 2. *Meta analysis of BGMT studies. Comparison of autologous stem cell transplantation (autoSCT) versus chemotherapy. Analysis according to the intention to treat (ITT).*

Comparison of different therapeutic modalities

One hundred thirteen patients under the age of 46 years had an HLA-identical sibling donor and were allocated to receive alloBMT (Group I patients); 100 of the Group I patients actually underwent alloBMT. The main characteristics (sex, FAB morphology, initial leukocytosis, cytogenetics) of these patients did not differ significantly from those who could not undergo alloBMT (Group II). However, Group I patients were younger than Group II patients ($p<0.0001$). The 3-year cumulative incidence of Group I patients who relapsed (RI) or survived without disease (LFS) was 24 ± 9 % and 58 ± 9%, respectively (Figure 1). The 3-year survival was 63 ± 9%. For Group II patients, the 3-year LFS, RI and survival were 37 ± 6%, 60 ± 6%, and 46 ± 6%, respectively (Figure 1). The difference between Group I and Group II was statistically significant for LFS ($p<0.004$), RI ($p<0.0001$) and survival ($p<0.0001$). Multivariate analysis confirmed that Group I patients had a significantly better outcome than Group II patients.

Among the 289 Group II patients included in BGM 84 and BGMT 87 studies, 112 were randomized to receive autoSCT (n=54) or chemotherapy (n=58). As shown in Figure 2, there was no statistically significant difference between autoSCT and chemotherapy, although the results of autoSCT looked better. Among patients undergoing autoSCT, there was no significant difference between patients undergoing either PBPC transplantation or autologous bone marrow transplantation.

Comparison of the three different studies

Some characteristics of patients enrolled in BGM 84, BGMT 87 and BGMT 91 studies were similar (Table 1). However, the BGM 84 patients were significantly younger ($p<0.006$) and had a higher proportion of favorable cytogenetic abnormalities. In these consecutive studies, the results of alloBMT and autoSCT were equivalent (Figure 3). In BGMT 87 study, the results of chemotherapy were significantly better than those obtained in the BGM 84 study (Figure 3), perhaps because of the introduction of high dose Ara-C in the chemotherapeutic protocol of BGMT 87 study. The toxicity of intensification treatment was less in the BGMT 91 study where the mortality rate was only 0.5% (1/42) compared with 7% (6/93) observed in the BGMT 87 study ($p<0.05$). This could be due to the administration of G-CSF after chemotherapy or to the use of a lower dose of Ara-C (or both) in the BGMT 91 study.

DISCUSSION

Most of the results observed in these studies are similar to those reported in the literature.[1] Using a standard "3+10" induction chemotherapy, a CR rate of 77% was obtained, a result that compares favorably with the CR study reported elsewhere in young adult patients treated with a combination of Ara-C and anthracycline.[1] As mentioned by Zittoun et al.,[2] some CR patients could not be included in the subsequent comparison of alloBMT versus autoSCT versus chemotherapy for various reasons such as early relapse, liver abnormalities, persisting infection, pulmonary or renal insufficiency; however, the "drop-out" was lower in the BGMT studies than in the other reports.[2,9]

The results of alloBMT observed in the three BGMT studies were equivalent and are comparable to those observed in most single center studies; they look superior to those reported from international registries (see review in [10]). At least three factors could explain these favorable results: first, the patients were transplanted in major transplant centers and it has been reported that the center size influences the outcome of transplanted patients[11]; second, alloBMT was performed very early after only two courses of chemotherapy (induction + consolidation) possibly contributing to a decrease in the extrahematological toxicity of alloBMT; third, a very high proportion of patients fulfilling the inclusion criteria for alloBMT were able subsequently to undergo alloBMT, so the results of the patients assigned to alloBMT were not jeopardized by the usual poor prognosis of excluded patients.

The results of autoSCT that we have observed in our consecutive studies are similar to those reported elsewhere.[12] Interestingly, they were not superior in BGMT 87 and BGMT 91 studies—in which patients received an additional course of intensive chemotherapy before autoSCT and a truly myeloablative

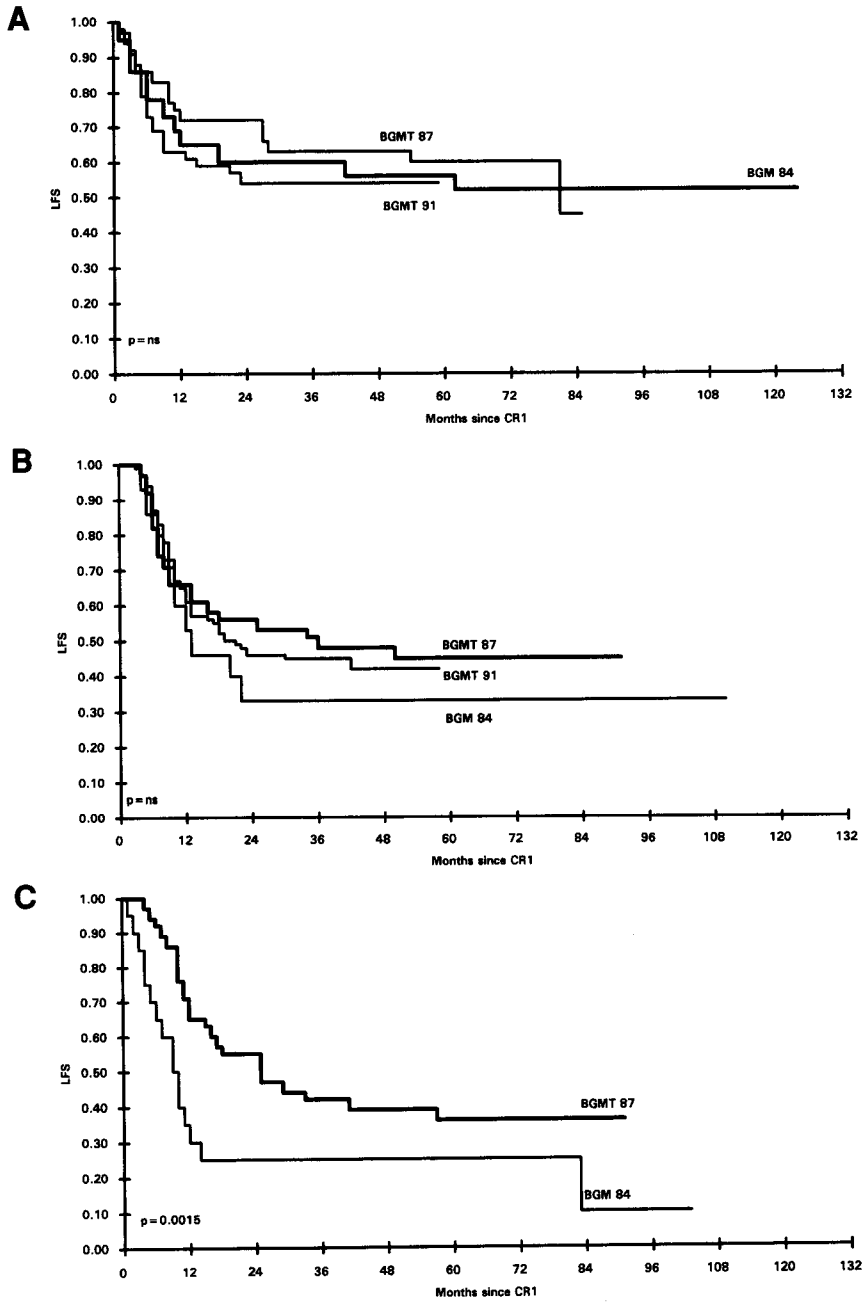

Figure 3. *Leukemia-free survival of patients intended to receive allogeneic bone marrow transplantation (alloBMT) (A), autologous stem cell transplantation (autoSCT) (B) or chemotherapy (C) in BGM 84, BGMT 87 and BGMT 91 studies.*

preparative regimen—when compared with those of the BGM 84 study in which the patients were less heavily treated. However, as the number of patients included in the BGM 84 study was very low, this finding must be interpreted very cautiously. The results of autoSCT in BGMT 87 and BGMT 91 studies were equivalent, indicating that the introduction of G-CSF (after intensive chemotherapy and before stem cell collection) does not increase the relapse incidence. They are also similar to those of BGMT 91, a study in which autologous stem cells were collected before intensification, suggesting that in vivo purging of stem cells to be transplanted does not decrease the relapse incidence. Finally, the analysis of the patients undergoing autoSCT showed that there were no statistically significant differences in LFS, RI or survival of patients treated with either bone marrow transplantation or PBPC.[13]

The results of chemotherapy were significantly inferior in the BGM 84 study when compared to BGMT 87 and BGMT 91 studies. This is in line with many other reports showing that intensification of chemotherapy (with high dose Ara-C in most cases) decreases the relapse rate and prolongs survival.[14,15]

Our studies indicate (but do not prove conclusively) that alloBMT produces results superior to those of other therapeutic modalities. Taken together with the many similar findings of others (see review in [10]), it can be stated that although alloBMT has not yet been shown to be clearly superior to autoSCT or chemotherapy, conversely autoSCT or chemotherapy have not been shown to be equivalent to alloBMT. Therefore, we continue to recommend alloBMT for patients under the age of 45 years having an HLA-identical sibling donor. Some authors who do not share this opinion have proposed restricting alloBMT to patients who present with bad prognostic factors (high risk cytogenetic abnormalities, more than one course of chemotherapy to induce CR) or to restrict alloBMT for patients who relapse. This latter proposition is mainly based upon the results of the Seattle study of alloBMT for AML patients in early relapse[16] but these results have not yet been reproduced elsewhere.

Few studies have compared autoSCT with alloBMT. As in the BGMT 91 study, they usually show that alloBMT is significantly better than autoSCT for preventing leukemic relapse, but due to the higher transplant-related mortality rate observed after alloBMT, the proportion of long-term survivors without recurrent leukemia is not statistically different in most studies. Many cooperative randomized studies have prospectively compared autoSCT and chemotherapy. The EORTC study is the only one showing autoSCT to be significantly better than chemotherapy to prevent relapse and prolong survival.[2] This result needs to be confirmed as the statistical significance observed in this latter study could have been due not to the superiority of autoSCT, but to the unexpectedly poor results in the chemotherapy arm. Further studies are also needed to better define the optimal protocol for autoSCT (such as source of stem

cells, in vivo or in vitro purging and conditioning regimens). The current BGMT 95 Study addresses one of these questions.

REFERENCES

1. Foon KA, Gale RP: Therapy of acute myelogenous leukemia. *Blood Reviews* 6:15–25, 1992.
2. Zittoun RA, Mandelli F, Willemze R et al.: Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. *N Engl J Med* 332:217–23, 1995.
3. Harousseau JL, Pignon B, Witz P, Tellier Z, Cahn PY et al.: Treatment of acute myeloblastic leukemia in adults. The GOELAM experience. *Hematol Cell Ther* 38:419–429, 1996.
4. Reiffers J, Gaspard MH, Maraninchi D et al.: Comparison of allogeneic or autologous bone marrow transplantation and chemotherapy in patients with acute myeloid leukaemia in first remission: A prospective controlled trial. *Br J Haematol* 72:57–63, 1989.
5. Blaise D, Marininchi D, Archimbaud E et al.: Allogeneic bone marrow transplantation for acute myeloid leukemia in first remission: A randomised trial of a busulfan-cytoxan versus cytoxan-total body irradiation as preparative regimen: A report from the Groupe d'Etudes de la Greffe de Moelle Osseuse. *Blood* 79:2578–2582, 1992.
6. Reiffers J, Stoppa AM, Attal M, Michallet M, Blaise D et al.: Allogeneic versus autologous stem cell transplantation versus chemotherapy in patients with acute myeloid leukemia in first remission: The BGMT 87 study. *Leukemia.* In press
7. Kaplan EL, Meier P: Non-parametric estimation from incomplete observations. *Am Statist Assoc* 53:457–481, 1958.
8. Suciu S: The value of BMT in AML patients in first remission. A statistician's viewpoint. *Ann Hematol* 62:41–44, 1991.
9. Bergman E, Little C, Gee T et al.: Reasons that patients with acute myelogenous leukemia do not undergo allogeneic bone marrow transplantation. *N Engl J Med* 16:156–160, 1992.
10. Reiffers J, Marit G, Cony-Makhoul P, Montastruc M et al.: HLA-identical sibling bone marrow transplantation for acute nonlymphoblastic leukemia. *Clin Bone Marrow Transplantation,* 24:185–190, 1994.
11. Horowitz MM, Przepiorka D, Champlin RE et al.: Should HLA-identical sibling bone marrow transplants for leukemia be restricted to large centers? *Blood* 79:2771–2774, 1992.
12. Gorin NC, Dicke K, Lowenberg B: High-dose therapy for acute myelocytic leukemia treatment strategy: What is the choice? *Ann Oncol* 4(suppl 1):59–80, 1993.
13. Reiffers J: Incidence of relapse following blood stem cell transplantation for acute myeloid leukemia in first remission. *Bone Marrow Transplant* 17(5): 899–901, 1996.
14. Wolff SN, Herzig RH, Fay JW et al.: High-dose cytarabine and daunorubicin as consolidation therapy for acute myeloid leukemia in first remission: Long-term follow-up

and results. *Clin Oncol* 7:1260–1267, 1989.

15. Mayer RJ, Davis RB, Karp JE et al.: Intensive postremission chemotherapy in adults with myeloid leukemia. *N Engl J Med* 6:896, 1994.
16. Clift RA, Buckner CD, Appelbaum FR et al.: Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: A randomized trial of two irradiation regimens. *Blood* 76:1867–1871, 1990.

AUTOTRANSPLANTS FOR ACUTE MYELOGENOUS LEUKEMIA (AML) IN NORTH AMERICA

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More than 1900 patients receiving autotransplants for AML since 1989 have now been reported to the ABMTR-NA. These transplants were performed by 138 teams predominantly in the United States and Canada but also South America and a small number of overseas countries. Patient characteristics and early mortality from 1989 to 1995 are shown in Table 1.

Table 1 shows that since 1989 there has been a significant increase in the use of PBSC as hemopoietic support and a significant decrease of in vitro treatment of grafts. 100-day mortality has also significantly improved (p=0.01). Three year probabilities of survival (95% confidence interval) for patients transplanted in first and second complete remission (CR) are 53 (49–57)% and 36 (31–41)%, respectively. Three year probabilities of leukemia-free survival are 50 (46–54)% and 36 (31–41)% for first and second CR, respectively.

Results will soon be available from detailed ABMTR analyses comparing allogeneic versus autologous transplants and purged versus unpurged transplants for patients with AML in first and second remission.

Table 1.

	1989–90	*1991–2*	*1993–4*	*1995*
average number of patients transplanted per year	218	302	308	260
median age, years (range)	32 (1–66)	33 (1–75)	36 (1–69)	36 (1–71)
disease stage at transplant				
first CR (%)	48	50	53	60
second CR (%)	30	26	25	19
other (%)	22	24	22	21
graft type				
bone marrow (%)	97	92	70	50
peripheral blood stem cell (%)	<1	1	19	38
BM + PBSC (%)	3	7	11	12
in vitro treatment of graft (%)	70	62	44	20
100-day mortality (%)	18	16	13	8

CHEMOPURGED AUTOLOGOUS BONE MARROW TRANSPLANTATION VERSUS INTENSIFIED CHEMOTHERAPY FOR ACUTE MYELOID LEUKEMIA IN CHILDREN: PEDIATRIC ONCOLOGY GROUP STUDY 8821

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ABSTRACT

We conducted a randomized study of autologous bone marrow transplantation (autoBMT) versus intensive chemotherapy (ICT) in the treatment of newly diagnosed acute myeloid leukemia (AML) in patients under 21 years of age. All patients received remission induction with one course of daunorubicin (D), cytarabine (A), and thioguanine (T) and one course of high-dose cytarabine (HdA; 3 g/m^2 q12 h × 3 d), and consolidation with one course of etoposide (VP) and azacytidine (AZ). Patients in remission after HdA were offered allogeneic BMT (alloBMT) if they had matched sib donors or were eligible for randomization to either ICT (sequential courses of D/HdA, DAT, VP/AZ, HdA, DAT, and VP/AZ) or autoBMT (busulfan 16 mg/kg and cyclophosphamide 200 mg/kg followed by infusion of 4-hydroperoxycyclophosphamide [4HC; 100 g/mL]-treated marrow). Of 649 evaluable patients, 552 (85%) attained CR; of these, 89 (16%) underwent alloBMT and 42 (8%) were ineligible for randomization (18 institutional nonprotocol autoBMT, 5 secondary AML, 11 relapses or deaths before randomization, 8 other reasons). Of 421 patients in CR eligible for randomization, 189 (45%) were nonrandomly assigned to ICT (111 parental refusal, 64 insufficient funds, 14 lack of available BMT beds); 232 (55%) were randomized to ICT (117) or autoBMT (115). For the entire group, the 3-year event-free survival (EFS) from

randomization was 34 ± 2.5%. Intention-to-treat analysis showed similar EFS after ICT (36 ± 5.8%) or autoBMT (38 ± 6.4%) (p=0.20). On the basis of actual treatment received, EFS was greater after autoBMT (53%) than ICT (38%) (p=0.39; Mantel-Byar test). The EFS after alloBMT was 52 ± 8% (p=0.06 and p=0.01 versus ICT and autoBMT, respectively). The lower relapse rate after autoBMT (31 versus 58% after ICT) was offset by higher treatment-related mortality (TRM; 15 versus 2.7%). No relapses occurred in the autoBMT group beyond 18 months post-randomization but occurred as late as 40 months after ICT. These findings support continued evaluation of intensive therapies for AML and strategies to reduce autoBMT-associated TRM.

INTRODUCTION

Despite advances in chemotherapeutic regimens, including the use of intensive postremission chemotherapy, the event-free survival in childhood acute myeloid leukemia (AML) remains 30 to 40%.[1–5] In contrast, the event-free survival in children who receive allogeneic bone marrow transplants (BMTs) from HLA-matched siblings exceeds 50%.[6,7] Although encouraging results have been reported after autologous BMT (autoBMT) with chemopurged[8,9] or unpurged[9–12] marrow in adults and children with first-remission AML, no previous studies have compared chemopurged autoBMT with intensive combination chemotherapy for childhood AML as a multicenter prospective randomized trial. We report a randomized prospective study from the Pediatric Oncology Group (POG) that evaluated autoBMT with 4-hydroperoxycyclophosphamide (4HC)-purged marrow versus intensive chemotherapy (ICT) in newly diagnosed childhood AML. We also compared the results of allogeneic BMT (alloBMT) from HLA-matched siblings to those of autoBMT or ICT.

PATIENTS AND METHODS

Patients 21 years of age and under with newly diagnosed AML were eligible for this study. All patients received standard remission induction therapy with daunorubicin (45 mg/m^2/day × 3 days), cytarabine (100 mg/m^2/day × 7 days as a continuous infusion), and thioguanine (100 mg/m^2/day × 7 days), followed by high-dose cytarabine (3 g/m^2 q12 hr × 6 doses). Patients who entered remission were then eligible for randomization to autoBMT or ICT. Patients were not eligible for randomization if they had secondary AML, Down syndrome, or isolated chloroma, or if they were eligible to receive alloBMT from an HLA-matched sibling. After randomization, all patients on study received one course of consolidation chemotherapy with etoposide (250 mg/m^2/day × 3 days) and azacytidine (300 mg/m^2/day × 2 days). Those who were randomized or assigned to the ICT regimen then received six additional courses of chemotherapy, administered at three-week intervals if tolerated: daunorubicin (45 mg/m^2) plus high-dose cytarabine;

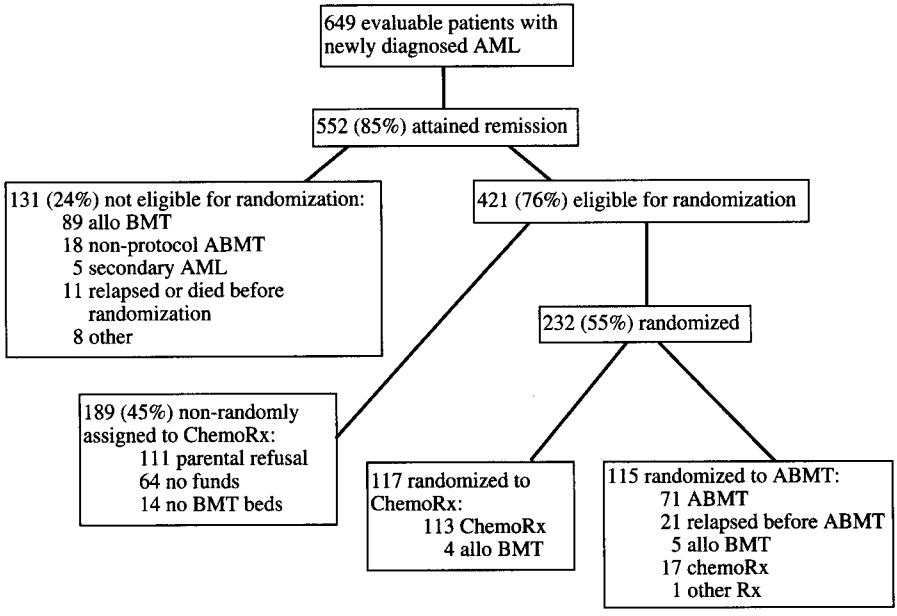

Figure 1. *Treatment assignments and randomizations of pediatric patients with first-remission AML.*

daunorubicin (45 mg/m^2 × 2 days), cytarabine (100 mg/m^2/day × 5 days as a continuous infusion), and thioguanine (100 mg/m^2/day × 5 days); etoposide and azacytidine, as described above; high-dose cytarabine; daunorubicin (2 days) plus cytarabine and thioguanine (5 days each); and etoposide and azacytidine.

Patients who were randomized to autoBMT underwent marrow collection six to 12 weeks after etoposide-azacytidine consolidation. An attempt was made to collect at least 3×10^8 nucleated marrow cells per kilogram. The marrow buffy coat was incubated ex vivo for 30 minutes with 100 g/mL of 4HC at an incubation hematocrit of 5 to 10%, as described previously,[8] and cryopreserved in the vapor phase of a liquid nitrogen freezer. The pre-transplant conditioning regimen consisted of oral busulfan (1 mg/kg/dose q 6 hr × 16 doses) and intravenous cyclophosphamide (50 mg/kg/day × 4 days).[8] There was no dose adjustment of busulfan on the basis of body surface area or individual patient busulfan pharmacokinetics profiling.[13] Forty-eight hours after the last dose of cyclophosphamide, the autologous marrow cells were thawed and infused through a central venous catheter at a rate of 10 mL/min to 15 mL/min.

RESULTS

Between June 1988 and March 1993, 649 patients were enrolled on this study; a summary of patient assignments and randomizations is shown in Figure 1. Five

Table 1. Summary of pediatric patients receiving autoBMT for AML

number assigned to autoBMT	115
number actually given autoBMT	71
interval from:	
randomization to marrow collection	49 days (range, 21–106)
marrow collection to autoBMT	17 days (range, 9–39)
marrow cell dose ($\times 10^8$/kg)	2.4 (range, 0.9–5.0)
time to ANC $>0.5\times 10^9$/L and platelets $>50\times 10^9$/L	43 days (range, 11–445)

hundred fifty-two (85%) of those enrolled on study attained remission. One hundred and thirty-one patients (24%) of those who attained remission were not eligible for randomization: 89 underwent alloBMT from HLA-matched siblings; 18 received autoBMTs on institutional non-POG protocols; five patients had secondary AML; 11 patients relapsed or died before randomization; and eight patients were removed from study for other reasons.

Even though 421 of the 552 patients in remission were eligible for remission, only 232 (55%) actually were randomized to autoBMT or ICT. One hundred eighty-nine (45%) of otherwise eligible patients were nonrandomly assigned to ICT because of parental refusal to randomization (111 patients), insufficient funds for the autoBMT procedure (64 patients), or lack of available BMT beds (14 patients). Of the 232 patients who proceeded to randomization, 115 were randomized to autoBMT and 117 to ICT. Details of the autoBMT patient group are shown in Table 1.

When evaluated on an intention-to-treat basis, the three-year event-free survival for the entire patient group was 34% ± 2.5%. The event-free survival was 38% ± 6.4% for the autoBMT group and 36% ± 5.8% for the ICT group ($p=0.20$). In contrast, the event-free survival after alloBMT was 52% ± 8% ($p=0.01$ versus autoBMT and $p=0.06$ versus ICT) (Figure 2). The actuarial relapse rates for the three groups were 31% after autoBMT, 58% after ICT, and 20% after alloBMT. The difference in relapse rates between autoBMT and ICT was statistically significant ($p<0.001$). Although relapses were observed as late as 40 months in the ICT group, no patients in either the autoBMT or alloBMT group have relapsed later than 18 months post-transplant.

Only 71 (62%) of the 115 patients randomized to autoBMT actually received that treatment. The other 44 patients did not undergo autoBMT because of pre-transplant relapse (21 patients), proceeding to alloBMT (five patients), decision to receive ICT instead (17 patients), or undergoing other therapy (one patient). However, 113 (97%) of the 117 patients randomized to ICT actually remained on that treatment arm; only four patients went on to alloBMT. The treatment-related

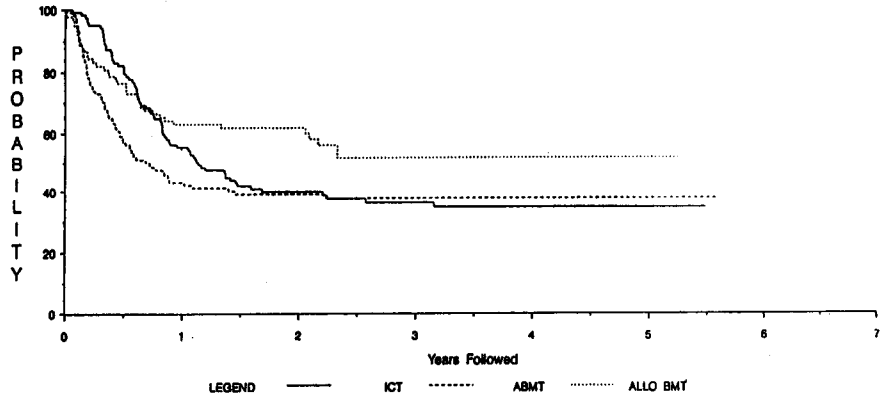

Figure 2. *Intention-to-treat analysis of event-free survival of pediatric AML patients treated with alloBMT (n=89), ICT (n=117), or autoBMT with 4HC-purged marrow (n=115).*

mortality in the actual treatment groups was significantly different (p=0.005). Eleven of the 71 autoBMT patients (15%) died with sepsis (eight patients), hepatic veno-occlusive disease (one patient), or hemorrhage (two patients); in contrast, three of 113 ICT patients (2.7%) died from sepsis (two patients) or hemorrhage (one patient). When analyzed on the basis of actual treatment received, the event-free survival was 52.5% in the autoBMT group and 38% in the ICT group. Using the Mantel-Byar test to assess the prognostic significance of autoBMT, these differences are not statistically significant (p=0.39).

DISCUSSION

This randomized study did not show any benefit of autoBMT with chemopurged marrow in children with first-remission AML. The intention-to-treat analysis showed similar event-free survival after either autoBMT or ICT; the significantly lower relapse rate after autoBMT was contrasted with a significantly greater post-autoBMT mortality. These observations suggest that methods to decrease the procedure-related mortality might ultimately lead to a superior event-free survival after autoBMT compared with chemotherapeutic regimens. Our results are also superior to those reported after either chemotherapy or autoBMT with unpurged marrow, where event-free survival was 27% after chemotherapy and 21% after autoBMT.[14] The significantly greater event-free survival that we observed in children who received alloBMT from histocompatible siblings is in contrast to a Children's Cancer Group (CCG) study, which showed no difference between alloBMT and autoBMT for first-remission childhood AML,[15] and the

United Kingdom Medical Research Council AML 10 trial, which showed no benefit of either alloBMT or autoBMT in increasing relapse-free survival after four courses of intensive chemotherapy.[16]

The outcomes of autoBMT and ICT, when analyzed on the basis of actual treatment received, showed a difference, albeit not statistically significant, in event-free survival. The event-free survival (52.5%) in those who actually received autoBMT is similar to that of alloBMT in our series and to those reported in the CCG study.[15] Although these analyses must be interpreted with caution, because they are not based on intention to treat, they nevertheless emphasize the potential utility of autoBMT in first-remission childhood AML. An alternative strategy, for which there are encouraging preliminary results in a single-institution study,[17] entails collection of autologous marrow in first remission AML, to be used for transplantation in the event of relapse. A new POG study is addressing this approach in childhood AML.

This study demonstrates the difficulties inherent in randomized multicenter trials comparing autoBMT and ICT for AML. Only 55% (232 of 421) eligible patients actually were randomized, and only 62% (71 of 115) of those randomized to the autoBMT arm actually received autoBMT. Similar problems in randomization and proceeding with autoBMT after randomization have been reported in a European adult AML trial[12] and in the MRC AML 10 trial in children.[16] Nevertheless, we stress the value and importance of the randomized prospective clinical trial, despite these logistical challenges, to compare the therapeutic effectiveness of autoBMT and chemotherapeutic regimens in AML. Future studies must also address the cost-benefit issues in randomized clinical trials of transplantation versus chemotherapy for first-remission AML.

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REFERENCES

1. Weinstein HJ, Mayer RJ, Rosenthal DS et al.: Chemotherapy for acute myelogenous leukemia in children and adults: VAPA update. *Blood* 62: 315–319, 1983.
2. Creutzig U, Ritter J, Riehm H et al.: Improved treatment results in childhood acute myelogenous leukemia: A report of the German cooperative study AML-BFM-78. *Blood* 65:

298–304, 1985.

3. Steuber CP, Civin C, Krischer J et al.: A comparison of induction and maintenance therapy for acute nonlymphocytic leukemia in childhood: Results of a Pediatric Oncology Group study. *J Clin Oncol* 9: 247–258, 1991.
4. Woods WG, Ruymann FB, Lampkin BC et al.: The role of timing high-dose cytosine arabinoside intensification and of maintenance therapy in the treatment of children with acute nonlymphocytic leukemia. *Cancer* 66: 1106–1113, 1990.
5. Ravindranath Y, Steuber CP, Krischer JP et al.: High-dose cytarabine for intensification of early therapy of childhood acute myeloid leukemia: A Pediatric Oncology Group study. *J Clin Oncol* 9: 572–580, 1991.
6. Sanders JE, Thomas ED, Buckner CD et al.: Marrow transplantation for children in first remission of acute nonlymphoblastic leukemia: An update. *Blood* 66: 460–462, 1985.
7. Bostrom B, Brunning RD, McGlave P et al.: Bone marrow transplantation for acute nonlymphocytic leukemia in first remission: Analysis of prognostic factors. *Blood* 65: 1191–1196, 1985.
8. Yeager AM, Kaizer H, Santos GW et al.: Autologous bone marrow transplantation in patients with acute nonlymphocytic leukemia, using ex vivo marrow treatment with 4-hydroperoxycyclophosphamide. *N Engl J Med* 315: 141–147, 1986.
9. Gorin NC, Aegerter P, Auvert B et al.: Autologous bone marrow transplantation for acute myelocytic leukemia in first remission: A European survey of the role of marrow purging. *Blood* 75: 1606–1614, 1990.
10. McMillan AK, Goldstone AH, Linch DC et al.: High-dose chemotherapy and autologous bone marrow transplantation in acute myeloid leukemia. *Blood* 76: 480–488, 1990.
11. Löwenberg B, Verdonck LJ, Dekker AW et al.: Autologous bone marrow transplantation in acute myeloid leukemia in first remission: Results of a Dutch prospective study. *J Clin Oncol* 8: 287–294, 1990.
12. Zittoun RA, Mandelli F, Willemze R et al.: Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. *N Engl J Med* 332: 217–223, 1995.
13. Yeager AM, Wagner JE Jr, Graham ML et al.: Optimization of busulfan dosage in children undergoing bone marrow transplantation: A pharmacokinetic study of dose escalation. *Blood* 80: 2425–2428, 1992.
14. Amadori S, Testi AM, Arico M et al.: Prospective comparative study of bone marrow transplantation and postremission chemotherapy for childhood acute myelogenous leukemia. *J Clin Oncol* 11: 1046–1054, 1993.
15. Woods WG, Kobrinsky N, Buckley J et al.: Intensively timed induction therapy followed by autologous or allogeneic bone marrow transplantation for children with acute myeloid leukemia or myelodysplastic syndrome: A Children's Cancer Group pilot study. *J Clin Oncol* 11: 1448–1457, 1993.
16. Stevens RF, Hann IM, Burnett AK et al.: Improved outcome in paediatric acute myeloid leukaemia: Results of the MRC AML 10 trial. *Med Pediatr Oncol* 23: 172, 1994 (abstract).
17. Petersen FB, Lynch MH, Clift RA et al.: Autologous marrow transplantation for patients with acute myeloid leukemia in untreated first relapse or in second complete remission. *J Clin Oncol* 11: 1353-1360, 1993.

AUTOLOGOUS BONE MARROW TRANSPLANTATION FOR ACUTE MYELOID LEUKEMIA IN REMISSION OR FIRST RELAPSE USING MONOCLONAL ANTIBODY-PURGED MARROW

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ABSTRACT

We report on 131 patients with acute myeloid leukemia (AML) who underwent autologous bone marrow transplantation (autoBMT) with monoclonal antibody (mAb)-purged marrow between August 25, 1984 and May 16, 1996. One-hundred-and-eight patients were in complete remission (CR) (CRT: 24; CR2/3: 84) and 23 were in first relapse at the time of autoBMT. In the case of transplants performed at first relapse (R1), marrow was previously harvested and purged in first CR. Marrow was purged with complement and two mAb (PM-81, anti-CD15 and AML-2-23, anti-CD14) before February 20, 1995, and, subsequently, with one mAb (PM-81), and cryopreserved. Preparative regimens included busulfan (16 mg/kg) and cyclophosphamide (CY) (120 mg/kg) (n=72), CY (120 mg/kg over two days) with total body irradiation (TBI) (1200 cGy in six fractions) (n=36), and busulfan (16 mg/kg) plus etoposide (60 mg/kg) (n=11). Median age of all patients was 38 years (range 2–59). The current status of each patient was verified.

We examined outcomes in groups of patients by remission status at the time of autoBMT and by preparative regimen. For patients in CR1 (n=21) two-year disease-free survival (DFS) and overall survival (OS) were both 52% (95% confidence interval [CI] of 35–79%). Two-year DFS for patients in CR2/3 treated with CY/TBI (n=27) was 26% (CI: 14–49%) and OS was 30% (CI: 17–53%). DFS for patients in CR 2/3 treated with BU/CY (n=43) was 41% (CI: 29–59%) and OS was 50% (CI: 36–68%). Two-year DFS and OS for patients in R1 (n=23) was 26% (13–52%) and 38% (22–65%), respectively.

The effect of cytogenetic subgroup, history of extramedullary disease, or history of either myelodysplastic syndrome or therapy-related AML was analyzed for patients in CR2/3 to determine impact on survival. We found no statistically significant difference in DFS or OS in patient groups with respect to selected cytogenetic findings nor in the potentially prognostic subgroups.

The effect of mAb purging on $CD34^+$ cell collection and transplantation was studied in 48 patients. The number of $CD34^+$ cells collected in CR1 was similar to the number collected in CR2 (1.8×10^6/kg versus 1.2×10^6/kg respectively) (p=0.67). No significant difference was noted in the number of $CD34^+$ cells harvested in patients with and without cytogenetic abnormalities (1.7×10^6 versus 1.2×10^6) (p=0.55). Patients with t(15;17) had fewer $CD34^+$ cells (p=0.02). No significant difference was noted between the number of $CD34^+$ cells harvested in those patients with primary and secondary AML. Patients with extramedullary disease had increased $CD34^+$ cell yields (p=0.006).

AutoBMT performed in CR or R1 results in excellent 2-year DFS and OS. Remissions are durable as only three relapses (of 37 pts at risk) have occurred later than two years following autoBMT. While autoBMT with mAb-purge marrow performed either at relapse or in remission can be a curative therapy, the value of purging will be addressed in a randomized clinical trial comparing autoBMT with purged and unpurged marrow.

INTRODUCTION

Autologous bone marrow transplantation (autoBMT) is an effective therapy for patients with AML. The results of several randomized and nonrandomized studies have shown that substantial proportions of patients who undergo autoBMT in complete remission (CR) remain disease-free for many years and are, therefore, probably cured of their disease. The relative value of autoBMT compared with allogeneic BMT (alloBMT) and to intensive consolidation chemotherapy (without BMT) is still somewhat uncertain since overall survival has been found to be similar with all three therapies, allowing for salvage therapy of patients who did not undergo BMT in first remission.[1] Another issue is the role of marrow purging in autoBMT. Gorin et al. report that purged marrow is superior to unpurged marrow in patients in CRT, but not in CR2.[2] Two ongoing studies in the United States with purged marrow.[3,4] In this report we update our 12-year experience with monoclonal antibody (mAb)-purged marrow in patients with AML in remission or in first relapse (R1).

PATIENTS AND METHODS

Patients

Patients less than 65 years of age with a Karnofsky performance status of 80–100%, and an expected survival of greater than 2 months were eligible for this protocol. Patients had the diagnosis of AML and were either in first CR (CR1), second or third CR (CR2/3), or in R1. All patients met previously published eligibility criteria for major organ functions.[3] Leukemia blast cells obtained at

Table 1. Clinical characteristics of patients

					FAB subclass*		
CR	*N*	*Median age*	*M/F*	*M1/M2*	*M3*	*M4/5*	*M6/7*
1st	24	45	15/9	12	3	6	1
2nd/3rd	84	36	47/37	36	13	31	1
1st Rel.	23	34	12/11	10	1	10	1
Totals	131	38	74/57	58	17	47	3

**FAB subclass was unavailable for six patients.*

diagnosis or at relapse were required to express the antigens reactive with PM-81 and/or AML-2-23 on >20% of cells. The study was approved by the Institutional Review Board of the respective institutions and a signed informed consent was obtained from each patient before study entry.

This study included 131 AML patients ranging in age from 2 to 59 who were in first, second or third CR or R1 at the time of transplant between August 25, 1984, and May 16, 1996 (Table 1). Two patients underwent two transplants with mAb-purged marrow. All but 13 patients had de novo AML at the time of initial diagnosis. Eight patients had a myelodysplastic syndrome before the diagnosis of AML, and five had secondary AML. Four patients were transplanted at the Scripps Clinic, three patients at University of Alabama, three patients at Children's Hospital of Pittsburgh, one patient at Children's Hospital of Orange County, 59 patients at the Dartmouth-Hitchcock Medical Center, 12 patients at Bowman-Gray School of Medicine, one patient at Shadyside Hospital (Pittsburgh), three patients at Shands Hospital Cancer Center (Gainesville, FL), two patients at St. Jude's Hospital (Memphis, TN), four patients at the Medical Center of Delaware, four patients at the University of Iowa Hospitals, four patients at Miami Children's Hospital, ten patients at Stanford University Medical Center, two patients from Harris Methodist Hospital (Fort Worth), and 19 patients at the University of Pittsburgh Medical Center. The FAB subclasses of the cases were as follows: M1/M2, 58; M3, 17; M4/M5, 47; M6/M7, 3; Unknown, 6.

Marrow harvesting and purging

Bone marrow was harvested and purged as previously described.[3] Purging was performed on the Haemonetics cell processor for patients treated after May 1987 at the Dartmouth-Hitchcock Medical Center (DHMC, Hanover, NH), Bowman Gray Medical Center (Winston-Salem, NC), and at the University of Pittsburgh. Before that date purging was performed at the DHMC, Scripps Clinic (La Jolla, CA) and Children's Hospitals (San Diego, CA), and the Medical Center of Delaware

(Newark, DE) the marrow cells were treated in plastic or Teflon vessels (Savillex, Minnitonka, MN) with gentle shaking. For these treatments, two separate incubations with mAb and C′ were performed as described previously.[5] Effective February 20, 1995, the mAb AML-2-23 was discontinued from the purging method after a study revealed no added benefit over PM-81 alone. Following treatment, the cells were cryopreserved as described.[3] Samples of untreated and mAb-treated marrow cells from each patient were analyzed for colony forming units as previously described[3] and $CD34^+$ cell numbers by flow cytometry.

Preparative regimens

Thirty-six patients were treated with the following preparative regimen: Cy – (60 mg/kg IV for 2 days) (days -5 to -3) and fTBI – (200 cGy twice daily for 3 days, total dose of 1,200 cGy) (days -2 to 0). In 1988, the preparative regimen changed from Cy/fTBI to Bu/Cy2. Seventy-two patients were treated with Bu (4 mg/kg/day orally for 4 days (days -8 to -5) and Cy2 (60 mg/kg/day intravenously for 2 days) (days -4 and -3). Eleven patients were treated with Bu (16 mg/kg/day) and VP-16 (60 mg/kg/day).

Statistical methods

We estimated rates of relapse, overall survival and disease-free survival (DFS) following autoBMT using the Kaplan-Meier method in patients transplanted before March 20, 1996 with follow-up to August 1, 1996.

RESULTS

Timing of bone marrow harvest and transplant

The median time between documentation of CR1 and bone marrow harvest was 97 days (with a range of 6 days–12 months) followed by the median time of 126 days (with a range of 24–420 days) between the harvest and the autoBMT. The median time between documentation of CR2/3 and bone marrow harvest was 60 days (with a range of 1–510 days) followed by the median time of 53 days (with a range of 33–391 days) from harvest to autoBMT.

$CD34^+$ cell counts

The effect of mAb purging on $CD34^+$ cell collection and transplantation in patients with AML was studied in 48 patients undergoing autoBMT. The number of $CD34^+$ cells collected in CR1 was similar to the number collected in CR2 (1.8×10^6/kg versus 1.2×10^6/kg, respectively) (p=0.67). Neuraminidase significantly reduced the number of $CD34^+$ cells available for transplantation by one-and-a-half logs. The mean number of $CD34^+$ cells harvested in CR1 in 20 patients was 2.9×10^6/kg. Following neuraminidase treatment and mAb purge, the number of

$CD34^+$ cells remaining for transplantation was 0.6×10^6/kg. In the group of patients harvested in CR1 whose marrow was not exposed to neuraminidase (9 patients), a mean of 3.6×10^6 $CD34^+$ cells/kg were harvested and post mAb purge 2.4×10^6/kg $CD34^+$ cells were transplanted. Similar findings were noted in patients transplanted in CR2. A total of 19 patients had cytogenetic abnormalities. No significant difference was noted in the number of $CD34^+$ cells harvested in the patients with and without cytogenetic abnormalities (1.7×10^6 versus 1.2×10^6) (p=0.55). Patients with t(l5;17) had fewer $CD34^+$ cells (p=0.02). Eight patients were diagnosed as having either therapy-related AML or a history of myelodysplastic syndrome (MDS). No significant difference was noted between the number of $CD34^+$ cells harvested in patients with primary versus secondary AML. Patients with extramedullary disease had increased $CD34^+$ cell yields (p=0.006).

Engraftment

Median observed recovery times for neutrophils to 0.5×10^9/L were 32, 37 and 41 days for the CR1, CR2/3 and R1 patients, respectively. Median times to reach platelet counts of greater than 20×10^9/L and greater than 50×10^9/L independent of platelet transfusions were 67 and 178 days (CR1), 105 and 242 days (CR2/3) and 163 and 222 days (R1). Median times in days to reach a hemoglobin concentration of greater than 10 gm/deciliter independent of red cell transfusions were 122, 105 and 191 for CR1, CR2/3 and R1 patients, respectively.

Toxicity

Seventeen patients died within 100 days of autoBMT from nonrelapse-related causes. Thirteen were in CR2/3 at time of transplant; nine were prepared with the Cy/TBI regimen and four were prepared with the Bu/Cy regimen. Four patients were in R1 and prepared with the Bu/Cy regimen.

Relapse

Eleven of 21 patients in CR1 relapsed at a median of 172 days post-autoBMT. Thirty-two of 70 CR2/3 patients relapsed at a median time of 136 days. Eleven patients transplanted in R1 relapsed at a median of 257 days.

Survival

Although this report is based on a subject number of 131, recent follow-up at this time was unavailable on six patients. Thus, the survival data are based on 125 patients with confirmed recent status updates. The two- and three-year DFS figures for the CR1 group are both 52% (95% CI: 0.35–0.79). The two and three year DFS figures for the CR2/3 group are 34% (CI: 25–46%) and 30% (CI: 21–43%). Analysis of CR2/3 patients by preparative regimen (Bu/Cy2 versus Cy/fTBI) is demonstrated in Figure 1. Patients receiving the Bu/Cy2 regimen have a three-year

Table 2. Summary of survival of 125 patients undergoing autoBMT with mAb-purged marrow

CR	*N*	*Prep. reg.*	*DFS**	*OS**
1	21	Cy/TBI or Bu/VP	52 (35–79)	52 (35–79)
2/3	43	BU/CY	41 (29–59)	50(36–68)
	27	CY/TBI	26 (14–49)	30 (17–53)
R1	23	Bu/Cy or Cy/TBI	26 (13–52)	38 (22–65)

**Percent at two years*

DFS of 38% (CI: 26–56%). Those receiving the Cy/TBI regimen have a three year DFS of 22% (CI: 11–45%). Overall survival at three for the Bu/Cy2 group is 47% (CI: 33–65%), while that of the Cy/TBI group is 25% (CI: 13–49%).

Six of the 23 patients transplanted in R1 remain disease-free with a median relapse-free survival post-autoBMT of 44 months (range 16–84 months). The two- and three-year actuarial DFS is 26% (CI: 13–52%).

Effect of cytogenetics on survival

We examined outcomes in groups of patients defined by cytogenetic abnormalities in their AML cells at diagnosis. Patients with favorable cytogenetics were defined as those with t(15;7), t(8;21), and abnormality of chromosome 16 (e.g., inversion 16). Patients with intermediate cytogenetics were those with normal karyotypes. Patients with unfavorable cytogenetics were defined as those with losses of chromosomes 5 or 7, trisomy 8 and abnormal 11q23. A trend to improved DFS for patients with favorable cytogenetics was seen when patients in these three groups were compared (Figure 2).

DISCUSSION

BMT after high-dose chemotherapy and/or radiation therapy offers the potential for complete elimination of occult leukemia cells during CR, and is probably the only curative treatment for patients with AML after first relapse. Encouraging results have been reported with alloBMT, but the majority of patients with AML cannot undergo this therapy due to lack of an HLA-matched donor and/or age over 55. This updated report continues to show that autoBMT is a viable alternative.

Due to the possibility that reinfused marrow may be contaminated with residual malignant cells after autoBMT, we are studying ex vivo purging to eliminate residual neoplastic cells from the graft. Although no randomized studies directly comparing autoBMT with and without marrow purging have been reported, long-term survival for AML patients after autoBMT using various methods for removing occult leukemia cells has been documented.[6–8] A recent analysis of European data has shown a benefit of mafosfamide purging for patients

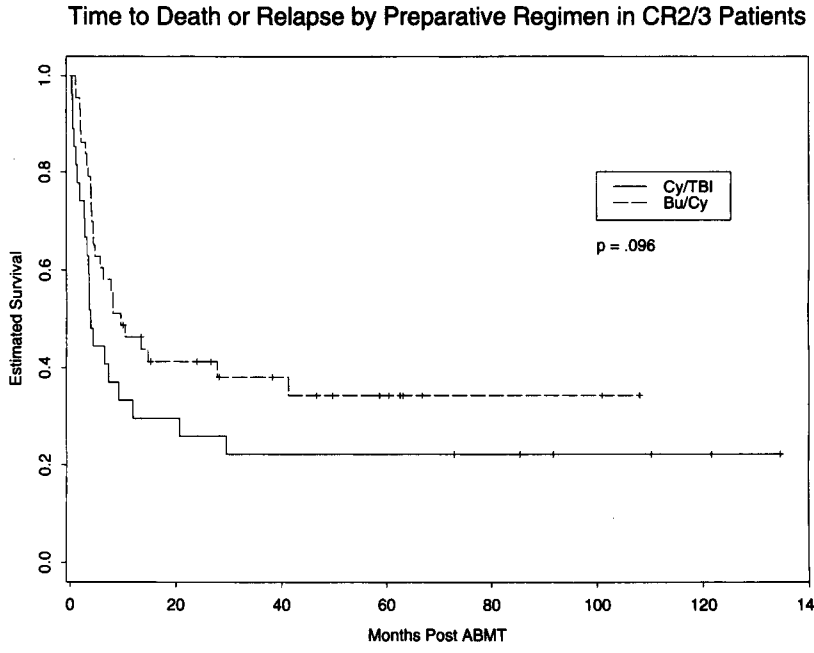

Figure 1. *DFS of patients transplanted in CR2/3 according to preparative regimen.*

transplanted in first CR within six months of attaining CR.[6] Chao et al.[7] published a phase II trial showing that patients who received purged bone marrow (4-MC and/or etoposide) had an actuarial DFS of 57% compared with a DFS of 32% in patients who received unpurged bone marrow. Yeager et al.[4] have reported favorable results similar to alloBMT with 4-HC marrow purging in patients with AML who underwent autoBMT. Miller et al.[8] compared purged versus nonpurged autoBMT outcomes using data from the Autologous Bone Marrow Transplant Registry (ABMTR) and found a benefit for purging in both CR1 and CR2.

Data from Brenner et al.[9] using the neomycin-resistance gene as a marker for AML relapse suggests that autologous marrow harvested from leukemia patients in clinical remission may harbor malignant cells capable of contributing to relapse. This evidence suggests that effective marrow purging may be essential for improving the outcome of autoBMT for AML.

This report updates our ongoing study of autoBMT in AML with mAb and C-mediated purging. The trend toward long-term DFS is evident for those patients transplanted in CR2/3 who were conditioned with Bu/Cy2. These improved results with Bu/Cy2 compared to Cy/fTBI are due both to a decreased relapse rate with this regimen Bu/Cy2 and a decreased number of toxic deaths. However, other

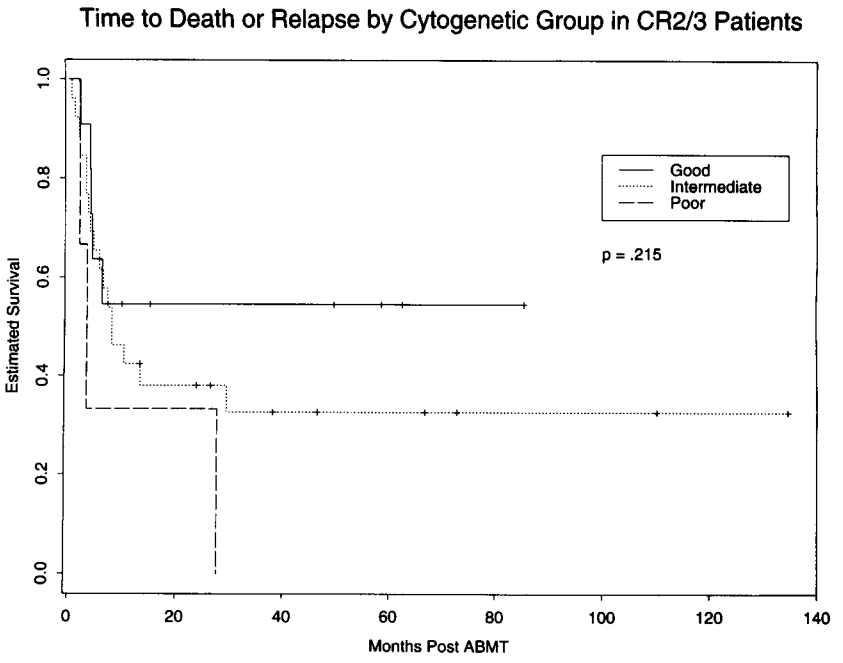

Figure 2. *DFS of patients transplanted in CR2/3 according to cytogenetically-defined subgroups. Good risk was defined as those with t(l5;17), t(8;21) or inversion 16. Intermediate was defined as normal or other cytogenetics. Poor risk was defined as those with –5, –7, +8 or abnormal 11q23.*

factors including improved supportive care may have contributed.

This brief report does not focus on issues of engraftment times and quality. However, we have recently examined the value of $CD34^+$ cell enumeration in predicting engraftment outcomes. This study demonstrates that the clinical remission status at time of harvest, presence of cytogenetic abnormalities and pre-existence of MDS or other malignancy did not significantly influence the numbers of $CD34^+$ cells that could be harvested from the BM in this group of patients with AML. The predictive value of $CD34^+$ cell counts for engraftment is under evaluation.

These data compare well to alternative approaches to autoBMT in AML such as the use of 4-HC and to alloBMT for patients at similar risk for relapse.[10] Many patients have been in remission for a longer time since autoBMT than the length of their previous remissions. In addition, relapse two years after autoBMT is uncommon.[3,6,8] The precise role of marrow purging will be tested in a Phase III study comparing the outcomes of autoBMT using purged and unpurged marrow.

REFERENCES

1. Zittoun RA, Mandelli F, Roel W et al.: Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. *N Engl J Med* 332:217–223, 1995.
2. Gorin NC, Aegerter P, Auvert B et al.: Autologous bone marrow transplantation for acute myelocytic leukemia in first remission: A European survey of the role of marrow purging. *Blood* 75:1606, 1990.
3. Selvaggi KJ, Wilson J, Mills LE et al.: Improved outcome for high risk acute myeloid leukemia patients using autologous bone marrow transplantation and monoclonal antibody purged bone marrow. *Blood* 83;1698–1705, 1994.
4. Yeager AM, Kaizer H, Santos GW et al.: Autologous bone marrow transplantation in patients with acute non lymphocytic leukemia, using ex vivo marrow treatment with 4-hydroperoxycyclophosphamide. *N Engl J Med* 315:141, 1986.
5. Howell AL, Fogg-Leach M, Davis BH et al.: Continuous infusion of complement by an automated cell processor enhances cytotoxicity of monoclonal antibody sensitized leukemia cells. *Bone Marrow Transplant* 4:317–322, 1989.
6. Gorin NC, Labopin M: European survey on 1688 autografts for consolidation of acute leukemia: Further evidence that marrow purging with mafosfamide is effective in acute myelocytic leukemia (AML). *Blood* 76:50, 1990.
7. Chao NJ, Stein AS, Long GD et al.: Busulfan/Etoposide—initial experience with a new preparatory regimen for autologous bone marrow transplantation in patients with acute nonlymphoblastic leukemia. *Blood* 81:319, 1993.
8. Miller CB, Rowlings PA, Jones RJ et al.: Autotransplants for acute myelogenous leukemia (AML): Effect of purging with 4Hydroperoxycyclophosphamide (4 HC). Proceedings of ASCO 15:338, 1996.
9. Brenner MK, Rill DR, Moen RC et al.: Gene-marking to trace origin of relapse after autologous bone marrow transplantation. *Lancet* 341:85, 1993.
10. Buckner CD, Clift RA: Clinical studies of allogeneic marrow transplantation in patients with acute nonlymphoblastic leukemia. Seattle Marrow Transplant team. *Bone Marrow Transplant* 3:82, 1989.

GENE-MARKING STUDIES IN ACUTE MYELOID LEUKEMIA

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ABSTRACT

Gene-marking has been used to determine if harvested marrow in patients with acute myeloid leukemia receiving autologous transplantation is contaminated with malignant cells and whether attempts to remove such cells have been successful. In first generation studies, four of twelve patients who received marked marrow relapsed and marked leukemic cells were found in three cases, demonstrating that residual malignant cells in infused marrow can contribute to disease recurrence. In follow-up studies, double-marking with distinguishable retroviral vectors allows intrapatient comparison of two purging methodologies. Fifteen patients with acute myeloid leukemia in first remission have received autografts of marrow purged with 4HC and either IL2 or CD15 antibodies. One patient has relapsed and marker genes were not detected in malignant cells at the time of relapse, so information is not yet available on the efficacy of purging. However, these double-marking studies have provided information on the effects of these purging methods on long-term engraftment.

INTRODUCTION

A number of studies suggest that autologous bone marrow transplantation (autoBMT) appears to result in an improvement in survival in patients with acute myeloid leukemia (AML) transplanted in first remission, with relapse remaining the major cause of treatment failure.[1,2] The possibility that reinfused leukemic cells may contribute to relapse has led to extensive evaluation of techniques for purging marrow to eliminate residual malignant cells,[3,4] although it has been unclear whether these procedures are necessary. One means of resolving this issue is to mark the marrow at the time of harvest with genetic markers. If the recipient of such marrow should subsequently relapse, detection of marked cells provides unequivocal evidence that the residual malignant cells in marrow are a source of leukemic recurrence.

FIRST GENERATION MARKING STUDIES

The initial study to address this question in patients with AML began at St. Jude Children's Research Hospital in September 1991, and closed in March 1993. Nucleated bone marrow ($>1.5\times10^8$ cells/kg of body weight) was taken from the posterior iliac crest and two-thirds were cryopreserved immediately. The remaining third was separated on a Ficoll gradient to produce a mononuclear cell fraction that was transduced with either the LNL6 or the closely related G1N retroviral vector for 6 hours in the absence of growth factors.[5] Both these vectors encode the neomycin resistance gene, which can subsequently be detected in transduced cells either genotypically by PCR or phenotypically because the transferred gene confers resistance to the neomycin analogue G418. At the time of marrow reinfusion, both transduced and unmanipulated marrow cells were thawed and reinfused through the patient's central venous line. To assess the efficiency of gene transfer and expression in marrow progenitor cells both pre- and post-transplantation, we obtained mononuclear cells from peripheral blood or bone marrow, and cultured them in methylcellulose as previously described with or without G418[6]. PCR was used to detect the transferred Neo^R gene in individual colonies or in bulk populations as previously described.[6]

Twelve patients were enrolled on this study and follow-up ranged from 44–60 months. Four patients have relapsed. To definitively prove that the marker gene is in leukemia cells, it is necessary to have a collateral leukemia-specific marker so that both this marker and the marker gene can be shown in the same cell. For example, in one of the AML patients who relapsed the malignant blasts co-expressed CD34 and CD56, a combination not found on normal hemopoietic cells, and had a complex t(1:8:21) translocation resulting in generation of an AML1/ETO fusion transcript that could be identified by RT-PCR. We were therefore able to sort blasts expressing CD34 and CD56 and show co-expression in this bulk population and in G418-resistant colonies derived from sorted cells of both a leukemia-specific marker (the AML:ETO fusion protein) and the transferred Neo^R gene.[7] In three patients, the malignant cells have contained the marker gene, while the fourth patient was uninformative.[8] Although the Neo^R gene could be detected by PCR in his marrow mononuclear cells, his blasts did not have a leukemia-specific marker, so it was not possible to determine if the PCR signal emanated from normal or malignant cells.

These data show definitively that marrow harvested in apparent clinical remission may contain residual tumorigenic cells that may contribute to relapse of leukemia. One implication of these observations is that effective purging of harvested marrow is one component for improving the outcome of autoBMT.

Because this genetic marking technique also marks normal progenitors, it provides an opportunity to evaluate the kinetics of reconstitution post transplant and determine the relative contribution of the engrafting marrow to short- and long-term hemopoietic and immune reconstitution.[6] The marker gene has continued to be detected and expressed for up to five years in both marrow clonogenic cells and their mature progeny, including peripheral blood T and B cells and neutrophils. The level of transfer varied and was highest in marrow clonogenic hemopoietic progenitors. In peripheral blood cells, expression was variable between the different lineages and was higher by an order of magnitude in myeloid cells than in T lymphocytes, with the lowest level of transfer in B lymphocytes. These observations suggest that autologous marrow makes a substantial contribution to long-term reconstitution following autologous transplantation.

SECOND GENERATION MARKING STUDIES

We have begun second generation studies in patients receiving autoBMT for AML using two gene markers to compare two different purging techniques.[9] The two retroviral vectors used differ in 3′ noncoding sequence, so it is possible to design primers which will amplify fragments which can be discriminated by size. In this study, one-third of the marrow is frozen unpurged as a safety backup. The remaining marrow is split into two aliquots which are marked with G1Na or LNL6 and then randomly assigned to two purging techniques. We initially purged one aliquot with 4HC and one aliquot with IL2[10] (in collaboration with Dr. H. Klingemann). Recently, CD15 antibodies[11] (provided by Dr. E. Ball) have been substituted for IL2 in the second purging arm. At the time of transplant both purged aliquots are reinfused. If the patient should subsequently relapse, detection of one or both marker genes in malignant cells will allow us to learn if either of these purging techniques is effective.

Fifteen patients have been treated on this protocol as of September 1996. Five patients have received transplants with both 4HC and IL2 purged aliquots and three with both 4HC and CD15 purged aliquots. Seven patients received marrow purged with 4HC alone, due to either insufficient number of cells harvested or unavailability of one clinical grade retroviral vector. Follow-up currently ranges from 1–35 months with a median of 24 months. Only one patient has relapsed so far and was noninformative as marked malignant cells were not detected. Transfer has been seen into normal progenitors, albeit at a lower level than in the studies using unpurged marrow. The double-marking component has allowed comparison of reconstitution of normal progenitors after the two purging

procedures. In peripheral blood the signal from the 4HC purged fraction has been consistently stronger and longer lived than that from the IL2 purged fraction, implying that the 4HC purged fraction is making a greater contribution to hemopoietic reconstitution. As a consequence, the second purging technique has been changed to use CD15 antibodies.

SAFETY OF GENE-MARKING

As the marking procedure confers no benefit to individual patients, safety is an important consideration. Patient follow-up in the two gene-marking studies is now over 600 patient months and no adverse effects attributable to gene-marking have occurred. To determine if there was any difference in leukemia-free survival in recipients of marked and unmarked marrow, we compared survival curves of patients with AML in first remission enrolled in marking studies with patients treated on the same chemotherapy and autoBMT protocol who received unmarked marrow due to unavailability of clinical grade supernatant or refusal of marking component. The comparison of survival curves was undertaken using the logrank test, and Kaplan-Meier plots are provided in Figure 1. There is no significant difference in leukemia-free survival between recipients of marked marrow (64% at 3 years) or unmarked marrow (49% at 3 years). Similarly, when the cumulative incidence of relapse was estimated,[12] there was no significant difference between the risk in recipients of marked marrow (18% at 3 years) and unmarked marrow (50% at 3 years) (p=0.06).

DISCUSSION

Genetic marking of autologous marrow has unequivocally shown that residual malignant cells may be one source of subsequent relapse after autograft for AML. Similar observations have been made in a study of adult patients with chronic myeloid leukemia,[13] where marked cells co-expressing *BCR-ABL* and Neo^R were found at the time of relapse. In contrast, marked malignant cells were not found at relapse in a study of adult patients receiving autoBMT for acute myeloid or lymphoid leukemia.[14] Failure to detect marked leukemic cells may reflect a difference between adult and pediatric patients, or it may be because only 10% of marrow was marked and the efficiency of marking was low.

In the purging study there are no data as yet on whether purging is efficacious in removing cells that may contribute to relapse. A potential problem with this study is that only a positive result is definitive so that a relapse with marked leukemic cells would indicate that the purging technique employed was not successful in removing all clonogenic blasts. Moreover, as the number of residual malignant cells in harvested marrow becomes smaller the efficiency of marking

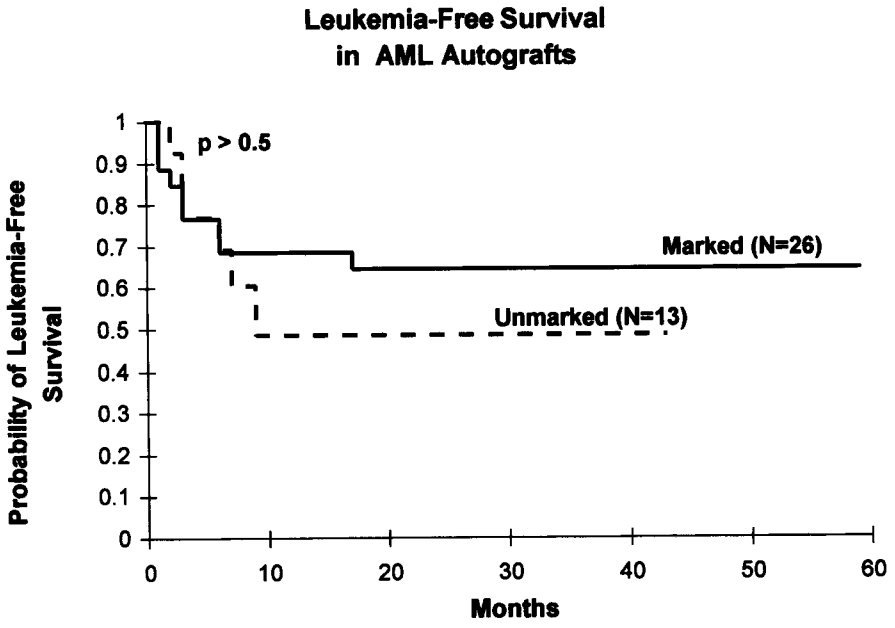

Figure 1. *Leukemia-free survival in AML autografts. Leukemia-free survival was estimated using the logrank test and Kaplan-Meier plots. Patients with AML in first remission who received an autoBMT with marked marrow were compared with control patients who received unmanipulated marrow after the same chemotherapy and transplant regimen. There is no significant difference in leukemia-free survival between recipients of marked marrow (64% at 3 years) or unmarked marrow (49% at 3 years).*

may become limiting. Rather than indicating the true efficacy of purging, a negative result may therefore reflect either a low number of residual leukemic cells in marrow that were not marked or a relatively small contribution to relapse from the infused marrow. This problem could be overcome by use of more efficient marking techniques. Although this double-marking study has not yet produced data on the efficacy of purging, it has allowed us to compare hemopoietic reconstitution by the two fractions and to demonstrate that the contribution from the 4-HC purged fraction is consistently greater than that from the IL2 purged fraction. Similar double-marking studies may allow comparison of different sources of hemopoietic stem cells to determine the optimum source for transplantation.

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REFERENCES

1. Santos GW, Yeager AM, Jones RJ: Autologous bone marrow transplantation. *Ann Rev Med* 40:99–112, 1989.
2. Zittoun RA, Mandelli F, Willemze R et al.: Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. *N Engl J Med* 332:217–223, 1995.
3. Gorin NC, Aegerter P, Auvert B et al.: Autologous bone marrow transplantation for acute myelocytic leukemia in first remission: A European survey of the role of marrow purging. *Blood* 75:1606–1614, 1990.
4. Yeager AM, Kaizer H, Santos GW et al.: Autologous bone marrow transplantation in patients with acute nonlymphocytic leukemia, using ex vivo marrow treatment with 4-hydroperoxycyclophosphamide. *N Engl J Med* 315:141–147, 1986.
5. Brenner M, Mirro J, Jr., Hurwitz C et al.: Autologous bone marrow transplant for children with AML in first complete remission: Use of marker genes to investigate the biology of marrow reconstitution and the mechanism of relapse. *Hum Gene Ther* 2:137–159, 1991.
6. Brenner MK, Rill DR, Holladay MS et al.: Gene-marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. *Lancet* 342:1134–1137, 1993.
7. Brenner MK, Rill DR, Moen RC et al.: Gene-marking to trace origin of relapse after autologous bone marrow transplantation. *Lancet* 341:85–86, 1993.
8. Heslop HE, Rooney CM, Brenner MK: Gene-marking and haemopoietic stem-cell transplantation. *Blood Rev* 9:220–225, 1995.
9. Brenner MK, Krance R, Heslop HE et al.: Assessment of the efficacy of purging by using gene marked autologous marrow transplantation for children with AML in first complete remission. *Hum Gene Ther* 5:481–499, 1994.
10. Klingemann HG, Neerunjun J, Schwulera U, Ziltener HJ: Culture of normal and leukemic bone marrow in interleukin-2: Analysis of cell activation, cell proliferation, and cytokine production. *Leukemia* 7:1389–1393, 1993.
11. Selvaggi KJ, Wilson JW, Mills LE et al.: Improved outcome for high-risk acute myeloid leukemia patients using autologous bone marrow transplantation and monoclonal antibody-purged bone marrow. *Blood* 83:1698–1705, 1994.
12. Gray RJ: A class of K-sample tests for comparing the culmulative incidence of a competing risk. *Annals of Statistics* 16:1141–1154, 1988.
13. Deisseroth AB, Zu Z, Claxton D et al.: Genetic marking shows that Ph^+ cells present in autologous transplants of chronic myelogenous leukemia (CML) contribute to relapse after autologous bone marrow in CML. *Blood* 83:3068–3076, 1994.
14. Cornetta K, Srour EF, Moore A et al.: Retroviral gene transfer in autologous bone mar-

PARENTERAL (IV) BUSULFAN/CYCLOPHOSPHAMIDE (BUCY) AS TRANSPLANTATION CONDITIONING THERAPY

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Pretransplant conditioning with Bu/Cy is becoming popular. Unfortunately, its use is limited by serious normal organ toxicity, most importantly liver toxicity, due to unpredictable intestinal drug absorption. A parenteral Bu formulation should standardize the drug's bioavailability and, hopefully, improve the toxicity profile with increased safety of the Bu-based conditioning regimens. We solubilized Bu for parenteral use in a combination of dimethylacetamide and PEG400. This formulation was very hypertonic; administration through a central venous catheter would be mandatory to achieve the dilution that would make parenteral administration safe. Bu dissolved in this vehicle retained full in vitro cytotoxic activity. Preclinical pharmacokinetic and toxicology studies in beagles did not reveal any serious extrahematopoietic organ toxicity at dose levels up to 1.0 mg/kg in a multiple dose study (16 doses given over 2 hours every 6 hours). The parenteral Bu was then used in a clinical phase I study (0.08–0.8 mg/kg); Bu was given IV over two hours by pump (one dose), followed by 15 oral doses at 1 mg/kg every 6 hours, after which Cy was administered (60 mg/kg daily for two days) followed by marrow or peripheral stem cell support. The parenteral Bu has so far been tolerated without acute toxicity. The pharmacokinetic studies show cytocidal Bu concentrations in the blood for four hours following infusion at the lowest dose level, and for 6 hours at higher levels. A kinetic analysis suggests that the pharmacokinetics of parenteral Bu can be best fitted to an open two-compartment model. The data further suggest that the bioavailability of oral high-dose Bu is limited to approximately 60%, based on a comparison of the pharmacokinetics of the oral 1 mg/kg dose to that of two higher dose levels of the parenteral formulation (0.4 and 0.8 mg/kg, respectively). Based on our findings, we suggest that IV Bu will become an important alternative to oral Bu for pretransplant conditioning therapy.

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

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ABSTRACT

Since 1987, 62 patients with acute myelogenous leukemia (AML) in CR2 have undergone autologous bone marrow transplant (autoBMT) at the Johns Hopkins Oncology Center with marrows purged with 4-hydroperoxycyclophosphamide (4HC). All patients received busulfan (4 mg/kg/day for 4 days) and cyclophosphamide (50 mg/kg/day for 4 days) as the preparative regimen. Mononuclear cells collected from Ficolled marrow were treated with 60 μg/mL of 4HC. Marrow was collected in second remission in all patients. Actuarial disease-free survival (DFS) at 8 years is 37% (median follow-up of 50 months) with relapse being the major cause of failure (relapse rate 58%). Duration of neutropenia was prolonged with a median day to absolute neutrophil count >500/mm^3 of 42 days (range 20 to 272); however, only two patients with neutropenia greater than 40 days died of aplasia-related causes. Overall transplant-related mortality was 13%. AutoBMT with marrow purged with 4HC in patients with AML in complete remission (CR)2 is effective. However, many patients are unable to achieve a second CR. Therefore, high risk patients should be transplanted in first CR. Standard and good risk patients should be considered for BMT as part of a clinical trial or have marrow harvested and purged in first CR for use in subsequent relapse.

INTRODUCTION

While conventional chemotherapy offers the potential for cure in patients with newly diagnosed acute myeloid leukemia (AML),[1,2] cure is rare with conventional dose chemotherapy alone in patients who have relapsed. Allogeneic bone marrow transplantation (alloBMT) has been the treatment of choice in patients with relapsed AML if a suitable matched sibling donor is available.[3,4] AlloBMT offers the advantage that a second remission need not be

obtained,[5] as well as the potential benefit of the graft-versus-leukemia (GVL) effect.[6] However, many patients do not have a suitable sibling donor or are too old to undergo alloBMT; autologous BMT (autoBMT) is an option for these patients.[7–13]

Potential drawbacks of autologous BMT are contamination of the autologous marrow graft with occult leukemia and the absence of the immunologic GVL effect associated with alloBMT.[6] Gene-marking studies have shown that tumor in the graft contributes to relapse.[14] Different strategies have been used to decrease leukemia contamination of marrow graft.[15–19] We have used 4-hydroperoxycyclophosphamide (4HC), a cyclophosphamide congener, to purge marrows in patients with acute myelogenous leukemia in second remission.

PATIENTS AND METHODS

Since 1987, 62 patients with AML in second complete remission have been treated with 4HC purged autologous BMT. Before 1988, mononuclear cells collected from a buffy coat preparation of marrow at a concentration of 20 million cells per mL were incubated with 100 micrograms/mL of 4HC for 30 minutes at 37°C. The red cell concentration of the buffy coat preparation varied greatly; this produced great variability in 4HC activity during purging since red cells contain aldehyde dehydrogenase, which inactivates 4HC.[20] The purging procedure was modified in 1988 to minimize the variability in 4HC activity. Red blood cells were removed from the autologous marrow graft by density centrifugation, before purging with 4HC. The dose of 4HC had to be decreased to 60 micrograms/mL when all the red cells were removed to approximate the average 4HC activity seen with 100 g/mL in the presence of red blood cells.

The characteristics of the 62 patients are shown in Table 1. Thirty percent of the patients were considered high risk as defined by having at least one of the following characteristics: chromosomal abnormalities other than t(8:21), t(15:17), or inverted 16; white blood count greater than 100,000/mm^3 at presentation; presence of extramedullary disease at diagnosis; secondary AML or preexisting myelodysplastic syndrome; or failure to achieve a complete remission with the first cycle of chemotherapy. The remaining patients were standard or good risk patients at diagnosis. Median age was 28 years (range 2–62) and median duration of CR1 was 18 months (range 2–60). After achieving a second remission, forty percent of the patients were consolidated before 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 per kilogram per day orally for four days), followed by cyclophosphamide (50 mg per kilogram IV daily for 4 days).

Table 1. Patient characteristics

age	
median	26 years
range	2–62 years
high risk at presentation	
yes	30%
no	70%
CR2 to BMT	
median	3 months
range	1–9 months
consolidation	
yes	40%
no	60%
duration of CR1	
median	18 months
range	2–60 months

RESULTS

Actuarial disease-free survival (DFS) and risk of relapse for all patients at 5 years are shown in figures 1 and 2. Median followup is 50 months. Actuarial DFS at 5 years is 37% with an estimated relapse risk of 58%. Median time to relapse was 6 months with two late relapses at 36 and 72 months. Survival after relapse was short; however, two patients are in long chemotherapy-induced third remissions at 2 and 5 years without a repeat BMT. Three relapsed patients underwent unrelated BMT. Two died early of transplant-related complications, and one patient is alive 4 months after unrelated transplantation.

The DFS of patients in second remission treated with autologous transplants was similar to several remission patients treated with allogeneic transplant from an HLA-identical sibling during the same time period (Figure 3). Risk of relapse was significantly higher in the autologous patients (Figure 4); however, the transplant-related mortality (mainly graft-versus-host disease) was higher in the allogeneic transplant recipients.

The nonrelapse mortality was 13% in the autologous transplant patients. The median day of transplant-related death was 40 days (range 6–272). Six patients died of preparative regimen toxicity (veno-occlusive disease of the liver and interstitial pneumonitis) and two patients died of infection (one early in aplasia and one on day 272 related to failure to engraft).

Patients required a median of 42 days (range 20 to greater than 272) to achieve a neutrophil count greater than 500/mm^3. The period of neutropenia did not contribute to excess mortality as only one of the patients with neutropenia lasting

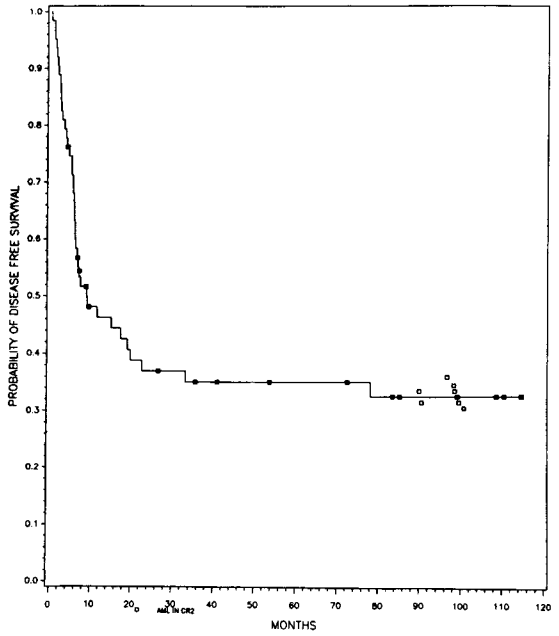

Figure 1. *DFS in 62 patients with AML in CR2 autotransplanted with 4HC purged marrow.*

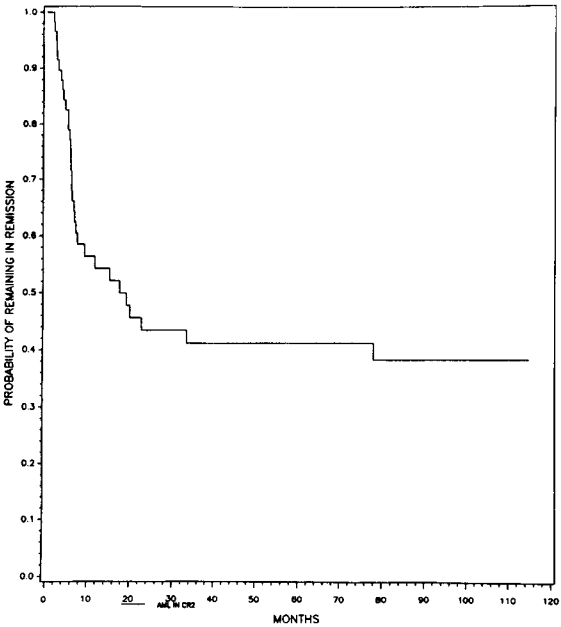

Figure 2. *Relapse-free survival in 62 patients with AML in CR2 autotransplanted with 4HC purged marrow.*

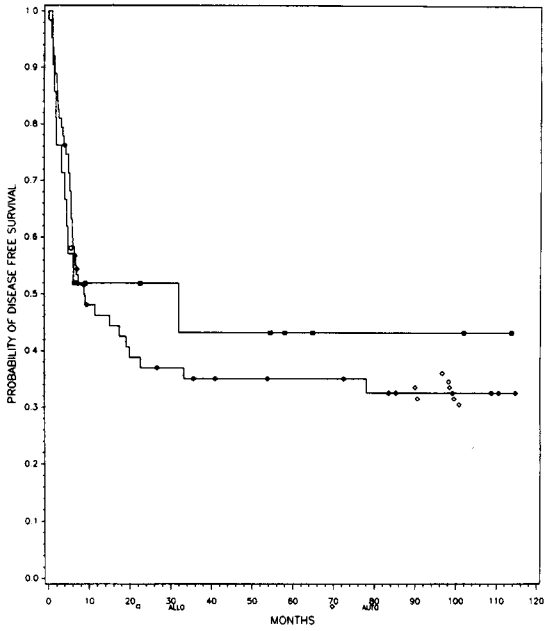

Figure 3. *DFS in patients receiving autologous transplants for AML in CR2 (—) compared with allogeneic transplants for AML in CR2 (—).*

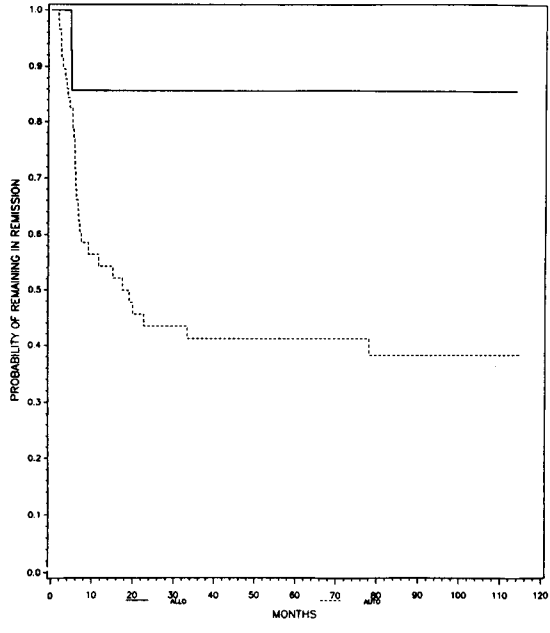

Figure 4. *Relapse-free survival in patients receiving autologous transplants for AML in CR2 (- - -) compared with allogeneic transplants for AML in CR2(—).*

more than 50 days died of nonrelapse mortality (sepsis related to failure to engraft on day 272). Platelet recovery was prolonged with only 30% of the patients recovering platelet independence before day 50. Median duration of platelet support was greater than 3 months. Despite prolonged thrombocytopenia, hemorrhage was not the primary cause of death in any patient.

DISCUSSION

In summary, autoBMT with 4HC purged marrow cures approximately 37% of patients autografted in CR2 using our current protocol. These results are comparable with alloBMT. AutoBMT requires that marrow be harvested in remission. As 50% of patients who relapse fail to achieve a second remission, lack of a suitable source of stem cells may prevent patients from proceeding to potentially curative therapy. Therefore, we recommend that patients with AML who do not have a sibling donor, are not candidates for, or refuse BMT in first complete remission have marrow harvested and purged with 4HC in first remission. Autologous BMT could then be performed in first early relapse.

REFERENCES

1. Geller RB, Burke PJ, Karp JE et al.: A two-step timed sequential treatment for acute myelocytic leukemia. *Blood* 74(5):1499–1506, 1989.
2. Schiller G, Gajewski J, Territo M et al.: Long-term outcome of high-dose cytarabine-based consolidation chemotherapy for adults with acute myleogenous leukemia. *Blood* 80(12):2977–2982, 1992.
3. Geller RB, Saral R, Piantadosi S et al.: Allogeneic bone marrow transplantation after high-dose busulfan and cyclophosphamide in patients with acute nonlymphocytic leukemia. *Blood* 73:2209–2218, 1989.
4. Welch HG, Larson EB: Cost effectiveness of bone marrow transplantation in acute nonlymphocytic leukemia. *N Engl J Med* 321(12):807–812, 1993.
5. Clift RA, Buckner CD, Appelbaum R et al. Allogeneic marrow transplantation during untreated first relapse of acute myeloid leukemia. *J Clin Oncology* 10(11):1723–1729, 1992.
6. Weiden PL, Sullivan KM, Flournoy N et al.: Antileukemic effect of chronic graft-versus-host disease. *N Engl J Med* 304(25):1529–1533, 1981.
7. Linker CA, Ries CA, Damon LE et al.: Autologous bone marrow transplantation for acute myeloid leukemia using busulfan plus etoposide as a preparative regimen. *Blood* 81(2):311–318, 1993.
8. McMillan AK, Goldstone AH, Linch DC et al.: High-dose chemotherapy and autologous bone marrow transplantation in acute myeloid leukemia. *Blood* 76(3):480–488, 1990.
9. Gress DE: Purged autologous bone marrow transplantation in the treatment of acute leukemia. *Oncology* August:40-43, 1990.

10. Meloni G, De Fabritiis P, Petti MC et al.: BAVC regimen and autologous bone marrow transplantation in patients with acute myelogenous leukemia in second remission. *Blood* 75(12):2282–2285, 1990.
11. Chopra R, Goldstone AH, McMillan AK et al.: Successful treatment of acute myeloid leukemia beyond first remission with autologous bone marrow transplantation using busulfan/cyclophosphamide and unpurged marrow: The British autograft experience. *J Clin Oncology* 9(10):1840–1847, 1991.
12. Yeager AM, Kaizer H, Santos GW et al.: Autologous bone marrow transplantation in patients with acute nonlymphocytic leukemia, using ex vivo marrow treatment with 4-hydroperoxycyclophosphamide. *N Engl J Med* 315(3):141–148, 1986.
13. Spinolo JA, Dicke KA, Horwitz LJ et al.: High-dose chemotherapy and unpurged autologous bone marrow transplantation for acute leukemia in second or subsequent remission. *Cancer* 66:619–626, 1990.
14. Brenner M, Krance R, Heslop HE et al.: Assessment of the efficacy of purging by using gene marked autologous marrow transplantation for children with AML in first complete remission. *Human Gene Therapy* 5(4):481–499, 1994.
15. Korbling M, Fliedner TM, Holle R et al.: Autologous blood stem cell (ABSCT) versus purged bone marrow transplantation (pABMT) in standard risk AML; influence of source and cell composition of the autograft on hemopoietic reconstitution and disease-free survival. *Bone Marrow Transplant* 7:343–349, 1991.
16. Ball ED, Mills LE, Cornwell III GG et al.: Autologous bone marrow transplantation for acute myeloid leukemia using monoclonal antibody-purged bone marrow. *Blood* 75(5):1199–1206, 1990.
17. Gulati S, Acaba L, Yahalom J et al.: Autologous bone marrow transplantation for acute myelogenous leukemia using 4-hydroperoxycyclophosphamide and VP-16 purged bone marrow. *Bone Marrow Transplant* 10:129–134, 1992.
18. Lemoli RM, Gasparetto C, Scheinberg DA et al.: Autologous bone marrow transplantation in acute myelogenous leukemia: In vitro treatment with myeloid-specific monoclonal antibodies and drugs in combination. *Blood* 77(8):1829–1836, 1991.
19. Lenarsky C, Weinberg K, Petersen J et al.: Autologous bone marrow transplantation with 4-hydroperoxycyclophosphamide purged marrows for children with acute non-lymphoblastic leukemia in second remission. *Bone Marrow Transplant* 6:425–429, 1990.
20. Jones RJ, Zuehlsdorf M, Rowley SD et al.: Variability in 4-hydroperoxycyclophosphamide activity during clinical purging for autologous bone marrow transplantation. *Blood* 70(5):1490–1494, 1987.

CHAPTER 2
ALL

CHOICES OF MARROW TRANSPLANTATION FOR ALL: TIMING, TOXICITY AND OUTCOME

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Patients with acute lymphoblastic leukemia (ALL) showing high-risk characteristics or following one or more relapses are unlikely to be cured with ongoing conventional chemotherapy.[1–5] While histocompatible related donor, usually sibling, transplantation has proved curative for 30–50% of patients and has been shown superior to chemotherapy in comparative analyses,[6–11] those lacking a matched sibling donor must seek other forms of treatment. Autologous bone marrow transplantation (autoBMT) using remission marrow, cryopreserved with or without purging, has been reportedly successful for 20–40% of patients.[12–20] AutoBMT is accompanied by relatively low (approximately 10%) transplant-related mortality but is followed by post-transplant relapse (40–70%) as the most frequent cause of treatment failure. Alternatively, allogeneic unrelated donor transplantation can be performed for 35–50% of patients for whom a suitable histocompatible donor can be identified before disease progression and subsequent relapse has occurred.[21–23] Unrelated donor (URD) transplantation yields more frequent post-transplant toxicity due to increased risks of rejection, graft-versus-host disease and post-transplant immunodeficiency complicated by opportunistic infection.[24–26] Conversely, URD transplantation yields a significantly superior protection against relapse, potentially due to the allogeneic graft-versus-leukemia effect accompanying the allograft.[23,27] Several factors impact the feasibility and efficacy of these two transplantation choices. Clinical decision-making must account for the comparative delays, toxicity and anti-leukemic effectiveness of these two transplant therapies to offer optimal choices to patients in different risk groups. Factors relevant to these clinical choices must all be considered in order to improve the results of overall therapy for patients with ALL.

TIMING OF TRANSPLANTATION

Autologous transplantation ostensibly offers little delay as a barrier to therapy for patients with ALL. Patients with suitable remission marrow, cellular enough for aspiration harvest, can undergo autografting promptly after achieving remission.[12,13,17,18] However, the consolidation and maintenance therapy usually applied to achieve and extend remission in ALL patients frequently results in marrow hypocellularity, which confounds satisfactory

marrow harvest shortly after achieving complete remission (CR). Withholding maintenance therapy, while facilitating marrow recovery and thus allowing harvest, may conversely increase the risk of clinical or subclinical relapse and thus potentially increase the leukemia burden in the graft and in the patient receiving the autoBMT. Previous analyses have identified the leukemia burden pretransplant measured either by leukemia precursor assays[19] or by molecular analyses as the strongest predictor of post-transplant relapse risk, again highlighting the importance of minimal residual disease as a critical factor predicting outcome post-autotransplantation for patients with ALL.

Unrelated donor (URD) allogeneic transplantation cannot be applied immediately upon achieving remission. Time for donor search, histocompatibility testing, third-party prior authorization approval for the costs of search and transplantation, as well as logistic arrangements for marrow harvest and transport all must be secured before the URD transplant can be effected.[28,29] Current experience in transplants facilitated by the National Marrow Donor Program (NMDP) reveal intervals of 1–26 months (median 3–6 months, 75% within 4.9 months) elapsed between initiation of a donor search and transplantation for patients with ALL. Because of short remission durations—especially for patients with relapsed ALL—this delay, even if a histocompatible donor is identified, sometimes results in a subsequent relapse before the unrelated transplant can take place.

At the University of Minnesota, we undertook a prospective strategic approach to facilitate identification of an URD and to expedite all phases of the search for patients with ALL.[28] Because of the expected greater anti-leukemic efficacy, we indicated URD allografts as first choice for all patients and allowed four months from initiation of the search for identification and arranging the URD transplant. Referring centers were encouraged to initiate referral and URD search at the time of relapse and administrative procedures were abbreviated and organized to expedite prior authorization approval and all steps in the search process. Consecutive referrals over 2 years (n=115) of patients with ALL were analyzed to assess the feasibility of this comparative URD versus autoBMT approach. For the patients referred, 40 had a histocompatible sibling available and 30 related donor transplants took place at a median of 9 weeks (4 to 19) following initial referral. For those lacking a related donor, 58 URD searches were initiated and 22 donors identified after a median of 10 weeks (range 1 to 33 weeks). Seven patients became unavailable for URD transplant during the search process even though they had donors identified. Patients were unavailable because of progressive leukemia, patient choice or alternative transplant elsewhere. Fifteen URD transplants took place at a median of 17 weeks (range 2 to 29) following initial referral. Nineteen percent of patients (38% of those searched) were unable to receive either graft alternative because of disease progression while search and transplant arrangements were underway. Therefore, despite this aggressive administrative and

organizational effort to improve the application of either autoBMT or URD allotransplants for patients with ALL, only 55 of the original 115 patients had transplants at our center: 15 unrelated, 10 autologous and 30 related donor allogeneic.[28]

OUTCOMES FOLLOWING AUTOLOGOUS VERSUS URD TRANSPLANTS FOR ALL

To evaluate the differential toxicity, protection against relapse and disease-free survival following these two alternative transplant approaches, we analyzed the results of six years of consecutive autologous transplants from the University of Minnesota and the Dana Farber Cancer Institute (n=214) compared to the first six years of URD allogeneic transplants facilitated by the NMDP (n=337). All transplants were performed between September 1987 and August 1993 with a minimum of 18 months follow-up for all patients. The data sets were merged and analysis performed in the Biostatistical Facility of the University of Minnesota Bone Marrow Transplant Database. Univariate Kaplan-Meier estimates of three endpoints (relapse, transplant-related mortality [TRM] and disease-free survival [DFS]) were compared using logrank test statistics. Cox model multivariable analysis was performed to assess the impact of clinical prognostic factors (age, remission number, gender and type of transplant) on overall outcome.

Patients undergoing autografts were younger (75% <18 years) compared with URD recipients (64% <18). Additionally, the autologous transplants were more frequently performed in CR1 and CR2 (70%) compared with URD grafts where only 38% were CR1 and CR2, 28% CR3 or CR4 and 25% underwent URD transplantation in relapse. Over 60% of patients in both cohorts were male.

Transplant-related mortality was significantly more frequent in recipients of URD grafts (50% URD versus 16% autologous). Multivariate analysis identified younger age and transplantation in CR1 or CR2 as associated with lesser risks of TRM across both the autologous and URD cohorts. Surprisingly, however, TRM after either URD or autotransplantation (in CR2 as an example) were similar in adults and children (URD: >18, 48% TRM versus ≤18, 44%; autologous: >18, 9% versus ≤18, 15%).

Despite this higher transplant-associated mortality, URD transplantation led to significantly greater protection against relapse in all cohorts divided by age or remission status. Overall, 21% of URD recipients relapsed compared with 59% of the autologous recipients. Multivariate analysis identified lower relapse risk in males and patients transplanted in CR1 and CR2 compared with later remission or to relapse.

These discordant results between the two transplantation options combined to yield distinctive chances of DFS after transplant in ALL patient cohorts identified by

different clinical characteristics. For patients transplanted in CR1, autotransplantation was somewhat superior with 42% surviving disease-free compared with 32% for those undergoing URD transplantation. Conversely, a notably higher risk of post-transplant relapse in the autologous recipients transplanted in CR2 reversed this difference yielding 42% DFS for URD recipients in CR2 compared with only 20% DFS after autotransplantation. Results were similar using both transplant techniques in patients transplanted in later remission or in relapse, though the pattern of greater toxicity with the allograft and greater relapse after the autograft remained. Multivariate analysis of factors influencing disease-free survival identified superior DFS for recipients of URD transplantation, children, males and those transplanted in CR1 or CR2.

DISCUSSION

These critical, yet practical, factors—delay before transplantation, age, remission status, anti-leukemic efficacy and transplant-related mortality—that differ in meaningful ways between autologous and URD allogeneic transplantation must all be considered carefully in making clinical decisions regarding the superiority of one transplant technique or the other.[30] Previous series may have allocated patients with expected higher relapse risk and thus less able to tolerate delay to the autologous treatment, thus selecting poorer risk patients for this inherently less potent anti-leukemic therapy. Since some reports have highlighted leukemia-associated factors such as lower leukocyte count at diagnosis and longer initial remission duration as important predictors of post-autotransplant outcome,[14,17,18] these selection factors may underestimate the true value of autologous transplant therapy for patients with ALL.

In contrast, clinical factors expected to amplify the known higher transplant-related mortality of URD transplantation (age, prior infectious history, compromised organ function) might discourage application of URD transplantation for clinically high-risk patient groups. At the same time, the several month delay involved in identifying a donor selects out of URD series all patients with brief remissions and thus preferentially includes patients with longer remissions able to be sustained until a donor is identified. Thus, some component of the superior anti-leukemic therapy observed after URD transplantation might be attributable to this favorable selection factor as well.

While the logistics and limited availability of URDs may make a formal randomized trial unfeasible, additional focused analyses of these comparative techniques may further amplify issues relevant to the choice of transplantation therapy and improve application of these two promising techniques. Advances in the efficacy and safety of both techniques may also yield superior treatment options for the broad population of patients with ALL.

REFERENCES

1. Rivera GK, Pinkel D, Simone JV et al.: Treatment of acute lymphoblastic leukemia–30 years' experience at St. Jude Children's Research Hospital. *N Engl J Med* 329:1289–1295, 1993.
2. Linker CA, Levitt LW, O'Donnell M et al.: Treatment of adult acute lymphoblastic leukemia with intensive cyclical chemotherapy: A follow-up report. *Blood* 78:2814–2822, 1991.
3. Hoelzer D, Thiel E, Loffler H et al.: Prognostic factors in a multicenter study for treatment of acute lymphoblastic leukemia in adults. *Blood* 71:123–131, 1988.
4. Hussein KK, Dahlberg S, Head D et al.: Treatment of acute lymphoblastic leukemia in adults with intensive induction, consolidation, and maintenance chemotherapy. *Blood* 73:57–63, 1989.
5. Henze G, Fengler R, Hartmann R et al.: Six year experience with a comprehensive approach to the treatment of recurrent childhood acute lymphoblastic leukemia (ALL-REZ BFM 85). A relapse study of the BFM Group. *Blood* 78:1166–1172, 1991.
6. Barrett AJ, Horowitz MM, Pollack BH et al.: Bone marrow transplants from HLA-identical siblings as compared with chemotherapy for children with acute lymphoblastic leukemia in second remission. *N Engl J Med* 331:1253–1258, 1994.
7. Horowitz MM, Messerer D, Hoelzer D et al.: Chemotherapy compared with bone marrow transplantation for adults with acute lymphoblastic leukemia in first remission. *Ann Intern Med* 115:13–18, 1991.
8. Barrett AJ, Horowitz MM, Gale RP et al.: Marrow transplantation for acute lymphoblastic leukemia: Factors affecting relapse and survival. *Blood* 74:862–871, 1989.
9. Weisdorf DJ, Nesbit ME, Ramsay NKC et al.: Allogeneic bone marrow transplantation for acute lymphoblastic leukemia in remission: Prolonged survival associated with acute graft-versus-host-disease. *J Clin Oncol* 5:1348–1355, 1987.
10. Chao NJ, Forman SJ, Schmidt GM et al.: Allogeneic bone marrow transplantation for high-risk acute lymphoblastic leukemia during first complete remission. *Blood* 8:1923–1927, 1991.
11. Weisdorf DJ, Woods WG, Nesbit ME et al.: Allogeneic bone marrow transplantation for acute lymphoblastic leukemia: Risk factors and clinical outcome. *Br J Haematol* 86:62–69, 1994.
12. Ritz J, Ramsay NK, Kersey JH: Autologous bone marrow transplantation for acute lymphoblastic leukemia. In: SJ Forman, KG Blume ED Thomas (eds): *Bone Marrow Transplant*, Blackwell Scientific Publications Inc., 1994, pp 731–742.
13. Weisdorf DJ: Autologous bone marrow transplantation for acute lymphoblastic leukemia. In: Atkinson K (ed) Clinical Bone Marrow Transplantation. Cambridge: Cambridge University Press, 1994.
14. Ramsay NKC, LeBien T, Nesbit M et al.: Autologous bone marrow transplant for patients with acute lymphoblastic leukemia in second or subsequent remission: Results of bone marrow treated with monoclonal antibodies BA-1, BA-2, and BA-3 plus complement. *Blood* 66:508–513, 1985.
15. Simonsson B, Burnett AK, Prentice HG et al.: Autologous bone marrow transplantation with monoclonal antibody purged marrow for high risk acute lymphoblastic leukemia. *Leukemia* 3:631–636, 1989.
16. Herve P, Labopin M, Plouvier E et al.: Autologous bone marrow transplantation for child-

hood acute lymphoblastic leukemia—a European survey. *Bone Marrow Transplant* 8(suppl):72–75, 1991.

17. Parsons SK, Castellino SM, Lehmann LE et al.: Relapsed acute lymphoblastic leukemia: Similar outcomes for autologous and allogeneic marrow transplantation in selected children. *Bone Marrow Transplant* 17:763–768, 1996.
18. Uckun FM, Kersey JH, Haake R et al.: Autologous bone marrow transplantation in high-risk remission B-lineage acute lymphoblastic leukemia using a cocktail of three monoclonal antibodies (BA-1/CD24, BA-2/CD9, and BA-3/CD10) plus complement and 4-hydroperoxy-cyclophosphamide for ex vivo bone marrow purging. *Blood* 79:1094–1104, 1992.
19. Uckun F, Kersey J, Haake R et al.: Pretransplantation burden of leukemic progenitor cells as a predictor of relapse after bone marrow transplantation of acute lymphoblastic leukemia. *N Engl J Med* 93:1296–1301, 1993.
20. Soiffer RJ, Roy DC, Gonin R et al.: Monoclonal antibody-purged autologous bone marrow transplantation in adults with acute lymphoblastic leukemia. *Bone Marrow Transplantation* 12:243–251, 1993.
21. Kernan NA, Bartsch G, Ash RC: Analysis of 462 transplantations from unrelated donors facilitated by the National Marrow Donor Program. *N Engl J Med* 328:593–602, 1993.
22. Beatty PG, Hansen JA, Longton GA et al.: Marrow transplantation from HLA-matched URDs for treatment of hematologic malignancies. *Transplantation* 51:443–447, 1991.
23. Davies SM, Shu XO, Blazar BR et al.: Unrelated donor bone marrow transplantation: Influence of HLA A and B incompatibility on outcome. *Blood* 86:1636–1642, 1995.
24. Bearman SI, Mori M, Beatty PG et al.: Comparison of morbidity and mortality after marrow transplantation from HLA-genotypically identical siblings and HLA-phenotypically identical unrelated donors. *Bone Marrow Transplant* 13:31–35, 1994.
25. Ochs L, Shu XO, Miller J et al.: Late infections following allogeneic bone marrow transplantation: Comparison of incidence in related and unrelated donor transplant recipients. *Blood* 86:3979–3986, 1995.
26. Ash RC, Casper JT, Chitambar CR et al.: Successful allogeneic transplantation of T-cell-depleted bone marrow from closely HLA-matched unrelated donors. *N Engl J Med* 322:485-494, 1990.
27. Horowitz MM, Gale RP, Sondel PM et al.: Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* 75:555-562, 1990.
28. Davies SM, Ramsay NKC, Weisdorf DJ: Feasibility and timing of unrelated donor identification for patients with ALL. BMT 17:737–740, 1996.
29. Stroncek D, Bartsch G, Perkins HA et al.: The National Marrow Donor Program. *Transfusion* 33:567–577, 1993.
30. Busca A, Anasetti C, Anderson G et al.: Unrelated donor or autologous marrow transplantation for treatment of acute leukemia. *Blood* 83:3077–3084, 1994.

THE ROLE OF BONE MARROW TRANSPLANTATION IN THE MANAGEMENT OF PH-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA IN ADULTS

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Despite improvement in the cure rate of adult acute lymphoblastic leukemia (ALL) there are still subgroups with a poor prognosis. The most inferior outcome is observed for adult and childhood patients with a Philadelphia chromosome (Ph) or the corresponding molecular rearrangement *BCR-ABL*.[1–3] The incidence of Ph positive ALL increases with age. Whereas in children only 3% to 5% are Ph positive, the frequency rises to approximately 22% (weighted mean, range 9% to 33%) in adult ALL. At the molecular level the translocation t(9;22) is heterogeneous in ALL with respect to the breakpoints in the *BCR* gene. In adults, about one-third show a breakpoint in *m-BCR*, resulting in an 8.5 kb chimeric *BCR-ABL* messenger RNA and a 210-kD protein, $p210^{BCR\text{-}ABL}$, whereas two-thirds show breaks in the *m-BCR* leading to a 7.4 kb *BCR-ABL* messenger RNA and a 190-kD protein, $p190^{BCR\text{-}ABL}$.

RESULTS

Chemotherapy

With recent intensive induction regimens a complete remission (CR) rate of approximately 70% (range 44–86%) could be obtained in several protocols with a total of more than 600 Ph-positive adult ALL patients.[4] However, the leukemia-free survival (LFS) in almost all studies is very poor, with about 6% (weighted mean, range 0 to 16%). Thus, there is a discrepancy between the relatively high remission rate, which is only somewhat lower than the CR rates obtained for Ph-negative adult ALL patients (70–90%), and the great difference in outcome of <10% in Ph/*BCR-ABL* positive ALL versus 30 to 40% in the latter group. One of the reasons is that most of the Ph/*BCR-ABL* positive ALL patients with a complete remission in bone marrow and peripheral blood by morphological criteria are not in CR when investigated by cytogenetic or molecular methods. Thus, in several studies there was a wide discrepancy between CR defined morphologically and the detection of the *BCR-ABL* rearrangement by polymerase chain reaction (PCR) analysis. The finding of a positive *BCR-ABL*

bone marrow in patients with morphologic CR varied from 18%[5] to all patients after induction therapy.[6] This variation is unexplained, particularly since the cytostatic drugs and regimens used in these studies are very similar, but small patient numbers or a different sensitivity of detection techniques may contribute. From the data it also remains unclear whether a particular regimen is superior to others or whether recent high-dose regimens such as high-dose cytosine arabinoside or high-dose methotrexate have an effect on the long-term outcome in adult Ph-positive ALL. Thus, from the poor results with chemotherapy alone it is unequivocally accepted that all patients with Ph-positive ALL, adults as well as children, are candidates for bone marrow transplantation (BMT) in first complete remission.

Bone marrow transplantation

The fact that the majority of Ph-positive ALL patients after induction are not in molecular remission not only signifies a poorer outcome after chemotherapy alone—since all patients eventually relapse but may also have an adverse impact for BMT. Thus, if this residual disease cannot be eliminated by the conditioning regimen, by a possible graft-versus-leukemia (GVL) effect or by maintenance therapy, a higher relapse rate is to be expected.

Allogeneic BMT

In the first published series by the Californian group,[7,8] an encouraging survival rate of 42% was achieved with allogeneic BMT (alloBMT) for adult Ph-positive ALL patients in first CR (CR1). A recent update by this group demonstrated that these cures are durable with a LFS of 46% at up to 10 years.[9] In a retrospective analysis of the largest series of alloBMT in adult Ph/*BCR-ABL*-positive ALL patients by the IBMTR,[10] good results were also obtained with a survival of 38% in CR1 patients and encouraging results, also, for patients beyond CR1 (Table 1).[9–22] Similar or even superior results were published in recent years from smaller series of adult Ph/*BCR-ABL*-positive ALL patients.[11,16,18] However, in a prospective multicenter adult ALL trial in which all adult Ph/B*CR-ABL* positive patients were considered for a BMT in CR1, the results are not as encouraging and at least the intent-to-treat analysis including all adult patients with a donor are inferior.[4] Also, the results for alloBMT in second CR (CR2), with a LFS of 34% are promising (Table 1) when compared with chemotherapy. The lower LFS rate after allogeneic transplantation in CR1, CR2 or later stages compared with that for Ph/*BCR-ABL*-negative ALL is due to a higher relapse incidence possibly explained by the high proportion of patients not in molecular CR at transplantation and higher treatment-related mortality. The latter is probably not disease-related but may be due to the significantly higher age of these patients.

Table 1. Results of bone marrow/PBSC transplantation in adult Ph/*BCR-ABL*-positive ALL patients

	N	*Treatment-related mortality*	*Relapse incidence*	*LFS*
allogeneic				
CR1	75	32%	34%	40%
CR2 or more	49	35%	40%	34%
relapsed/refractory	24	47%	66%	17%
autologous				
CR1	37	17%	44%	37%
CR2	18	9%	73%	13%
mismatched/unrelated donor	17*			68%

**Data from Refs. 9–22.*

Autologous BMT

Autologous bone marrow transplantation (autoBMT) has been performed in several series, mostly with small numbers of patients. The results are summarized in Table 1. For the autotransplanted patients in CR1, the LFS rate is 37% and thus not inferior to the results for allogeneic BMT in CR1. However, observation time in several of these series is short and a large proportion is still Ph/*BCR-ABL*-positive and thus may eventually relapse. The relapse rate is higher than after allogeneic BMT and the treatment related mortality, as is to be expected, is significantly lower. Overall, the results of autoBMT in CR1 and in CR2 are also superior to those which can be obtained by chemotherapy alone. In several series purging of bone marrow was used either with MoAbs + complement +4HC,[12] MoAbs (CD10) + complement[13] or MoAbs + magnetic beads.[20] It is difficult to draw conclusions as to whether the survival rates in these series (9%, 80%, 30%) are better than those in the series without purging. However, it was demonstrated by RT-PCR that the tumor burden in the transplant can be reduce; e.g., a 3-log depletion of Ph/*BCR-ABL*-positive cells was obtained by means of immunomagnetic bead purging using a cocktail of anti-CD10, -CD10 and -AB4 mAbs although completely RT-PCR negative grafts could not be achieved by purging of bone marrow.[4]

Autologous peripheral blood stem cell transplantation (PBSCT)

In Ph/*BCR-ABL*-positive ALL patients in first complete remission—defined by conventional morphological criteria—the level of residual *BCR-ABL* cells found in

peripheral blood was 1–2 log less than in bone marrow samples.[18,19] This offers the possibility of collecting peripheral blood stem cells after mobilization with a lower Ph/*BCR-ABL* blast cell contamination. Thus, in the study by Carella,[19] five of 10 PBSC harvests were cytogenetically Ph-negative in contrast to bone marrow, which was positive in all patients. However, the samples were tested by cytogenetic analysis only and may have still been *BCR-ABL* positive, as suggested by the fact that all except one patient relapsed. Also, in a Belgian study,[18] three of seven patients had *BCR-ABL* negative peripheral blood without purging. The lower tumor load in the peripheral blood cells seems, however, a better starting point to achieve a Ph/*BCR-ABL*-negative transplant by purging. Thus, in a study by Martin[20] the tumor load could be reduced to undetectable levels in four patients. Whether the *BCR-ABL* negative peripheral blood transplants that can now be obtained also result in better survival remains open, since very few patients have been transplanted in this setting. These studies, however, suggest that the relapse may be mainly caused by the leukemic cells remaining in the patient. Thus, one can focus on better eradication of the tumor load in the patient.

Unrelated matched or mismatched BMT

Unrelated matched or mismatched BMT is an alternative therapeutic approach to autologous transplantation for patients without an HLA-related compatible sibling. The few results reported so far for Ph/*BCR-ABL*-positive ALL patients are encouraging (Table 1) with LFS rates up to 68%. This is true for Ph/*BCR-ABL*-positive ALL children reported in these series. The number of Ph/*BCR-ABL*-positive ALL adults so far reported for unrelated BMT is surprisingly low and it remains to be seen whether the results are similarly encouraging as in children. These promising results for unrelated BMT in children may to some extent be the result of patient selection and must be confirmed in larger numbers of patients. The reason for its superiority to allogeneic HL-A,B-DR sibling BMT, and particularly autoBMT, may be that in the unrelated setting a more potent GVL reaction is responsible for the eradication of Ph/*BCR-ABL*-positive leukemic blast cells.

Maintenance therapy

Since minimal residual disease is observed in most cases after autoBMT and in single cases after alloBMT, a maintenance approach with α-interferon alone or combined with interleukin-2 (IL-2) has been applied. The rationale to use α-interferon in the treatment of Ph/*BCR-ABL*-positive ALL patients is based on similarities to CML in which α-interferon can give hematological remissions and occasionally cytogenetic remissions. Alpha-interferon has been successful in only a few patients with Ph/*BCR-ABL*-positive ALL.[23,24,25] In a single case when α-interferon was given after unrelated alloBMT,[22] LFS was sustained. In the autologous BMT setting when α-interferon combined with IL-2 was used as

maintenance, the results were discouraging. Although an in vitro immuno response[26] could be obtained there was apparently no benefit from α-interferon and IL-2 in terms of LFS.

CONCLUSIONS

In adult Ph/*BCR-ABL*-positive ALL bone marrow/PBSC transplantation is at present the only curative option. All adults in first CR or even in a good partial remission are candidates for alloBMT. As the majority lack a matched sibling, autologous or unrelated BMT are therapeutic options. Since in the autologous setting the tumor load is lower in peripheral blood and negative autografts can be obtained by purging, PBSCT might be superior to autoBMT. Unrelated BMT has promising results in Ph/*BCR-ABL*-positive ALL in children that may be due to a GVL effect. The very small number of patients reported with unrelated BMT in Ph/*BCR-ABL*-positive ALL suggests selection and the remaining question is how to compare the efficacy of autologous BMT versus unrelated BMT. For practical reasons it might be more realistic to use stratification instead of a randomized comparison. Autologous transplantation is a more suitable option for the large proportion of elderly patients than an unrelated approach. Furthermore, due to the time elapsed for an unrelated donor search and the high early relapse rate, an autologous BMT seems advisable if a suitable donor cannot be found in the first three months after achievement of CR. Thus, the practical approach at present in several study groups is to prepare for autologous transplantation and to search for a donor in parallel. If a donor is found within three months the patient would undergo an unrelated BMT, but if the search is unsuccessful, the patient would proceed with an autologous transplant.

REFERENCES

1. Bloomfield CD, Secker-Walker LM, Goldman AL et al.: Six-year follow-up of the clinical significance of karyotype in acute lymphoblastic leukemia. *Cancer Genet Cytogenet* 40:171–185, 1989.
2. Hoelzer D: Treatment of acute lymphoblastic leukemia. Semin Hematol 31:1–15, 1994.
3. Cortes JE, Kantarjian HM: Acute lymphoblastic leukemia. A comprehensive review with emphasis on biology and therapy. *Cancer* 76:2393–2417, 1995.
4. Hoelzer D, Martin H, Atta J: In preparation.
5. Nickelsen M, Schoch R, Schafhausen P et al.: Reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of chromosomal abnormalities in patients with t(9;22), t(15;17), t(8;21) and inv(16) at diagnosis and in remission. *Br J Haematol* 93(S2):204, 1996.
6. van Rhee F, Marks DI, Lin F et al.: Quantification of residual disease in Philadelphia-positive acute lymphoblastic leukemia: Comparison of blood and bone marrow. *Leukemia* 9:329–335, 1995.

7. Forman SJ, O'Donnell MR, Nademanee AP et al.: Bone marrow transplantation for patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood* 70:587–588, 1987.
8. Blume KG, Schmidt GM, Chao NJ et al.: Bone marrow transplantation for acute lymphoblastic leukemia. In: Gale RP and Hoelzer D (eds.) *Acute Lymphoblastic Leukemia*, Alan R Liss, Inc. pp. 279–283, 1990.
9. Chao NJ, Blume KG, Forman SJ, Snyder DS: Long-term follow-up of allogeneic bone marrow recipients for Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood* 85:3353–3354, 1995.
10. Barrett AJ, Horowitz MM, Ash RC et al.: Bone marrow transplantation for Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood* 79:3067–3070, 1992.
11. Gehly GB, Bryant EM, Lee AM et al.: Chimeric BCR-abl messenger RNA as a marker for minimal residual disease in patients transplanted for Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood* 78:458–465, 1991.
12. Brennan C, Weisdorf D, Kersey J et al.: Bone marrow transplantation (BMT) for Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph'+ALL). *Proc ASCO* 10:222, 1991.
13. Miyamura K, Tanimoto M, Morishima Y et al.: Detection of Philadelphia chromosome-positive lymphoblastic leukemia by polymerase chain reaction: Possible eradication of minimal residual disease by marrow transplantation. *Blood* 79:1366–1370, 1992.
14. Mandelli F, Annino L, Ciolli S et al.: Philadelphia chromosome (Ph 1) positive acute lymphoblastic leukemia (ALL) in adult: Interim results of GIMEMA ALL 0288 pilot study. *Blood* 82(suppl 1):58a, 1993.
15. Preti HA, O'Brien S, Giralt S et al.: Philadelphia chromosome-positive adult acute lymphocytic leukemia: Characteristics, treatment results, and prognosis in 41 patients. *Am J Med* 97:60–65, 1994.
16. Stockschläder M, Hegewisch-Becker S, Krüger W et al.: Bone marrow transplantation for Philadelphia chromosome-positive acute lymphoblastic leukemia. *Bone Marrow Transplant* 16:663–667, 1995.
17. Dunlop LC, Mehta J, Treleaven J, Powles R: Results of bone marrow transplantation in Philadelphia chromosome-positive acute lymphoblastic leukaemia. *Br J Haematol* 89(Suppl 1):18, 1995.
18. Stryckmans P, Marie JP, Muus P et al.: Evaluation of the quality of peripheral blood progenitors (PBP) for autologous transplantation in adult Philadelphia positive (Ph+) acute lymphoblastic leukemia (ALL): A pilot study. *Blood* 86(S1):783a, 1995.
19. Carella AM, Frassoni F, Pollicardo N et al.: Philadelphia-chromosome-negative peripheral blood stem cells can be mobilized in the early phase of recovery after a myelosuppressive chemotherapy in Philadelphia-chromosome-positive acute lymphoblastic leukaemia. *Br J Haematol* 89:535–538, 1995.
20. Martin H, Atta J, Zumpe P et al.: Purging of peripheral blood stem cells yields BCR-ABL-negative autografts in patients with BCR-ABL-positive acute lymphoblastic leukemia. *Exp Hematol* 23:1612-1618, 1995.
21. Casper J, Camitta B, Ash R et al.: Bone marrow transplantation for Philadelphia chromosome positive (Ph+) acute lymphocytic leukemia (ALL) using alternative donors. *Blood*

80(Suppl 1):65a, 1992.

22. Klemperer MR, Barbosa JL, Grana N et al.: Successful interferon-α (INF-α) therapy of Philadelphia chromosome (Ph+) acute lymphoblastic leukemia (ALL) in molecular relapse after matched unrelated bone marrow transplantation (BMT). *Blood* 84(S1):712a, 1994.
23. Ohyashiki K, Ohyashiki JH, Tauchi T et al.: Treatment of Philadelphia chromosome-positive acute lymphoblastic leukemia: A pilot study which raises important questions. *Leukemia* 5:611–614, 1991.
24. Haas OA, Mor W, Gadner H et al.: Treatment of Ph-positive acute lymphoblastic leukemia with α-interferon. *Leukemia* 2:555, 1988.
25. Harousseau JL, Guilhot P, Casassus P et al.: Treatment of Philadelphia chromosome positive ALL with interferon alpha. *Haematology and Blood Transfusion* 37:240–242, 1996.
26. Martin H, Bergmann L, Bruecher J et al.: Immunotherapy with IL-2 and α-IFN after autologous BMT in patients with BCR-ABL-positive ALL. *Ann Hematol* 68 (Suppl 1):A40, 1994 (abstr).

CHAPTER 3
CML

MOBILIZATION AND AUTOGRAFTING OF PHILADELPHIA-NEGATIVE PERIPHERAL BLOOD PROGENITOR CELLS EARLY IN CHRONIC MYELOGENOUS LEUKEMIA

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INTRODUCTION

In patients who cannot receive allografting, the only chance to cure chronic myelogenous leukemia (CML) relies almost entirely on the presence of normal progenitor cells in the leukemic cell population. The first evidence that Ph-negative progenitors exist was demonstrated in vitro by the cytogenetic analysis of hematopoietic colonies grown from CML marrow cells.[1] More recently, the persistence of normal hematopoiesis in CML was provided by the experience with interferon-alpha (IFN-α)[2] and by mobilization of Ph-negative progenitors into the blood of CML patients in the early phase of recovery after chemotherapy.[3] These observations suggest that normal progenitor cells may be suppressed, and that after the debulking of neoplastic cells by interferon or chemotherapy, normal cells re-emerge and repopulate the marrow.

This paper summarizes the results with mobilization therapy and transplantation of Ph-negative peripheral blood progenitor cells (PBPC) in 26 patients in chronic phase in the first weeks from diagnosis and not pretreated with IFN-α.

METHODS

Between May 1993 and October 1996, 26 patients with CML were entered on our treatment protocol, all within 12 months of diagnosis. All but three patients had 100% Ph-positive metaphases in marrow cells; one had 90% and two had 80%. All patients had received prior hydroxyurea only. This protocol was begun after the white blood cell count was reduced by hydroxyurea to $<20\times10^9$/L. The

treatment regimen in 15 patients was idarubicin 8 mg/M^2/days on day 1-5, arabinosylcytosine 800 mg/M^2 by 2-hour infusion on days 1–5 and etoposide 150 mg/M^2/day by 2-hour infusion on days 1–3 (ICE protocol). In the last 11 patients we used the mini-ICE protocol (3 days instead of 5 days therapy) and all were initially managed in an outpatient setting. All patients received a prophylactic antibiotic regimen consisting of IV ceftazidime, netilmicine and fluconazole beginning when the white count (WBC) was less than 2×10^9/L after chemotherapy. Anti-emetic treatment with ondansetron was given by continuous infusion beginning 12 hours before starting the ICE or mini-ICE protocols through the last day of therapy. The patients treated with mini-ICE entered our Department when WBC was $\leq2\times10^9$/L after chemotherapy and all were managed in single rooms.

Beginning 8 days after the end of chemotherapy, rh-G-CSF was administered daily until the total neutrophil count was greater than 1.0×10^9/L for three consecutive days. Leukaphereses were performed generally when the WBC exceeded 0.8×10^9/L combined with the appearance in the peripheral blood of CD34$^+$ cells rising above our threshold of detection (>0.05% of viable mononuclear cells). The next leukaphereses were mostly performed when the WBC reached 1.0×10^9/L and the procedure was performed daily until the total CD34$^+$ cells collected were at least $\geq2\times10^6$/kg, which usually occurred when the WBC exceeded 3.0×10^9/L. In some patients WBC recovery after mobilizing chemotherapy was so rapid that the first leukapheresis was performed when white count was well over 0.8×10^9/L. The leukapheresis product was analyzed each day for the number of mononuclear cells, CFU-GM, CD34$^+$ cells and cytogenetics. Samples of each product were later analyzed for LTC-IC in 19 patients, and CD34$^+$Thy1$^+$Lin$^-$ cells; the latter was possible in 18 of the patients and was performed using four-color flow cytometry.[4] The total leukapheresis product for each patient was frozen using the Planer R-203 instrument. When the peripheral blood count had fully recovered a sample of bone marrow was evaluated for cytogenetic analysis.

RESULTS

All 26 patients completed the mobilization protocol. All patients achieved nadir white counts of $<0.5\times10^9$/L and platelets $<20\times10^9$/L. Toxicity was graded according to the standard WHO criteria and consisted mainly of mild mucositis (grade 1–2) and diarrhea (grade 2) in the majority of patients treated with ICE protocol. Two had grade 3 mucositis and diarrhea. In contrast to those on the ICE protocol, the patients treated with mini-ICE tolerated the therapy very well and only mild mucositis was seen in two. Nausea and vomiting were well controlled with our anti-emetic regimen.

White count recovery was adequate to initiate leukapheresis at 12 to 28 days (median 17 days); in no patient did it take longer than 4 weeks to recover baseline blood counts. The Philadelphia chromosome was absent in PBPC in 16 of the 26 patients on repeated sampling; in the other ten patients, Ph-positive PBPC ranged from 2% to 100% (median, 26%). In this small series of patients the stratification according to Sokal index was not predictive for cytogenetic response.

Bone marrow analyzed on recovery in 19 patients showed no decrease in Ph-positive metaphases in nine patients and decreases to 15–40% Ph-positive metaphases in four, 5–10% metaphases in five and no evidence of Ph-positive metaphases in one patient.

AUTOTRANSPLANT RESULTS

To date, 16 patients have been autografted: 13 patients with entirely Ph-negative PBPC and three other patients with minimal Ph-positive contamination. The ages of the autografted patients ranged from 30 to 60 years. The preparative regimen was idarubicin 50 mg×1 (day –11) and etoposide 800 mg/M^2/day×2 (days –8 and –7) followed by single-dose total body irradiation (6 patients) or high-dose busulfan (4 mg/kg/daily for 4 days) (10 patients). The minimum number of progenitor cells we required to proceed with autografting was: CFU-GM $\geq 2\times10^4$/kg and CD34$^+$ $>2\times10^6$/kg. Evidence of marrow engraftment (ANC $>0.5\times10^9$/L and platelets $>25\times10^9$/L) was attained in all patients in a median of 13 days (range 9–17) and 17 days (range 8–106), respectively. After engraftment, IFN-α was begun earlier after transplant and given daily for five days per week together with IL-2 at 2×10^6 daily for five days every eight weeks. Recombinant interleukin-2 (rIL-2) has been demonstrated to have an antitumor effect in several animal models and pilot clinical trials of leukemia.[5–7]

Cytogenetic analysis of bone marrow samples was performed approximately 2 months after infusion, and follow-up analyses were performed every 2 months. Seven patients continue in full cytogenetic remission at 2 to 24 months (median, 17 months) after Ph-negative PBPC infusion and two other patients maintain major cytogenetic remission at 12 and 15 months after autografting. No patient has experienced late graft failure or has required a second transplant.

DISCUSSION

This pilot study involved patients given chemotherapy early in CML without prior exposure to interferon therapy. Regimen-related toxicity during the mobilization phase was low, particularly with the mini-ICE protocol; hematopoietic recovery was rapid, and a higher yield of Ph-negative and progenitor cells was obtained in comparison to patients who had previously received interferon, partic-

ularly for more than one year. In the apheresis product, significant numbers of $CD34^+$ $Thy1^+$ Lin^- cells and LTC-IC were found only in those patients with entirely Ph-negative collections. Considering that 20% of IFN-α pretreated patients experienced relevant nonhematological toxicity, the ICE protocol has been recently modified by reducing the days of treatment from 5 to 3 (mini-ICE protocol) in the attempt to reduce such toxicity in this group of patients. No differences in cytogenetic response or progenitor yield were found between the ICE and mini-ICE protocols, but in the latter no patient mobilized at diagnosis experienced relevant nonhematological toxicity; besides, the median duration of hospitalization was shorter in mini-ICE (ICE 30 days; mini-ICE 17 days, $p=0.001$).[8]

There is limited experience to date using Ph-negative cells in an attempt to increase the curative potential of autografting in CML. Approaches to obtaining such cells have involved the use of repeated courses of intensive chemotherapy, in vitro purging or long-term culture. These promising studies have been limited to selected patients to date; in two studies only 25% of candidates were ultimately eligible for transplant.[9,10] With investigative approaches, the risk of graft failure may be significant.[9,10] All 16 patients transplanted in our preliminary study experienced marrow engraftment, suggesting that the chance of graft failure is minimized with cells mobilized with this approach. Graft failures reported by other groups occurred most often in patients transplanted after the first year.[10,11] Our experience suggests that in previously treated patients, mobilization of Ph-negative cells is still possible, but that lower numbers of progenitors may be obtained. Though we do not have quantitation of LTC-IC or $CD34^+$ $Thy1^+$ Lin^- cells in these patients, we have noted the increased risk of slow count recovery from chemotherapy, and poor engraftment after transplant. The correlation of interferon treatment with slower engraftment was also noted by the European Bone Marrow Transplant Group.[9] The minimum number of precursor cells necessary to ensure engraftment in man is not yet known, but our data for LTC-IC and $CD34^+$ $Thy1^+$ Lin^- cells suggest that high levels of precursor cells may be mobilized early in the disease, and that there is durable engraftment with the numbers of cells we infused.

There has been predominantly Ph-negative hematopoiesis in many of our patients for months post-grafting and with no hematologic relapse to date, although follow-up is short. We, as most groups, have implemented post-graft immunotherapy based on the likelihood of minimal residual disease. Its role, as well as the best preparative regimen for transplant (TBI or alkylating agents), cannot be determined in a pilot study of this size. Combination therapies of rIL-2 and interferon have been shown to function synergistically in augmenting cytolytic activity in mice, both in vitro and in vivo.[12–14] Both rIL-2 and IFN-α were able to increase the suppressed NK cytolytic activity of lymphocytes isolated from CML patients.[15,16] In vitro, the combination of both cytokines had a synergistic effect and potentiated NK-mediated cytotoxicity in CML patients to normal levels.[17] The

efficacy of this combination was recently confirmed in 10 patients in chronic and advanced phase of CML.[18]

In conclusion, our data suggest that early mobilization of peripheral blood progenitor cells is a technique worth developing further. Morbidity was lowest and higher numbers of precursors were obtained in patients treated within the first year of diagnosis, without receiving prior interferon therapy.

REFERENCES

1. Chevernick PA, Ellis LD, Pan SF et al.: Human leukemic cells: In vitro growth of colonies containing the Philadelphia chromosome. *Science* 174:1134, 1971.
2. Giralt S, Kantarjian H, Talpaz M: The natural history of chronic myelogenous leukemia in the interferon era. *Semin Hematol* 32:152, 1995.
3. Carella AM, Podestà M, Frassoni F et al.: Collection of 'normal' blood repopulating cells during early hemopoietic recovery after intensive conventional chemotherapy in chronic myelogenous leukemia. *Bone Marrow Transplant* 12:267, 1993.
4. Murray L, Chen B, Galy A et al.: Enrichment of human hematopoiesis stem cell activity in the $CD34^+$ $Thy1^+$ Lin^- subpopulation from mobilized peripheral blood. *Blood*, 85:368, 1995.
5. Ackerstein A, Kedar E, Slavin S: Use of recombinant interleukin-2 in conjunction with syngeneic bone marrow transplantation in mice as a model for control of minimal residual disorders. *Blood* 78:1212, 1991.
6. Foa R, Fierro MT, Tosti S et al.: Induction and persistence of complete remission in a resistant acute myeloid leukemia padent after treatment with recombinant interleukin-2. *Leuk Lymphoma* 1:113,1990.
7. Slavin S, Ackerstein A, Weiss L: Adoptive immunotherapy in conjunction with bone marrow transplantation-amplification of natural best defence mechanisms against cancer by recombinant IL-2. *Nat Immun Cell Growth Regul* 7:180, 1988.
8. Carella AM, Frassoni F: ICE, mini-ICE or high dose hydroxyurea to mobilize Philadelphia (Ph1)-negative PBPC in chronic myelogenous leukemia. *Br J Haematol* 95:212, 1996.
9. Reiffers J, Goldman J, Meloni G.: Autologous stem cell transplantation in chronic myelogenous leukemia: A retrospective analysis of the European Group for Bone Marrow Transplantation. *Bone Marrow Transplant* 14:(Suppl. 3):51, 1994.
10. Barnett MJ, Eaves CJ, Phillips GL et al.: Autografting with cultured marrow in chronic myeloid leukemia: Results of a pilot study. *Blood* 84:724, 1994.
11. Hoyle C, Gray R, Goldman J: Autografting for patients with CML in chronic phase: an update. *Brit J Haematol* 86:76, 1994.
12. Brunda MJ, Tarnowsld D & Davatelis V: Interaction of recombinant interferons with recombinant interleukin-2. Differential effects on natural killer cell activity and interleukin-2 activated killer cells. *Int J Cancer* 37:787, 1986.
13. Igio M, Sakurai M, Tamura T et al.: In vivo anti-tumor activity of multiple injections of recombinant interleukin-2, alone and in combination with three different types of recombinant interferon, on various syngeneic murine tumors. *Cancer Res* 48:260, 1988.
14. Kuribayashi K, Gillis S, Kern DE et al.: Murine NK cell cultures: effects of interleukin-2 and interferon on cell growth and cytotoxic activity. *J Immunol* 126:2321, 1981.

15. Verfaillie C, Kay N, Miller W et al.: Diminished A-LAK cytotoxicity and proliferation accompany disease progression in chronic myelogenous leukemia. *Blood* 76:401, 1990.
16. Hauch M, Gazzola MV, Small T et al.: Anti-leukemia potential of interleukin-2 activated natural killer cells after bone marrow transplantation for chronic myelogenous leukemia. *Blood* 75:2250, 1990
17. Dabbolkar M, Tatake R, Amin K et al.: Modulation of natural killer and antibody-dependent cellular cytotoxicity by interferon and interleukin-2 in chronic myeloid leukemia patients in remission. *Oncology* 46:123, 1989.
18. Nagler A, Ackerstein A, Barak V et al.: Treatment of chronic myelogenous leukemia with rhIL-2 and interferon-α-2a. *J Hematother* 11:31, 1994.

MECHANISMS OF IMMUNE ESCAPE: STUDIES IN LEUKEMIC RELAPSE AFTER BMT

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ABSTRACT

The treatment of leukemia by an allogeneic bone marrow stem cell transplant (alloBMT) represents a unique opportunity to study and exploit clinically the alloreaction of the donor immune system against a malignant process. Although in vitro experiments and animal studies implicate T cells and natural killer (NK) cells, the in vivo graft-versus-leukemia (GVL) reaction remains poorly characterized. In patients receiving BMT for chronic myelogenous leukemia (CML), there is clinical and experimental evidence for $CD4^+$ T lymphocyte suppression of leukemia by cytokine release and by direct killing of $CD34^+$ leukemia cells through the fas (CD95) pathway. The reasons for leukemic relapse after BMT are not well defined. Relapse may occur when there is defective immune control of residual leukemia, or when, by clonal evolution, the leukemia escapes control. While several lines of evidence point to a relationship between relapse and immune competence after BMT, we have recently found that reduced immunogenicity is a frequent feature of leukemia relapsing after BMT. Inability of the leukemia to stimulate T cell responses and a reduced susceptibility to T cell cytotoxicity were associated with downregulation of surface molecules critical for stimulator/responder and effector/target cell interactions. Further definition of the relapse process after BMT should improve our ability to use combinations of T cell therapy and cytokines to prevent and treat the reemergence of leukemia after BMT.

INTRODUCTION

Although it is now clear that the graft-versus-leukemia (GVL) effect plays a central role in the cure of leukemia following allogeneic bone marrow transplantation (alloBMT), little is known about the way the immune system interacts with the residual leukemia that escapes destruction by the myeloablative preparative regimen. Knowledge from in vitro studies in man and from animal transplant experiments indicates that natural killer (NK) cells, $CD4^+$ and $CD8^+$ T cells can all be GVL effectors, but the details have not been elucidated. Whether a leukemia cure (defined

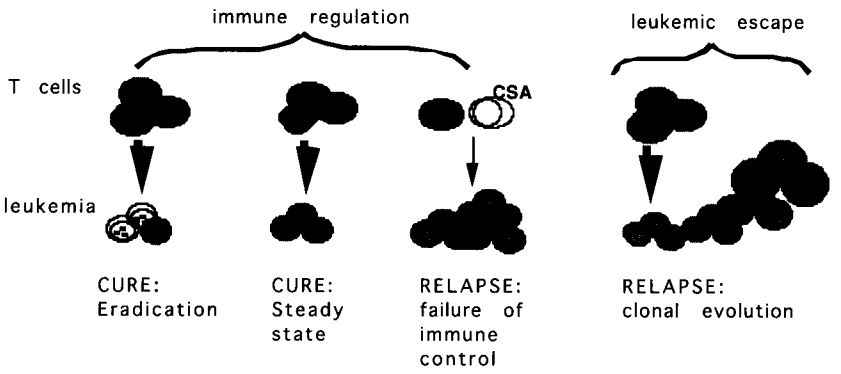

Figure 1. *Interaction of donor immune system with residual leukemia.*

as greater than 5 years leukemia-free survival) is achieved by once-and-for-all eradication of the last malignant cell, or whether leukemia is suppressed but never completely eliminated is not known. This latter process would require maintenance of cure by a lifelong persistence of GVL immunity. There are clinical and experimental data to support both possibilities.[1] We know even less about the reasons for relapse post-transplant. The several possible scenarios are as follows (Figure 1):

1) Failure of the immune system at any time to effectively control residual disease. There are unfortunately numerous examples of failure to cure high risk and advanced malignancies despite the use of T cell replete transplants or donor lymphocyte transfusions (DLT) to treat post-transplant relapse. Such leukemias appear to be uncontrolled by even the most powerful alloreaction.

2) A decrease in donor immune competence leading to escape of residual disease. The interaction of immunosuppressive agents with relapse (discussed below) illustrates the existence of a balance between the immune system and leukemia.

3) Clonal selection in residual leukemia. Leukemia mutations less susceptible to suppression by the donor immune system would be automatically favored.

We describe here studies (largely in allograft recipients with chronic myelogenous leukemia [CML]) to further elucidate interactions of allogeneic lymphocytes with leukemia. Our results emphasize a role for $CD4^+$ T cells in the GVL reaction and suggest that immune escape is a common reason for leukemic relapse after BMT. Characterizing deficiencies in the stimulatory and target properties of leukemia cells should improve rational approaches to prevention and treatment of relapse after BMT by combining DLT with cytokines to improve leukemia cell susceptibility.

RESULTS

Interaction of alloreacting CD4+ T cell clones and CML

There is growing evidence that $CD4^+$ lymphocytes play a central role in the GVL reaction after allogeneic bone marrow transplantation for CML, not only as initiators of the immune response but also as effectors of GVL[1]. Leukemia cells from a patient with CML were used to generate $CD4^+$ $CD8^-$ T cell lines from an unrelated responder sharing HLA-A2 and -DR4 with the stimulator.[2] Clones showed specificity for the patient's leukemia and unresponsiveness to K562 cells and third party CML cells. Some clones showed preferential cytotoxicity to CML cells, while some to phytohemagglutinin (PHA) stimulated blasts from the same stimulator. Fifteen leukemia-specific clones were further studied. There was a broad correlation between cytotoxicity to CML cells and colony inhibition. However, even weakly cytotoxic lines were inhibitory to CML colony-forming units granulocyte/macrophage (CFU-GM). This effect was partly mediated by the supernatant from the T cell line; four of five supernatants tested inhibited the growth of CFU-GM. Antibody neutralization studies demonstrated the presence of gamma interferon and tumor necrosis factor alpha in the supernatants. Interestingly, there was greater suppression of CML CFU-GM when compared with CFU-GM from normal individuals. One supernatant from a noncytotoxic T cell line stimulated CFU-GM and was demonstrated by antibody neutralization tests to contain interleukin-3 and GM-CSF. These data indicated that the CML CFU-GM in particular appears to be highly susceptible to attack by alloreacting $CD4^+$ cells exerting both cell and cytokine-mediated antileukemia effects. These mechanisms may be responsible for a GVL effect in CML by a Th1 type of cytotoxic $CD4^+$ cell (Figure 2).

Cytotoxic effect of CD4 cells on CML cells and their progenitors is mediated via the fas pathway

To further investigate the interaction of $CD4^+$ cells with CML progenitors we generated HLA-DR1 restricted $CD4^+$ cytotoxic T cell clones specifically recognizing tuberculous purified protein derivative (PPD).[3] Proliferative and cytotoxic $CD4^+$ T cell responses to cells exposed to PPD were induced using patient's CML cells and B-LCL's but not PHA-blasts. Antigen presentation was blocked by antibodies to HLA-DR1 but not to MHC class I, and by treatment with chloroquine and brefeldin. This indicated that CML cells used a classic MHC class II antigen processing pathway to present PPD antigens to $CD4^+$ T cells. Cytotoxicity to CML was shown by antibody blocking studies to be mediated mainly through the fas-antigen, which is present on $CD34^+$ CML cells.[4] These experiments indicated that alloreacting $CD4^+$ T cells alone are sufficient to mediate GVL effects following allogeneic BMT for CML.

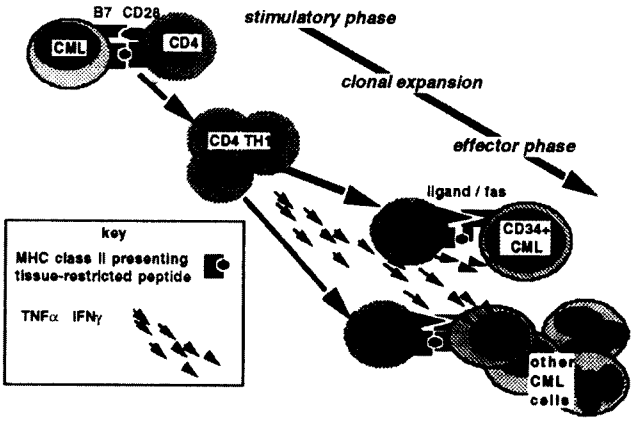

Figure 2. *Role of CD4 cells in GVL response to CML.*

Correlation of relapse with immune recovery after BMT

Fifteen patients undergoing unmanipulated BMT for CML in chronic or accelerated phase were studied to correlate lymphocyte recovery with relapse and GVHD. Lymphocyte phenotype was studied on three occasions up to 9 months after BMT together with spontaneous (NK) and IL-2 stimulated (LAK cell) cytotoxic function. Five patients had a cytogenetic or hematologic relapse between 9–24 months post BMT. Seven developed grade O-I acute GVHD and eight developed acute GVHD grade II-IV. All patients survived at least 9 months after BMT. Although there were individual variations in the speed and completeness of recovery of lymphocyte numbers, the group of five patients who ultimately relapsed with CML had consistently low numbers of $CD8^+$, $CD16^+$, $CD56^+$ cells and to a lesser extent $CD4^+$ cells. Wilcoxon rank sum analysis was used to test significance. Circulating $CD8^+$ and NK cell but not $CD4^+$ cell numbers remained significantly lower in five patients who relapsed compared with those remaining in remission after BMT (mean 0.03 versus 0.32×10^9/L, p=0.002 for $CD8^+$ cells; mean 0.03 versus 0.11×10^9/L, p=0.002 for NK cells). In contrast, $CD4^+$, $CD8^+$ and NK cell numbers did not correlate with grade II or more acute GVHD (p=NS). Thus the differences in circulating lymphocyte counts could not be explained by differences caused by GVHD or its treatment. Spontaneous NK cytotoxic function rose to within the normal range in the first month after BMT, while LAK function remained low during the study period in all patients. However, three of the four patients relapsing after BMT had the lowest NK function on all occasions tested: mean cytotoxicity against K562 target of <10% at an E:T ratio of 50:1. These results link NK and T cell recovery with GVL rather than GVHD and implicate multiple effectors in the GVL response. They do not, however, clarify the relative contribution of NK versus T cell reactivity to the GVL response.

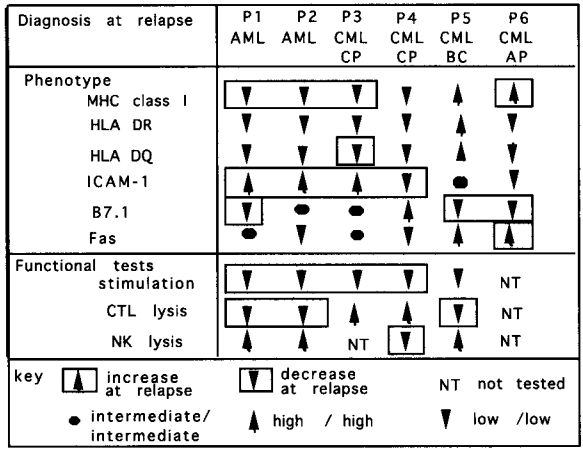

Table 1. Comparison of leukemias pre-BMT and at relapse

Immune escape of residual leukemia

Relapse due to escape of residual leukemia from the donor's immune control following BMT is a possibility that has been largely unexplored. We therefore compared the immunogenicity of leukemia cells before BMT and at relapse in recipients of allogeneic BMT from an HLA-matched sibling donor.[5] Compared with their pre-BMT counterparts, relapsed leukemia cells from 2/2 AML and 2/3 CML patients induced significantly lower proliferation of HLA mismatched lymphocytes from a normal individual. In the third patient with CML, both the pretransplant and the relapsed leukemia were poor stimulators. Cytotoxic T lymphocytes generated by a healthy unrelated responder against pretransplant leukemia efficiently lysed pretransplant leukemia cells in all patients. However, relapsed leukemia from 3/5 patients showed reduced susceptibility to CTL lysis. Pre- and post-BMT leukemia samples were also tested for their susceptibility to NK-mediated lysis using fresh mononuclear cells from a normal individual as a source of NK cells. Susceptibility to NK lysis was seen in all pre- and post-transplant leukemias with the exception of one patient who developed NK resistance at relapse. Immunophenotypic analysis of pretransplant and relapsed leukemia cells was carried out for HLA class I, HLA-DR and DQ, CD54 (ICAM-1), CD80 (B7.1) and CD95 (Fas) molecules. A correlation was observed between stimulatory capacity and lytic susceptibility, with down-regulation of one or more molecules in the relapsed cells. These findings suggest that immune escape may be a relatively common cause of leukemic relapse following BMT (Table 1).

MHC class II expression was typically low in leukemias both pre- and post-BMT. The change at relapse that could be associated with decreased immunogenicity were a downregulation of MHC class I expression (3 patients), loss of B7.1 expression (3 patients) and loss of ICAM-1 expression (1 patient). All leukemias at relapse showed one or more of these features. In one patient with AML relapsing after BMT, we found that incubation of the leukemia with IFNγ increased MHC expression and restored stimulatory ability and target susceptibility to cytotoxic lysis. These findings emphasize the need to study combinations of cytokines with donor lymphocyte transfusions (DLT) in the treatment of post-BMT leukemic relapse. Furthermore, in addition to boosting the immune response, they refocus our efforts on finding ways to modify the immunogenicity of the leukemia.

Treatment of relapse: Effect of combining DLT with cytokines

Twelve patients relapsing after BMT were studied. Relapse occurred between 2 and 28 months following BMT. To deliver optimum immune modulation to individual patients relapsing with a variety of leukemias, we used the following sequential strategy:

1) Karyotypic relapse of CML: Stop cyclosporin (CSA) for two months and reassess.
2) For patients relapsing off CSA, give 5×10^7 $CD3^+$ cells /kg DLT.
3) Evaluate at 2 months: If no response, repeat with 5×10^8 $CD3^+$ cells /kg.
4) Evaluate at 2 months: If no response, give 5×10^7 $CD3^+$ cells/kg with subcutaneous IFN-α 3×10^6 U daily or IL-2 16×10^6 U/m^2/day×5 days.
5) No response/progression: myeloablation and second BMT.

Patients relapsing with acute leukemia entered the schema at (4). Figure 3 illustrates the results. Twelve patients were studied. Three CML patients with karyotypic relapse responded to cessation of cyclosporin alone. They remain in continuous complete remission. Four in hematological relapse of CML in chronic phase (CP) or accelerated phase (AP) not receiving immunosuppression were given DLT without response. Two of three patients responded to DLT and IFN-α and are in stable remission. The third patient showed accelerating CML and received a second non-T cell depleted, G-CSF mobilized peripheral blood stem cell transplant from the same donor. He is now in a karyotype remission. A fourth patient failed both DLT and DLT + IL-2. He successfully achieved a remission following a second unmanipulated BMT but died of hepatic GVHD. Five patients with relapsed acute leukemia, 2 transformed myelodysplastic syndrome t(MDS), 2 acute myeloblastic leukemia (AML), 1 CML blast crisis (BC), were given DLT + IL-2. None responded. One is alive in remission following a second BMT, the others died of progressing leukemia. Overall 7/12 patients (55% actuarial) survive leukemia-free with a median remission duration of 9 months.

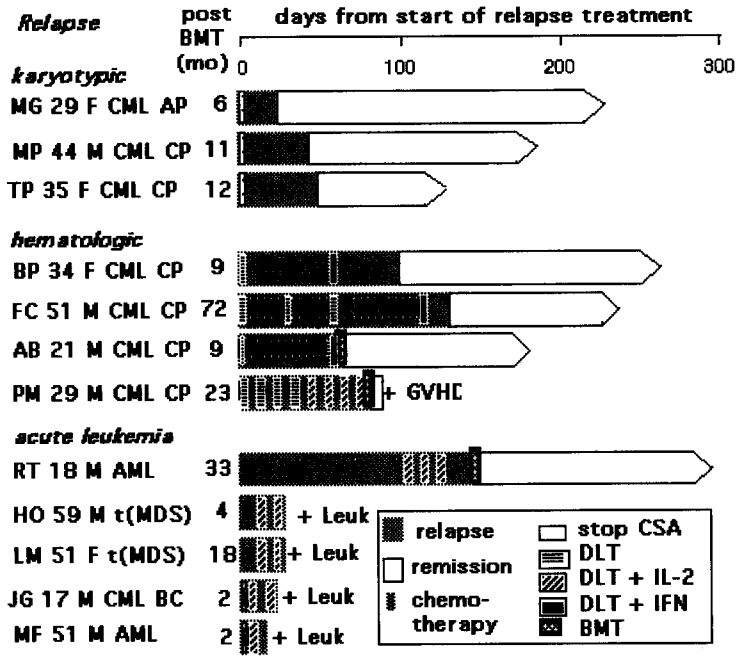

Figure 3. *Treatment of leukemia relapsing after BMT.*

DISCUSSION

These results suggest that a logical approach to the treatment of post-transplant relapse would be to tailor management according to the situation. In patients with slowly progressive disease the efficacy of stopping immunosuppression, followed if necessary by repeated DLT, can be evaluated. In nonresponders the use of combinations of cytokines with DLT should be explored in attempts to improve either immune reactivity or target susceptibility. In this small series, IFN-α appeared to offer more promise than IL-2. In vitro studies of leukemic immunogenicity such as those described above could be very helpful in selecting appropriate cytokines for treatment. Finally, our data confirm the general experience that rapidly progressing leukemias may be manageable only by myeloablative therapy and a second stem cell transplant followed by therapy with a cytokine chosen, on the basis of in vitro experiments, to have immunomodulatory properties. Although the treatment of leukemia relapsing after BMT can be daunting, it represents an opportunity to study GVL and to develop combined chemotherapy, T cell and cytokine treatment strategies.

REFERENCES

1. Barrett AJ, Malkovska V: Graft versus leukaemia: Understanding and using the alloimmune response to treat haematological malignancies. *Br J Haematol* 93:754–761, 1996.
2. Jiang YZ, Barrett AJ: Cellular and cytokine mediated effects of CD4-positive lymphocyte lines generated in vitro against chronic myelogenous leukemia. *Exp Hematol* 23:1167–1172, 1995.
3. Jiang YZ, Mavroudis D, Dermime S et al.: Alloreactive $CD4^+$ T lymphocytes can exert cytotoxicity to chronic myeloid leukaemia cells processing and presenting exogenous antigen. *Br J Haematol* 93:612–619, 1996.
4. Selleri C, Sato T, del Vecchio L et al.: Involvement of Fas-mediated apoptosis in the inhibitory effects of interferon-a in chronic myelogenous leukemia. (Submitted).
5. Dermime S, Mavroudis D, Jiang YZ et al.: Does immune escape from a graft-versus-leukemia effect play a role in the relapse of myelogenous leukemias following allogeneic bone marrow transplantation? (Submitted).

ENGRAFTMENT OF ALLOGENEIC HEMATOPOIETIC PROGENITOR CELLS WITH PURINE ANALOG CONTAINING CHEMOTHERAPY: HARNESSING GRAFT-VERSUS-LEUKEMIA WITHOUT MYELOABLATIVE THERAPY

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INTRODUCTION

The curative potential of allogeneic bone marrow transplantation (BMT) is mediated in part by an immune mediated graft-versus-leukemia effect (GVL).[1] Evidence for this phenomenon includes the observation that donor lymphocyte infusions can reinduce remissions in many patients with hematologic malignancies who have relapsed after allogeneic BMT (alloBMT).[2] Therefore, it is possible to exploit the GVL effect independently of the preparative regimen, at least in the post-transplant setting. The strategy of exploiting GVL as treatment for susceptible malignancies could be considered in patients ineligible for high-dose myeloablative treatment because of age or concurrent medical conditions.

The purine analogs fludarabine and 2-chlordeoxyadenosine (2CDA) have been shown to be extremely active against a variety of lymphoid malignancies.[3] These compounds are also immunosuppressive and in vitro can effectively inhibit the mixed lymphocyte reaction.[4,5] We performed a pilot trial to determine if nonmyeloablative chemotherapy in combination with purine analogs could be sufficiently immunosuppressive to allow allogeneic hematopoietic progenitor cells to engraft in patients considered ineligible for myeloablative therapy, because of advanced age or medical condition.

STUDY SCHEMA

Patients with acute leukemia or myelodysplasia beyond first complete remission, between the ages of 55 and 70, were considered eligible for this

study. Patients less than 55 years old not eligible for a conventional transplant because of a concurrent medical condition were also eligible. Patients required an HLA compatible related donor and a serum bilirubin <3.0 mg/dL, serum creatinine <2.0 mg/dL, cardiac ejection fraction of 40% or greater and a Zubrod performance status of ≤2. Patients and donors signed a written informed consent and the study was approved by the local institutional review board.

Donors received filgrastim 6 μg/kg subcutaneously twice daily starting 4–5 days before the first collection. Leukaphereses were done daily using conventional techniques for blood progenitor cell collection until $>3\times10^6$ $CD34^+$ cells/kg recipient were collected.[6] If stem cell collection was not feasible, donor bone marrow was harvested.

Eight patients without prior exposure to fludarabine received fludarabine 30 mg/m^2 intravenously daily at the same time over 30 minutes for four days with either Ara-C 2 gm/m^2 given intravenously over 4 hours, starting 4 hours after the beginning of the fludarabine infusion, and idarubicin 12 mg/m^2 intravenously for three days (n=7)[7] or melphalan 140 mg/m^2 (n=1). Seven patients with prior fludarabine exposure received Ara-C 1.0 gm/m^2 over 2 hours intravenously daily for five days and 2-CDA at a dose of 12 mg/m^2 daily by continuous intravenous infusion for five days beginning four hours after the first dose of Ara-C.[8] Cells were infused 2 days after the last dose of chemotherapy. All patients were treated in private rooms and received standard post transplant supportive care. Patients received filgrastim 5 μg/kg daily from day 0 until engraftment. Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine at a dose of 3 mg/kg/daily by continuous intravenous infusion changed to oral as soon as tolerated and methylprednisolone 1.0 mg/kg daily beginning on day +5 tapered over the following 8 weeks.

The primary objectives of this study were to evaluate the engraftment potential and the antileukemic effects of allogeneic hematopoietic progenitor cells. Chimerism and evidence of minimal residual disease were determined by conventional cytogenetics, restriction fragment length polymorphisms, and fluorescence in situ hybridization using published techniques.[9–11] Additional endpoints, as defined by conventional published criteria, included toxicity, the incidence of acute and chronic GVHD, response and survival as of August 8, 1996.[12–15]

RESULTS

From July 1995 to July 1996 fifteen patients with acute leukemia or MDS were treated (9 females and 6 males). Patient- and disease-related characteristics are summarized in Table 1.

Donors received a median of 5 days of filgrastim; eight underwent one apheresis procedure and five required two. The median $CD34^+$ cell yield

Table 1. Patient and disease characteristics

Variable	
n	15
sex	9 female
	6 male
age in years, median (range)	59 (27–71)
diagnosis	AML: 13
	MDS: 2
stage at transplant	refractory: 10
	untreated Relapse: 2
	CR#3: 1
	CMML: 1
	untreated 2ry AML: 1
number of prior therapies, median (range)	2 (0–4)
time to transplant	391 (50–1315)
donor type	6/6 sibling matched: 13
	5/6 sibling matched: 2
cell source	peripheral blood stem cells: 14
	bone marrow: 1

expressed as the number of $CD34^+$ cells per liter of blood processed during the first collection was 21.3×10^6 (range, 6.2–45.5). The median number of $CD34^+$ cells infused was 4.5×10^6/kg (range, 1.7–9.9).

The chemotherapy was well tolerated in 13 of the 15 patients. One patient died from multi-organ failure after a second dose of fludarabine, idarubicin and Ara-C, and another developed congestive heart failure that responded to diuretics and inotropic support.

Thirteen of 15 patients achieved an absolute neutrophil count (ANC) of $>0.5\times10^9$/L, a median of 10 days post transplant (range, 8–17). Ten patients achieved a platelet count of 20.0×10^9/L or greater without transfusions a median of 13 days post infusion (range, 7–78).

Acute GVHD occurred in three patients; one limited to skin alone, the other two involving skin and gut. GVHD responded to steroid therapy alone in two patients and required antithymocyte globulin in the third. Chronic GVHD has not occurred.

Thirteen of 15 patients cleared their peripheral blood blasts. Eight patients achieved remission with 6 having >90% donor cells at that time (between days 14 and 30). One patient achieved remission criteria without evidence of donor cell engraftment by cytogenetics or RFLP, and one patient could not be

assessed. Five patients have relapsed between 43 and 127 days after transplant (median, 65 days). Six of the 15 patients remain alive between 34 and 175 days after transplant (median, 100 days). Two patients remain in remission 34+ and 170+ days after transplant; the other four have active disease. None of the relapsing patients have responded to cyclosporine withdrawal or filgrastim therapy as treatment of relapse. The median survival for all 15 patients is 78 days (range, 0–175+). The actuarial 100 day survival was 66 ± 19% for patients achieving a complete remission and 21% ± 17% for patients not achieving this response (p=0.16 logrank).

SUMMARY AND CONCLUSION

Despite modern combination chemotherapy, most patients with acute leukemia relapse within the first two years of achieving a remission. Durable responses to salvage therapy are rare.[16] Myeloablative chemoradiotherapy with allogeneic progenitor cells can result in long-term, disease-free survival, but has usually been limited to younger patients with a relatively good performance status.[17,18]

The anti-leukemic effect seen with donor lymphocyte infusions administered to patients relapsing after allogeneic transplantation[2] suggests that if donor hematopoietic cells can engraft after standard-dose chemotherapy, it may be possible to exploit the graft-versus-leukemia effect without the potential morbidity associated with myeloablative therapy.

Fludarabine and 2-CDA combinations have been extensively studied in combination with Ara-C as salvage therapy for patients with acute leukemia. These regimens have been generally well tolerated.[7,8] The addition of allogeneic hematopoietic progenitor cells could potentially enhance hematopoietic recovery as well as provide a graft-versus-leukemia effect. This study demonstrates that such an approach is feasible: granulocyte recovery was prompt and three of four patients in remission 60 to 90 days post infusion had >90% donor cells as assessed by conventional techniques. Further studies with long-term follow-up will be necessary to determine if this approach improves leukemia-free survival in the setting of salvage therapy or as consolidation after an initial remission.

REFERENCES

1. Sullivan K, Weiden P, Storb R, Witherspoon R, Fefer A, Fisher L, Buckner C, Anasetti C, Appelbaum F, Badger C, Beatty P, Bensinger W, Berenson R, Bigelow C, Cheeves M, Clift R, Deeg H, Doney K, Greenberg P, Hansen J, Hill R, Longhran T, Martin P, Neiman P, Peterson F, Sanders J, Singer J, Stewart P, Thomas E: Influence of acute

and chronic graft-versus-host disease on relapse and survival after bone marrow transplantation from HLA-identical siblings as treatment of acute and chronic leukemia. *Blood,* 73:1720, 1989.

2. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, Ljungman P, Ferrant A, Verdonck L, Niederwieser D, van Rhee F, Mittermuller J, de Witte T, Holler E, Ansari H: Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood* 86:2041–2050, 1995.
3. Plunkett W, Sanders PP: Metabolism and action of purine nucleoside analogs. *Pharmacol Ther* 49:239–268, 1991.
4. Goodman ER, Fiedor PS, Fein S, Sung RS, Athan E, Hardy MA: Fludarabine phosphate and 2-chlorodeoxyadenosine: Immunosuppressive DNA synthesis inhibitors with potential application in islet allo-xenotransplantation. *Transplant Proc* 27:3293–3294, 1995.
5. Gorski A, Grieb P, Korczak-Kowalska G, WierzLicki P, Stepien-Sopniewska B, Mrowiec T: Cladribine (2-chlorodeoxyadenosine, CDA): An inhibitor of human B and T cell activation in vitro. *Immunopharmacology* 26: 197–202, 1993.
6. Anderlini P, Przepiorka D, Seong D, Miller P, Sundberg J, Lichtiger B, Norfleet F, Chan KW, Champlin R, Korbling M: Clinical toxicity and laboratory effects of granulocytecolony stimulating factor (filgrastim) mobilization and blood stem cell apheresis from normal donors, and analysis of charges for the procedures. *Transfusion* 36, 1996.
7. Estey E, Plunkett W, Gandhi V, Rios MB, Kantagian H, Keating MJ: Fludarabine and arabinosylcytosine therapy of refractory and relapsed acute myelogenous leukemia. *Leuk Lymphoma* 9:343–350, 1993.
8. Kornblau S, Andreeff M, Beran M, Escudier S, Kantagian H, Koller C, O'Brien S, Robertson LE, Estey E: 2-CDA + Ara-C is ineffective therapy for relapsed or refractory AML in adults. *Proc ASCO* 14:336, 1995.
9. Standing Committee on Human Cytogenetic Nomenclature, An international system for human cytogenetic nomenclature 20. (1978 ISCN) *Cytogenet Cell Genet* 21:309, 1978.
10. Pinkel D, Straume T, Gray JW: Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. *Proc Natl Acad Sci USA* 83:2934, 1986.
11. Yam P, Petz L, Knowlton R, Wallace R, Stock A, DeLange G, Brown V, Doris-Keller H, Blume K: Use of DNA restriction fragment length polymorphisms to document marrow engraftment and mixed hematopoietic chimerism following bone marrow transplantation. *Transplantation* 43:399, 1987.
12. Bearman SI, Appelbaum FR, Buckner CD, Petersen FB, Fisher LD, Clift RA, Thomas ED: Regimen related toxicities in patients undergoing bone marrow transplantation. *J Clin Oncol* 6:1562, 1988.
13. Glucksberg H, Storb R, Fefer A, Buckmer CD, Nerman PE, Clift RA, Lemer KG, Thomas ED: Clinical manifestations of graft versus host disease in human recipients of marrow from HLA matched sibling donors. *Transplantation* 18:295, 1974.
14. Schulman HM, Sullivan KM, Weiden PL, McDonald GB, Striker GE, Sale GE, Hackman R, Tsoi MS, Storb R, Thomas ED: Chronic graft-versus-host disease syn-

drome in man. A long term clinical pathologic study of 20 Seattle patients. *Am J Med* 69:204, 1980.

15. Kaplan E, Meier P: Non parametric estimation from incomplete observations. *J Am Stat Assoc* 53:457, 1958.
16. Keating M, Kantagian H, Smith T, Estey E, Walters R, Andersson B, Beran M, McCredie K, Freireich E: Response to salvage therapy amd survival after relapse in acute myelogenous leukemia. *J Clin Oncol* 7:1071–1080, 1989.

USE OF ROQUINIMEX AS AN IMMUNE MODULATOR IN PHILADELPHIA CHROMOSOME-POSITIVE CHRONIC MYELOGENOUS LEUKEMIA

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INTRODUCTION

Unmanipulated autologous bone marrow transplantation (BMT) for patients with chronic myelogenous leukemia (CML) offers, at most, survival to only 10% of patients. There are accumulating data, however, that of all hematopoietic neoplasms, CML may be the most susceptible to immune modulation. The increased relapse rate following T cell–depleted allografts is greatest in CML,[1,2] and allografts which have relapsed respond to donor T-lymphocyte infusions or to adoptive immunotherapy to a greater extent in CML than in acute myelogenous leukemia (AML).[3,4]

Roquinimex is a quinoline derivative that is orally active and has been shown to have a number of immunological properties. It increases natural killer (NK) cell number and activity, enhances the delayed-type hypersensitivity reaction, augments the proliferative response to T cell mitogens, increases monocyte activity, antagonizes cyclosporine, increases the production of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interferon-α (IFN-α), and raises serum level of interleukin-6 (IL-6). These findings provided a rationale for the use in the following study, which is still in progress, of roquinimex in CML.[5,6,7]

PATIENTS AND METHODS

The study was conducted at the University of Rochester Medical Center, Rochester, New York, in patients aged 18 to 65 years with Philadelphia-chromosome-positive (Ph) CML either in first chronic phase (CP1), accelerated phase (AP) or second chronic phase (CP2). Patients in frank blast crisis or those who were less than 45 years and have a histocompatible sibling donor were excluded, the former due to presumed lack of efficacy of autologous transplantation and the latter due to competing intramural protocol using allogeneic transplantation.

Most of the patients received significant myelosuppressive therapy before transplant and most were refractory to α-interferon. Prior to transplantation, patients

received hydroxyurea to reduce the white blood cell (WBC) count to less than 20,000/μL and to reduce the spleen size to less than 8 cm below the left costal margin. No other pre-transplant manipulation of the malignant clone was attempted. Patients were conditioned with busulphan 4 mg/kg/day for 4 days and cyclophosphamide 60 mg/kg for 2 days followed by reinfusion of unmanipulated Ph+ bone marrow ($>1\times10^8$ nucleated cells/kg) or peripheral blood stem cells. As soon as the neutrophil count had reached 100/μL, patients received roquinimex twice weekly at an initial starting dose of 0.05 mg/kg, escalating rapidly to a maintenance dose of 0.2 mg/kg within two weeks. This regimen was then continued for two years.

The primary endpoint of the study was the efficacy of the treatment regimen as measured by cytogenic conversion, the time to clinical progression and the overall survival from bone marrow transplantation. Other objectives were 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.

RESULTS

The study was initiated in March 1992, and 17 patients have entered the study until July 1996. All patients were cytogenetically positive for the Ph chromosome pre-transplant. Twelve were in CP1, 3 were in AP and 2 in CP2. The latter two patients presented in blast crisis, were successfully reinduced with high-dose chemotherapy consisting of daunorubicin 70 mg/m^2 for 3 days and cytosine arabinoside 200 mg/m^2 for 9 days,[8] and were then reinfused with marrow obtained during the second chronic phase as their source of stem cells. The patients in AP 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. All patients treated to date have tolerated the transplantation procedure well. Median time to engraftment of 500 neutrophils/μL was 15 (7–49) days. There was no transplant-related mortality, but five patients have died from progressive disease. All patients entering the study in CP1 have shown a hematological response and more than half have achieved a major cytogenetic response (>65% Ph-clones) at one year or beyond. Not surprisingly, the response rates were lower among patients in CP2; nevertheless, some have clearly benefited, including one who remained in very stable chronic phase for four years following transplantation and roquinimex therapy.

Virtually all patients have shown some moderate, but tolerable, adverse side effects following transplantation (Table 1). The toxicities did not require specific therapies; however, the bone marrow aplasia responded to a short course of corticosteroids and the hypothyroidism required replacement therapy.

Table 1. Toxicity (17 patients)

	Toxicity	*No. of patients*
skin	GVHD	7
	eccrine sweat gland necrosis	5
edema	orbit, ankles	9
gastrointestinal	reduced appetite	1
	pain	1
	acute abdomen	1
hypothyroidism		1
pleural effusion		1
bone marrow aplasia		1

DISCUSSION

These findings confirm that, following unmanipulated ABMT, and with continuing roquinimex therapy, engraftment with a Ph^- cell population can occur in previously Ph^+ CML patients.

It may be important to note that in some patients the cytogenetic response developed over time; it was thus not simply a case of post-transplant engraftment with Ph^- cells. Toxicity was moderate, but tolerable. The skin changes and bone marrow aplasia were consistent with graft-versus-host disease (GVHD) while the hypothyroidism may be attributable to the stimulation, by roquinimex, of autoimmune antibody production. Further confirmation of these encouraging early results is awaited together with more prolonged follow-up and increased accrual of patients.

REFERENCES

1. Horowitz MM, Gale RT, Sondel PM et al.: Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* 75:555–562, 1990.
2. Apperley JF, Jones L, Hale G et al.: Bone marrow transplantation for patients with chronic myeloid leukemia: T-cell depletion with Campath-1 reduces the incidence of graft-versus-host disease, but may increase the risk of leukemic relapse. *Bone Marrow Transplant* 1:53–66, 1986.
3. Fefer A, Sullivan KM, Weiden P et al.: Graft-versus-leukemia effect in man: The relapse rate of acute leukemia is lower after allogeneic than after syngeneic marrow transplantation. *Proc Clin Bio Res* 244:401–408, 1987.
4. Kolb HJ, Mittermuller J, Clemm C et al.: Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplantation. *Blood* 76:2462–2465, 1990.
5. Rowe JM, Nilsson BI, Simonsson B: Treatment of minimal residual disease in myeloid

leukemia–the immunotherapeutic options with emphasis on linomide. *Leuk Lymphoma* 11:321–329, 1993.

6. Kalland T: Regulation of natural killer progenitors. Studies with a novel immunomodulator with distinct effects-acting precursor level. *J Immunol* 144:4472–4476, 1990.
7. Larsson EL, Joke A, Stalhandskie T: Mechanisms of action of the immunomodulator. LS2616 on T-cell responses. *Int J Immunopharmacol* 9:425–431, 1987.
8. Kouides PA, Rowe JM: A dose-intensive regimen of cytosine arabinoside and daunorubicin for chronic myelogenous leukemia in blast crisis. *Leuk Res* 19:763–770, 1995.

IN VITRO SELECTION OF *BCR-ABL* NEGATIVE PROGENITORS BY TYROSINE KINASE INHIBITION

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ABSTRACT

We investigated the effect of genistein, a protein tyrosine kinase inhibitor, on the in vitro growth of CML and normal marrow-derived multipotent (CFU-Mix), erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) hematopoietic progenitors. Continuous exposure of marrow cells to genistein induced a dose-dependent suppression of CML and normal colony formation. Preincubation with genistein (200 μM, 1–18 hours) induced a time-dependent suppression of progenitor cell growth, while sparing a substantial proportion of long-term culture-initiating cells (LTC-IC) from CML (range: 91 ± 9% to 32 ± 3%) and normal marrow (range: 85 ± 8% to 38 ± 9%). Analysis of individual CML colonies for the presence of the hybrid *BCR-ABL* mRNA by reverse transcription polymerase chain reaction (RT-PCR) showed that genistein treatment significantly reduced the mean (±SD) percentage of marrow *BCR-ABL*$^+$ progenitors both by continuous exposure (76 ± 18% versus 24 ± 12%, $p \leq 0.004$) and by preincubation (75 ± 16 versus 21 ± 10, $p \leq 0.002$). Preincubation with genistein reduced the percentage of leukemic LTC-IC from 87 ± 12% to 37 ± 12% ($p \leq 0.003$). Analysis of nuclear DNA fragmentation by terminal deoxynucleotidyl transferase assay showed that preincubation of CML MNC-AC$^-$ and CD34$^+$ cells with genistein induced significant evidences of apoptosis. These observations demonstrate that genistein is capable of a) exerting a strong antiproliferative effect on CFU-Mix, BFU-E and CFU-GM while sparing the more primitive LTC-IC, and b) selecting benign hematopoietic progenitors from CML marrow.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder due to an acquired abnormality in a pluripotential hematopoietic stem cell.[1] CML is cytogenetically marked by the Philadelphia (Ph) chromosome, which originates from a reciprocal translocation between chromosomes 9 and 22, and molecularly marked by a chimeric *BCR-ABL* gene, resulting from juxtaposition of the ABL

proto-oncogene with the BCR gene.[2] The chimeric *BCR-ABL* gene expresses an 8.5-kb hybrid messenger RNA transcript giving rise to a 210-kD fusion protein ($p210^{BCR\text{-}ABL}$) with increased tyrosine kinase activity.[3] Dysregulated tyrosine kinase activity of the $p210^{BCR\text{-}ABL}$ fusion protein plays a key role in the pathogenesis of CML.[2–4] Although recombinant interferon-alpha can prolong survival of CML patients,[5] the only curative treatment for these patients remains allogeneic bone marrow transplantation, which is restricted to <25% of the patients.[6] Several experimental and clinical findings support a role for autologous bone marrow transplantation (ABMT) in CML.[7] To improve the therapeutic index of ABMT, several in vivo or in vitro strategies have been proposed.[8–14]

In the past decade, several natural or synthetic compounds able to inhibit the signaling cascades triggered by protein tyrosine kinases (PTKs) have been generated.[15] Inhibitors of PTK catalytic activity include genistein, quercetin, herbimycin A and tyrphostins. Genistein, a naturally occurring PTK inhibitor, has been shown to exhibit specific inhibitory activity against receptor and cytoplasmic tyrosine kinases, including epidermal growth factor receptor, $pp60^{v\text{-}src}$, $pp110^{gag\text{-}fes}$, platelet derived growth factor receptor, and c-*kit*.[16] Genistein induces differentiation and apoptosis of HL-60 and K562 cells,[17] and may also inhibit topoisomerases I and II.[18] Using established leukemic cell lines, it has been demonstrated that genistein inhibits $p210^{BCR\text{-}ABL}$ tyrosine kinase activity.[19] However, the effect of PTK inhibitors on primary CML cells has not been extensively investigated.

It therefore was the aim of the present study to evaluate the effect of genistein on the in vitro growth of marrow-derived CML and normal progenitors grown in methylcellulose in the continuous presence of or after a transient exposure to genistein. In addition, the capability of genistein to select in vitro for *BCR-ABL*– progenitors was investigated by detecting the *BCR-ABL* mRNA on single progenitors by reverse transcription polymerase chain reaction.

MATERIALS AND METHODS

Patients

Seven patients with Ph^+ CML were included in this study. Three patients were evaluated at diagnosis and prior to any treatment; the others had been diagnosed 3 to 55 months before the study and had received prior treatment with hydroxyurea, interferon-alpha or mafosfamide-purged ABMT. At the time of the study, all patients were in chronic phase.

Cell separation procedures

After informed consent, bone marrow was obtained from normal donors or CML patients by aspiration from the posterior iliac crest and mononuclear cells

(MNC) were separated by centrifugation on a Ficoll-Hypaque gradient. Monocyte-macrophage cells were removed by adherence to plastic for 60 minutes.

CFU-MIX, BFU-E and CFU-GM assay

The assay for multilineage colony-forming units (CFU-Mix), erythroid bursts (BFU-E) and granulocyte-macrophage colony-forming units (CFU-GM) was carried out as described elsewhere.[13] Briefly, 5×10^4 MNC-AC$^-$ cells were plated in 35 mm Petri dishes in 1-mL aliquots of Iscove's modified Dulbecco's medium (IMDM, Seromed, Berlin, Germany) containing: 30% FBS (Stem Cell Technologies, Vancouver, Canada); 10^{-4} M 2-mercaptoethanol (Gibco, Grand Island, NY); and 1.1% (w/v) methylcellulose. Cultures were stimulated with interleukin-3 (10 ng/mL, Sandoz, Basel, Switzerland), granulocyte colony-stimulating factor (10 ng/mL, Amgen Inc., Thousand Oaks, CA), granulocyte-macrophage colony-stimulating factor (10 ng/mL, Sandoz) and erythropoietin (3 U/mL, Amgen Inc.). Progenitor cell growth was evaluated after incubation (37°C, 5% CO_2) for 14–18 days in a humidified atmosphere. Colonies were scored according to previously published criteria.[13]

Long-term culture-initiating cell assay

The long-term culture-initiating cell (LTC-IC) assay was performed according to Sutherland et al.[20] Briefly, test cell ($3–5\times10^6$ MNC-AC$^-$) suspensions were seeded into cultures containing a feeder layer of irradiated (8000 cGy) murine M2-10B4 cells (3×10^4/cm^2, kindly provided by Dr. C. Eaves) engineered by retroviral gene transfer to produce human IL-3 and human G-CSF.[21] Test cells were resuspended in complete medium consisting of alpha-medium (Gibco) supplemented with fetal bovine serum (12.5%), horse serum (12.5%), L-glutamine (2 mM), 2-mercaptoethanol (10^{-4} M), inositol (0.2 mM), folic acid (20 μM) and freshly dissolved hydrocortisone (10^{-6} M). Cultures were fed weekly by replacement of half of the growth medium containing half of the nonadherent cells with fresh complete medium. After 5 weeks in culture, nonadherent cells and adherent cells harvested by trypsinization were pooled, washed and assayed together for clonogenic cells in standard methylcellulose cultures at an appropriate concentration (usually 5×10^4/mL). The total number of clonogenic cells present in 5-week-old LTC provides a relative measure of the number of LTC-IC originally present in the test suspension.[7] Absolute LTC-IC values were calculated by dividing the total number of clonogenic cells by 4, which is the average output of clonogenic cells per LTC-IC.

Cytogenetic analysis

Cytogenetic analysis and standard GTG- or QFQ-banding techniques were performed according to standard methods.[22] To exclude that genistein could induce

false negative results by blocking *BCR-ABL* gene expression, individual colonies were aspirated, divided into two aliquots and analyzed at both the cytogenetic and the molecular level.[13]

Detection of *BCR-ABL* mRNA in individual progenitors

BCR-ABL mRNA was detected by reverse transcription polymerase chain reaction (RT-PCR). Colonies were individually removed and transferred into microcentrifuge tubes containing 40 µL phosphate buffered saline (PBS). After adding guanidinium thiocyanate colonies were frozen at –70°C until nested RT-PCR was performed. Total RNA was extracted from thawed colonies according to Chomczynski and Sacchi with slight modifications.[23] Briefly, 500 µL of TRIzol (Gibco) and 5 µg of MS2 phage RNA (Boehringer Mannheim, Mannheim, Germany) as a carrier were added to each tube. After incubation for 5 minutes at room temperature, 100 µL of chloroform were added, each tube was centrifuged (12,000*g*, 15 minutes, 4°C), the upper aqueous phase aspirated and RNA precipitated. Total RNA from each colony was reverse transcribed to cDNA using 1.1 nmol of hexa random primer, 10 mmol/L DTT and 0.125 mmol/L dNTP (Gibco), 40 U of RNAse Inhibitor (Boehringer Mannheim) and 200 U of MLV reverse transcriptase (Gibco) in PCR-buffer (50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L $MgCl_2$). Total cDNA from each colony was divided into two aliquots, for detection of the *BCR-ABL* rearrangement and the internal ABL sequence, respectively. The first PCR amplification was performed using 10 µL of the reverse transcription mixture, 0.25 mmol/L dNTP, 0.57 µmol/L of each primer, 2 U of Taq polymerase (Gibco) in PCR-buffer (20 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$). Forty-five cycles, each consisting of 30 seconds at 94°C, 30 seconds at 60°C and 30 seconds at 72°C, were performed using a Perkin-Elmer Cetus DNA Thermal Cycler. The *BCR-ABL* primer sequences were:[24] 5′-GAA GAA GTG TTT CAG AAG CTT CTC CC-3′ (sense) and 5′-GAC CCG GAG CTT TTC ACC TTT AGT T-3′ (antisense). The ABL primer sequences were:[24] 5′-TTC AGC GGC CAG TAG CAT CTG ACT T-3′ (sense) and 5′-GAC CCG GAG CTT TTC ACC TTT AGT T-3′ (antisense). *BCR-ABL* internal nested primer sequences were: 5′-GTG AAA CTC CAG ACT GCT CAC AGC A-3′ (sense) and 5′-TCC ACT GGC CAC AAA ATC ATC ATA CAGT-3′ (antisense). The expected products generated by PCR were for *BCR-ABL*: 272 base pairs (bp) and 197 bp depending on the position of junction point within M-BCR and for ABL: 185 bp. Two negative controls consisting of RNA isolated from normal marrow CFU-GM and of sterile DEPC water (Diethylpyrocarbonate, Sigma, St Louis, MO) including all reagents without RNA were performed in each experiment. As positive control, RNA extracted from single K562 colonies was used. To ensure that RNA could be reverse-transcribed and subsequently amplified, control amplification using exon 2-exon 3 ABL sequence-specific

primers was performed in all samples. Only colonies with an internal positive control were considered evaluable.[25]

Nuclear DNA fragmentation

Nuclear DNA fragmentation was detected by terminal deoxynucleotidyl transferase (TdT) assay.[26] Briefly, the cells were fixed in PBS containing 4% paraformaldehyde, washed and permeabilized with 0.1% Triton X-100. After washing cells were resuspended in 50 μL of a solution containing 0.1 M sodium cacodylate, 1 mM $CoCl_2$, 0.1 mM dithiothreitol, 0.05 mg/mL bovine serum albumin, 10 U of TdT, and 0.5 nmoles FITC-conjugated biotin-16-deoxyuridine triphosphate. All chemicals and nucleotides were purchased from Boehringer Mannheim (Mannheim, Germany). The cells were incubated for 60 minutes at 37°C and analyzed by flow cytometry.

Genistein treatment

Genistein (Sigma) was diluted in dimethyl sulfoxide to prepare 1000-fold concentrated solutions that were used at a final concentration of 0.1% (v/v) to obtain the appropriate concentrations in culture. The effect of genistein was evaluated by continuous exposure and preincubation experiments. For continuous exposure experiments, MNC-AC^- (50,000/mL) were cultured throughout the entire incubation period in the presence of varying doses of genistein (1–100 μM). For each experiment appropriate controls with vehicle alone (DMSO 1 μL/dish) were set up. For preincubation experiments, MNC-AC^- (1×10^6/mL) were exposed (1–18 hours, 37°C, 5% CO_2) to either control medium (IMDM, 10% FBS) or medium containing genistein (50–200 μM). At the end of the incubation period, the cells were cultured to quantitate progenitor cell growth.

Statistical analysis

Four plates were scored for each data point per experiment and the results were expressed as the mean ± 1 standard error of the mean (±SEM). Statistical analysis was performed with the statistical package Statview (BrainPower Inc., Calabasas, CA) run on a Macintosh Performa 6300 personal computer (Apple Computer Inc., Cupertino, CA). The Student *t*-test or the Wilcoxon signed-rank test were used to test the probability of significant differences between samples. Genistein concentrations resulting in 50% inhibition (ID_{50}) of colony growth were calculated by linear regression analysis.

RESULTS

Effect of genistein on CML and normal progenitors

When CML (n=5) or normal (n=4) MNC-AC^- were exposed throughout the entire culture period to genistein (1–100 μM), a statistically significant, dose-

Table 1. PCR analysis of individual CFU-GM following continuous exposure to genistein

Case	*Untreated CFU-GM*	*PCR⁺ colonies (%)*	*Genistein-treated CFU-GM**	*PCR⁺ colonies (%)*
3	20/20#	100	6/20	30
4	12/20	60	4/19	21
5	13/20	65	2/20	10
7	25/31	81	3/8	37

**Bone marrow cells were continuously exposed to genistein (10–50 μM) added in methylcellulose culture on day 0.*

#Values represent number PCR+ colonies/number colonies tested.

dependent suppression of colony growth from multipotent and lineage-restricted progenitors was seen. Genistein doses causing 50% inhibition of CML and normal progenitors were not significantly different for CFU-Mix (27 μM versus 23 μM), BFU-E (31 μM versus 29 μM) and CFU-GM (40 μM versus 32 μM). Transient exposures (1–18 hours) of CML and normal marrow to genistein (200 μM) revealed a time-dependent suppression of CFU-Mix, BFU-E and CFU-GM growth, suggesting that the effect of the drug does involve toxicity. To investigate the effect of genistein on primitive progenitors, LTC-IC growth was evaluated. The percentages of CML-derived (n=5) LTC-IC surviving after a transient exposure to genistein (200 μM) for 1 (91 ± 9%), 2 (88 ± 6%) and 18 (32 ± 3%) hours were significantly higher ($p \leq 0.05$) than those of CML-derived CFU-Mix, BFU-E and CFU-GM.

BCR-ABL mRNA expression on single colonies

Table 1 shows that continuous exposure to genistein (10 to 50 μM) significantly reduced the mean (±SD) percentage of *BCR-ABL*⁺ progenitors (76 ± 18% versus 24 ± 12%, $p \leq 0.004$).

As shown in Table 2, preincubation of marrow cells with genistein resulted in a significant reduction of progenitors expressing *BCR-ABL* mRNA (75 ± 16 versus 21 ± 10, $p \leq 0.002$).

The capability of genistein to reduce the percentage of leukemic progenitors was also evaluated at the level of the primitive LTC-IC. In two out of five cases (Nos. 4 and 5), molecular analysis of CFU-GM produced by LTC-IC revealed a high percentage of *BCR-ABL*⁻ progenitors; therefore, the effect of genistein could be evaluated in only three cases (Nos. 3, 6, 7). As shown in Table 3, in these patients genistein treatment resulted in a marked reduction of *BCR-ABL*⁺ LTC-IC (87 ± 12% versus 37 ± 12%, $p \leq 0.003$). In two separate experiments colonies were individually harvested and split into two aliquots, one for cytogenetics and the other for RT-PCR. In genistein-treated cultures, 13 out of 60 colonies were found

Table 2. PCR analysis of individual CFU-GM following transient exposure to genistein

Case	*Untreated CFU-GM*	*PCR⁺ colonies (%)*	*Genistein-treated CFU-GM**	*PCR⁺ colonies (%)*
3	20/20#	100	6/20	30
4	12/20	60	6/20	30
5	13/20	65	2/15	13
6	12/18	67	4/17	24
7	25/31	81	1/15	7

**Bone marrow cells were preincubated with genistein (200 μM) for 2 or 18 hours.*
#Values represent number PCR⁺ colonies/number colonies tested.

Ph⁺ and 12/13 (92%) transcribed the *BCR-ABL* gene. These findings confirm that genistein-induced increase in the percentage of nonleukemic progenitors is not related to suppression of *BCR-ABL* transcription.

Nuclear DNA fragmentation

CML-derived MNC-AC⁻ or CD34⁺ cells were treated with genistein (200 μM, 18 hours) and analyzed for the presence of intracellular DNA fragmentation using terminal deoxynucleotidyl transferase assay. A significant portion of genistein-treated cells (38 ± 11% of the live gated population) was found to be in a progressive stage of apoptosis, whereas virtually no apoptotic cells were detected in the control samples (3 ± 2%).

DISCUSSION

PTKs play a crucial role in regulating hematopoietic cell proliferation.[4] Because PTKs participate in the establishment and progression of several malignant diseases, inhibitors of PTKs represent attractive antiproliferative agents. Genistein inhibits tyrosine phosphorylation events both at membrane level and distal to membrane-bound growth factor receptors,[16,27] induces apoptosis,[17] and inhibits topoisomerases I and II.[18] Genistein could prevent the phosphorylation of regulatory proteins, such as Grb-2 and Shc, that have the potential to stimulate Ras and might play a crucial role in the pathogenesis of CML.[4]

We demonstrate that genistein strongly inhibits marrow-derived CML as well as normal CFU-Mix, BFU-E, CFU-GM while sparing a substantial proportion of LTC-IC. Under the experimental conditions used in this study, both continuous and transient exposure of marrow cells to genistein induced a similar antiproliferative effect on leukemic and normal progenitors. This argues for a nonspecific inhibitory effect occurring through a complete shutoff of the PTK signaling pathways rather than a selective inhibition of *BCR-ABL* tyrosine kinase, as has been demonstrated

Table 3. PCR analysis of individual bone marrow CFU-GM produced by LTC-IC after 5 weeks in long-term culture

Case	*Untreated CFU-GM*	*PCR⁺ colonies (%)*	*Genistein-treated CFU-GM**	*PCR⁺ colonies (%)*
3	16/20#	80	7/20	35
6	30/30	100	10/20	50
7	16/20	80	4/16	25

**Bone marrow cells were preincubated with genistein (200 μM for 2 or 18 hours) and subsequently cultured in long-term.*

#Values represent number PCR⁺ colonies/number colonies tested.

for other PTK inhibitors.[28,29] DNA fragmentation analysis demonstrated that apoptosis mediates genistein-induced inhibition of progenitor cell growth.

Analysis of individual colonies for the expression of hybrid *BCR-ABL* mRNA revealed that genistein-induced growth inhibition was associated with suppression of leukemic CFU-GM and LTC-IC and reemergence of nonclonal progenitors. Overall, genistein induced a significant increase in the percentage of *BCR-ABL*⁻ primitive and committed progenitors, although a complete disappearance of leukemic progenitors was never detected. The percentage reduction of progenitors expressing the hybrid *BCR-ABL* mRNA is not caused by a suppression of *BCR-ABL* transcription, as has been described for interferon-alpha.[30] In fact, analysis of individual colonies by both cytogenetics and RT-PCR revealed similar percentages of Ph⁻ and *BCR-ABL*⁻ colonies generated from genistein-treated samples, thus excluding that genistein acts by suppressing *BCR-ABL* transcription.

The use of PTK inhibitors for marrow purging has been proposed.[29] Data reported herein demonstrate the possibility to select nonclonal LTC-IC as well as CFU-GM from CML marrow by means of a simple incubation with the PTK inhibitor genistein, thus suggesting that it may be feasible to select a population of benign progenitors from CML marrow that could be used for autografting patients without suitable allogeneic bone marrow donors. The toxicity of genistein to normal marrow progenitors could prevent its in vivo use for autografting purposes. However, other drugs, such as the cyclophosphamide derivative mafosfamide, which is at least as toxic as genistein to normal marrow progenitors but similarly to genistein spares LTC-IC, have been extensively used in vivo for autografting without evidences of clinically unacceptable damage of hematopoietic function.[11,31] An additional problem related to the in vivo use of genistein is represented by lot-to-lot changes in the biological activity of the molecule. In this respect, the recently described use of synthetic PTK inhibitors may represent an important alternative.[29]

The availability of several natural and synthetic PTK inhibitors will allow to screen for compounds with a more effective antileukemic action. Further investi-

gations are required to explore the therapeutic potential of PTK inhibitors alone or in combination with other agents, including biological response modifiers and antisense oligonucleotides, as well as their effects on highly purified hematopoietic cell subpopulations in order to improve selection of benign progenitors in CML.

REFERENCES

1. Fialkow PJ, Gartler SM, Yoshida A: Clonal origin of chronic myelocytic leukemia in man. *Proc Natl Acad Sci USA* 58:1468–1471, 1967.
2. Kurzrock R, Gutterman JU, Talpaz M: The molecular genetics of Philadelphia chromosome-positive leukemias. *N Engl J Med* 319:990–998, 1988.
3. Konopka JB, Watanabe SM, Witte ON: An alteration of the human c-abl protein in K562 leukemia cells unmasks associated tyrosine kinase activity. *Cell* 37:1035–1042, 1984.
4. Shannon K: The Ras signaling pathway and the molecular basis of myeloid leukemogenesis. *Current Opinion Hematol* 2:305–308, 1995.
5. The Italian Cooperative Study Group on Chronic Myeloid Leukemia: Interferon Alfa-2a as compared with conventional chemotherapy for the treatment of chronic myeloid Leukemia. *N Engl J Med* 330:820–825, 1994.
6. Goldman JM: Bone marrow transplantation for chronic myelogenous leukemia. *Curr Opin Oncol* 4:259–263, 1992.
7. Udomsakdi C, Eaves CJ, Swolin B et al.: Rapid decline of chronic myeloid leukemic cells in long-term culture due to a defect at the leukemic stem cell level. *Proc Natl Acad Sci USA* 89:6192–6196, 1992.
8. Daley GD, Goldman JM: Autologous transplant for CML revisited. *Exp Hematol* 21:734–737, 1993.
9. Reiffers J, Trouette R, Marit G et al.: Autologous blood stem cell transplantation for chronic granulocytic leukaemia in transformation: A report of 47 cases. *Br J Haematol* 77:339–345, 1991.
10. McGlave PB, Arthur D, Miller WJ et al.: Autologous transplantation for CML using marrow treated ex vivo with human interferon gamma. *Bone Marrow Transplant* 6:115–120, 1990.
11. Carlo-Stella C, Mangoni L, Almici C et al.: Autologous transplant for chronic myelogenous leukemia using marrow treated ex vivo with mafosfamide. *Bone Marrow Transplant* 14:425–432, 1994.
12. Skorski T, Nieborowska-Skorska M, Nicolaides NC et al.: Suppression of Philadelphia+ leukemia cell growth in mice by BCR/ABL antisense oligodeoxynucleotide. *Proc Natl Acad Sci USA* 91:4504–4508, 1994.
13. Carlo-Stella C, Mangoni L, Piovani G et al.: Identification of Philadelphia-negative granulocyte-macrophage colony-forming units generated by stroma-adherent cells from chronic myelogenous leukemia patients. *Blood* 83:1373–1380, 1994.
14. Barnett MJ, Eaves CJ, Phillips GL et al.: Autografting with cultured marrow in chronic myeloid leukemia: Results of a pilot study. *Blood* 84:724–732, 1994.
15. Levitzki A, Gazit A: Tyrosine kinase inhibition: An approach to drug development. *Science* 267:1782–1788, 1995.

16. Akiyama T, Ishida J, Nakagawa S et al.: Genistein, a specific inhibitor of tyrosine-specific protein kinase. *J Biol Chem* 262:5592–5595, 1987.
17. Traganos F, Ardelt B, Halko N et al.: Effects of genistein on the growth and cell cycle progression of normal human lymphocytes and human leukemic MOLT-4 and HL-60 cells. *Cancer Res* 52:6200–6208, 1992.
18. Okura A, Arakawa H, Oka H et al.: Effect of genistein on topoisomerase activity and on the growth of (Val 12)Ha-ras-transformed NIH cells. *Biochem Biophys Res Commun* 157:183–189, 1988.
19. Honna Y, Okabe-Kado J, Kasaukabe T et al.: Inhibition of abl oncogene tyrosine kinase induces erythroid differentiation of human myelogenous leukemia K-562 cells. *Jpn J Cancer Res* 81:1132–1136, 1990.
20. Sutherland HJ, Eaves CJ, Eaves AC et al.: Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. *Blood* 74:1563–1570, 1990.
21. Sutherland HJ, Eaves CJ, Lansdorp PM et al.: Differential regulation of primitive human hematopoietic cells in long-term cultures maintained on genetically engineered murine stromal cells. *Blood* 78:666–672, 1993.
22. Yunis JJ: New chromosome techniques in the study of human neoplasia. *Human Path* 12:540–549, 1981.
23. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987.
24. Martiat P, Maisin D, Philippe M et al.: Detection of residual BCR/ABL transcripts in chronic myeloid leukaemia patients in complete remission using the polymerase chain reaction and nested primers. *Br J Haematol* 75:355–358, 1990.
25. Melo JV, Kent NS, Yan X-H et al.: Controls for reverse transcriptase-polymerase chain reaction amplification of BCR-ABL transcripts. *Blood* 84:3984–3986, 1994.
26. Gorczyca W, Gong J, Darzynkiewicz Z: Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res* 53:1945–1953, 1993.
27. Rizzo MT, Boswell SH, Mangoni L et al.: Arachidonic acid induces c-jun gene expression in stromal cells stimulated by interleukin-1 and tumor necrosis factor: Evidence for a protein tyrosine kinase dependent process. *Blood* 86:2967–2975, 1995.
28. Anafi M, Gazit A, Gilon C et al.: Selective interactions of transforming and normal *abl* proteins with ATP, tyrosine-copolymer substrates, and tyrphostins. *J Biol Chem* 267:4518–4523, 1990.
29. Druker BJ, Tamura S, Buchdunger E et al.: Preclinical evaluation of a selective inhibitor of the Abl tyrosine kinase as a therapeutic agent for chronic myelogenous leukemia. *Blood* 86 (suppl. 1):601a, 1995.
30. Keating A, Wang X-H, Laraya P: α-interferon suppresses transcription of BCR-ABL in PH+ chronic myeloid leukemia (CML) progenitor cells. *Exp Hematol* 21:1073, 1993
31. Gorin NC, Aegerter P, Auvert B et al.: Autologous bone marrow transplantation for acute myelocytic leukemia in first remission: A European survey of the role of marrow purging. *Blood* 75:1606–1614, 1990.

BLOOD OR MARROW PROGENITORS AS AUTOGRAFT FOR CML

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ABSTRACT

Chronic myelogenous leukemia (CML) is a malignant disorder of the human hematopoietic stem cell characterized clinically by a massive expansion and premature circulation of malignant myeloid progenitors and precursors. The disease is due to the presence of the rearrangement between the *bcr* gene and the *c-abl* gene resulting in the Philadelphia chromosome. Although patients can survive for 3–5 years, the disease is invariably lethal. Therapies consist of interferon-alpha, which may "cure" 10–15% of patients and allogeneic transplantation which may "cure" 20–75% of patients, dependent on the disease state, age of the patients and type of donor graft. The invariably poor outcome in the absence of successful therapy with either interferon-alpha or allogeneic transplant has led several groups to examine the possibility of using autologous stem cells for transplantation. At the University of Minnesota, we have performed three sequential autotransplant studies. In the initial protocol, marrow was purged with interferon-gamma. Although this resulted in complete remissions in up to one-third of patients, engraftment was delayed and incomplete in up to one-third of patients. In a second cohort of patients, marrow collected after cyclophosphamide/granulocyte-macrophage colony stimulating factor (GM-CSF) mobilization was used. This led to major cytogenetic responses in 40% of patients, almost all of them with early chronic phase disease. In a recently opened study, patients underwent mobilization with mitoxantrone, cytosine arabinoside (Ara-C) and cyclophosphamide combined with granulocyte-CSF (G-CSF). Mobilized blood progenitors were then used for transplantation. Although this regimen is more toxic, it may increase the likelihood that the graft is Ph negative. However, all these grafts still contain *BCR/ABL* mRNA positive cells. Therefore, based on prior observations that the Ph negative stem cell in CML may be $CD34^+HLA\text{-}DR^-$, we have developed a large scale selection method that allows selection of $1\text{–}4\times10^5$ $CD34^+/HLA\text{-}DR^-$ cells/kg recipient body weight, which are *BCR/ABL* negative and suitable for transplantation. This approach will be used in early chronic phase CML patients. If ongoing studies demonstrate that the number of Ph negative $CD34^+$ cells present in mobilized blood products is higher than that in steady state marrow, the in vivo mobilization and ex vivo selection protocols could be merged.

INTRODUCTION

CML is the result of a monoclonal expansion of a transformed, pluripotent stem cell. Myeloid, erythroid and megakaryocytic[1] and less frequently lymphoid cells[2] arise from the leukemic clone. CML is characterized by a reciprocal translocation between chromosomes 9 and 22, termed the Philadelphia chromosome (Ph).[3] At the molecular level, the Ph is the result of a rearrangement between the *BCR* gene (break-point cluster region) located on chromosome 22 and the *ABL* gene located on chromosome 9.[4] Even though CML may have a prolonged course, the disease is invariably lethal. Allogeneic bone marrow transplantation (BMT) using related or unrelated HLA-matched or closely matched BM is the only curative treatment modality for CML.[5,6] Due to immunological disparity, this procedure results in significant mortality and morbidity caused by graft failure and graft-versus-host disease.[7] Furthermore, up to 60% of CML patients are ineligible for alloBMT because a suitable donor cannot be located or because of age.

These observations have led to clinical trials testing the hypothesis that autologous marrow or peripheral blood transplant after cytoreduction with combination high-dose chemotherapy or chemoradiotherapy may be an effective treatment for CML.[8–18] A recent meta-analysis of 200 autologous transplants performed as therapy for CML at eight institutions demonstrates that such therapy provides prolonged survival that may exceed that associated with conventional therapy.[13] The autologous transplant approach is associated with high primary engraftment rates, low transplant mortality and prompt return to normal activity levels in both younger and older recipients. Transplant in chronic phase rather than after onset of advanced disease provides better outcome.

AUTOGRAFTS: EX VIVO PURGING

At the University of Minnesota we have performed 44 autologous marrow transplants as therapy for CML[9] using autologous marrow subjected to ex vivo purging with 1000 U interferon-gamma. Although the overall clinical course of recipients is favorable, transplants are associated with several shortcomings. A greater than 30% incidence of graft failure was seen, suggesting that "purging" of autologous marrow with interferon-gamma in vitro may have a toxic effect on benign progenitors. Regeneration of 100% Ph positive hematopoiesis occurred in 66% of patients and restoration of Ph negative hematopoiesis following transplant was seen in only 26% of patients, suggesting that the interferon-gamma purging may not be an efficient method for selecting benign hematopoietic progenitors in most patients.

In Vancouver,[10] autologous marrow grafts are purged by long-term ex vivo culture. Eighty-seven patients were studied. These screening studies demonstrated that ex vivo marrow cultures of 22 of 87 patients resulted in elimination of the Ph

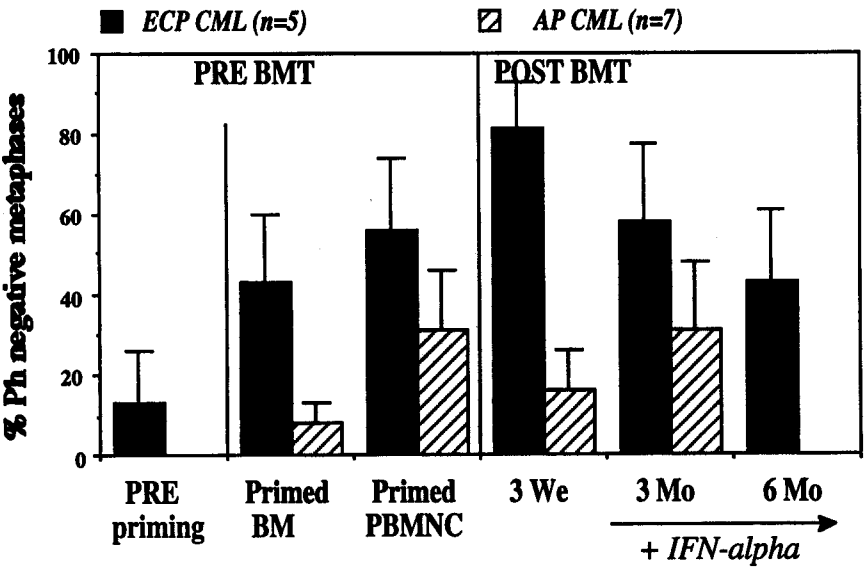

Figure 1. *Cytogenetics after transplants within marrow collected after cyclophosphamide/GM-CSF mobilization. Patients with Ph positive CML were treated with 4 g/m² cyclophosphamide and GM-CSF (250 μg/m²). When the ANC was >0.5×10⁹/L, marrow was harvested under general anesthesia and cryopreserved. Patients underwent conditioning with cyclophosphamide/TBI and were transplanted with the "mobilized" marrow graft. Cytogenetics were performed on marrow and blood before cyclophosphamide/GM-CSF mobilization, on the marrow harvest as well as on a blood sample obtained after mobilization and at 3 weeks, 3 months, 6 months, 9 months and 12 months after transplant.*

positive clone. These patients underwent autografting with ex vivo cultured marrow grafts. Graft failure was seen in 23% of patients. Thirteen of 22 patients achieved a complete cytogenetic response which lasted 4–36 months. Carlo Stella et al.,[11] used ex vivo incubation with mafosfamide to purge malignant progenitors. Twenty-two patients were screened. In 10 patients, >50% of stroma-adherent progenitors treated with mafosfamide were Ph negative, the minimum requirement for enrollment of patients in a subsequent autologous transplant protocol. These 10 patients underwent autografting with mafosfamide-purged marrow. All patients had timely recovery of peripheral counts. Six of 10 patients achieved a complete cytogenetic remission that lasted for 6.5 months.

These observations suggest that autologous marrow transplant therapy for CML may have a beneficial effect on outcome. However, improvements in the methods used to select hematopoietic progenitors used for autologous transplantation will be

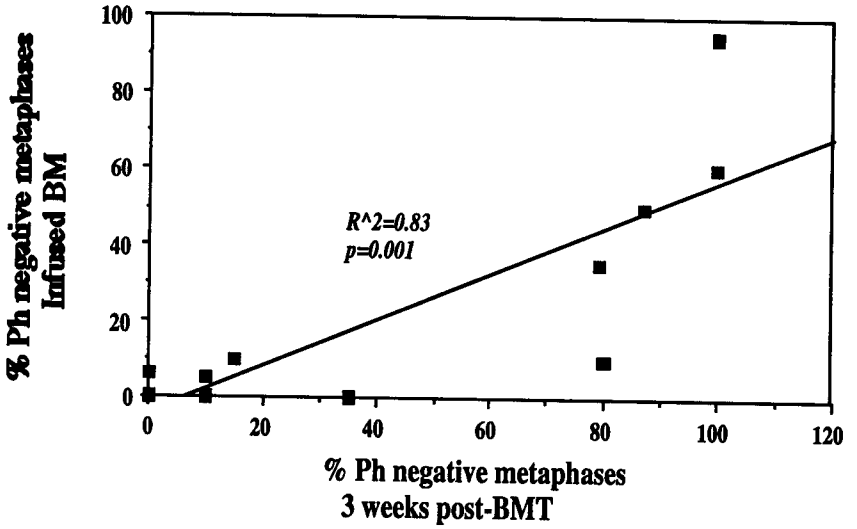

Figure 2. *The number of Ph negative metaphases present in the infused mobilized marrow cells correlates significantly with the Ph status 3 weeks after transplant. Patients with Ph positive CML were treated with 4 g/m^2 cyclophosphamide and GM-CSF (250 μg/m^2). When the ANC was >0.5×10^9/L, marrow was harvested under general anesthesia and cryopreserved. Patients underwent conditioning with cyclophosphamide/TBI and were transplanted with the "mobilized" marrow graft. Cytogenetics were performed on the marrow harvest obtained after mobilization and at 3 weeks after transplant. Statistics: The Spearman correlation coefficient was determined to compare the percent Ph negative metaphases present in the marrow inoculum and at 3 weeks after transplant.*

needed to a) induce a Ph negative state following transplant and b) result in timely multilineage engraftment.

AUTOGRAFTING: IN VIVO MOBILIZATION OF BENIGN PROGENITORS WITH CHEMOTHERAPY/CYTOKINES PRIOR TO AUTOGRAFTING

Treatment of CML patients with high-dose chemotherapy followed by harvest of marrow or peripheral blood as the hematopoietic compartment recovers from the high-dose therapy increases the relative and absolute number of benign primitive progenitors available for transplantation.[11,14–18]

In a second series of autologous transplants for CML at the University of Minnesota involving 15 patients, marrow progenitors and peripheral blood progenitors were collected after high dose cyclophosphamide (4 gm/m^2) and GM-

CSF priming. Mobilized PBMNC from 2/12 patients were 100% Ph negative and >50% Ph negative in an additional 3 of 12 patients. Marrow metaphases immediately after mobilization were 100% Ph negative in 1 patient and >50% Ph negative in 1 additional patient. Prompt engraftment was observed in all but 1 patient transplanted with mobilized marrow progenitors. Following transplant with the cyclophosphamide/GM-CSF mobilized marrow graft, recovering marrow demonstrated variable degrees of Ph positivity. A major cytogenetic response (>60% Ph negative metaphases) was seen in 5 of 12 patients evaluated thus far, four of whom had early chronic phase CML and one had late chronic phase CML (Figure 1). The number of Ph negative metaphases present in marrow obtained both 3 weeks and 3 months after transplant was highly correlated with the percentage of Ph negative cells infused (Figure 2). This indicates that use of a less contaminated graft may increase the likelihood of reestablishing Ph negative hematopoiesis and underlines the need for improved in vivo or ex vivo selection methods.

One possibility to increase the likelihood of obtaining a Ph negative graft is to intensify further the chemotherapeutic regimen used for mobilization. Several recently reported studies from other institutions have described the use of the ICE chemotherapy regimen (idarubicin 8 mg/m^2 for 2–5 days, cytosine arabinoside 800–1000 mg/m^2 for 5 days with or without etoposide 150 mg/m^2 for 5 days) or variants. These studies demonstrate that higher dose intensity regimens may mobilize benign, Ph negative progenitors in blood (and marrow) of between 25 and 50% of "good risk," early chronic phase CML patients.[11,14–18]

At the University of Minnesota, we have recently started to examine the effect of mobilization with mitoxantrone (8 mg/m^2, for two days), Ara-C (1 g/m^2, twice daily, day 1 and 2) and cyclophosphamide (4 gm/m^2, day 1) in combination with G-CSF (from day 5 until end of harvest), abbreviated MAC-G, on the mobilization of Ph negative cells in blood and marrow. Although this study has only recently been initiated and results are preliminary, some conclusions can be drawn. First, as has been shown by Carella and others, the higher intensity chemotherapeutic regimen, MAC-G, results in a more profound depletion of cells from the marrow. Therefore, a marrow harvest after the mobilization regimen could not be done. Second, almost 100% Ph negative cells can be collected from the blood of some patients with chronic phase CML. However, >50% of mobilizations do not result in Ph negativity in either marrow or blood. Third, reinfusion of the mobilized peripheral blood progenitors results in a significantly faster rate of engraftment (7–20 days, mean 11 days to ANC>500 for three days) than the cyclophosphamide/GM-CSF mobilized marrow or ex vivo interferon-γ purged marrow graft. However, the more intensive MAC-G mobilization regimen is associated with a higher morbidity due to infections at the time of mobilization. Thus, although these results are encouraging, benign progenitors cannot be collected from a large number of patients with CML suitable for autografting. Furthermore,

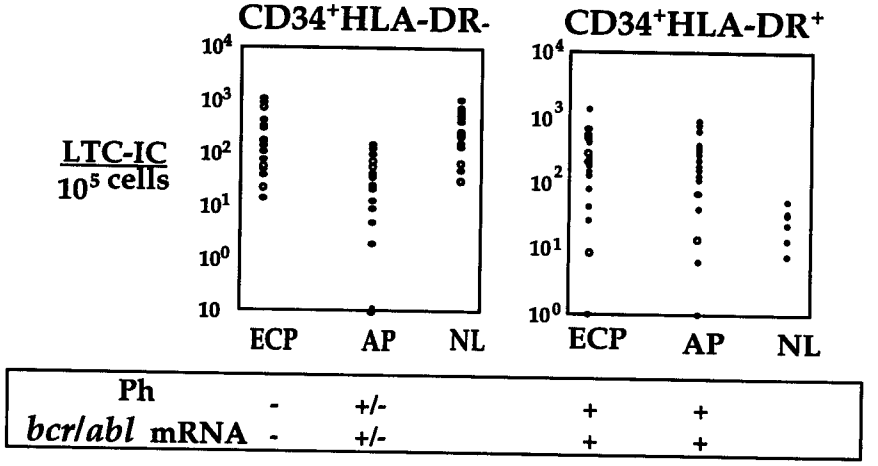

Figure 3. *$CD34^+HLA\text{-}DR^-$ cells from ECP CML patients contain large numbers of LTC-IC that are BCR/ABL mRNA And Ph negative. Marrows of patients with ECP CML, patients with AP CML and normal individuals were subjected to $CD34^+$ enrichment by either sequential counterflow centrifugation and immunomagnetic depletion of lineage committed cells or by avidin-biotin selection of $CD34^+$ cells. $CD34^+$ enriched cells were then sorted by FACS into a $CD34^+HLA\text{-}DR^-$ and $CD34^+HLA\text{-}DR^+$ cell population. FACS selected cells were then plated in long-term marrow culture for 5 weeks to enumerate the number of LTC-IC present. Cells obtained after the FACS sort or after 1 or 5 weeks of LTBMC were subjected to RT-PCR, cytogenetic exam or FISH to determine the presence or absence of BCR/ABL mRNA or Ph positive cells.*

preliminary studies indicate that in the vast majority of patients, BCR/ABL mRNA positive cells continue to be present in the mobilized product.

EX VIVO POSITIVE SELECTION OF PH AND BCR/ABL NEGATIVE PROGENITORS SUITABLE FOR TRANSPLANTATION (FIGURE 3)

Hematopoietic stem cells are defined as primitive progenitors that can provide long-term reconstitution of both myeloid and lymphoid cell lineages in a lethally irradiated host. We and others have identified primitive human hematopoietic progenitors capable of initiating and sustaining ex vivo long-term cultures for at least 12 weeks. These progenitors are lineage negative,[20–23] CD34 positive,[20–27] express low levels of Thy-1[23] and HLA-DR antigens,[20,22,24] but not CD38 antigens.[25] These cell populations contain cells that can differentiate in myeloid,[20–25] B-lymphoid,[25] natural killer cell[28,29] and T-lineages[24,25] when assayed in vitro or in xenogeneic recipients. Over the last several years, a number of groups

have examined the phenotypic characteristics of Ph negative and Ph positive primitive progenitors in the steady state marrow or blood of patients with CML.[30–34]

In initial studies, we selected lineage negative ($CD2^-CD11b^-CD15^-CD16^-CD19^-$) $CD34^+HLA\text{-}DR^-$ cells from BM of patients with early chronic phase (ECP, less than 1 year from diagnosis) CML, more advanced phase (AP) CML (chronic phase but more than 1 year after diagnosis and accelerated phase as defined by classical criteria) and normals by a multistep selection method including Ficoll-Hypaque separation, counterflow elutriation, sheep erythrocyte rosetting, immunomagnetic bead depletion followed by a four-parameter FACS sort[30]. These studies demonstrated that the total number of $Lin^-CD34^+HLA\text{-}DR^-$ cells, thought to contain the hematopoietic stem cell, recovered from 10 cc BM obtained from normals or patients with ECP CML was not significantly different. However, significantly fewer $Lin\text{-}CD34^+HLA\text{-}DR^-$ cells were present in 10 cc of AP CML marrow than in normal or ECP CML marrow. Normal and ECP CML $Lin^-CD34^+HLA\text{-}DR^-$ cells contained an equivalent number of primitive progenitors that can initiate and sustain ex vivo long-term cultures (LTC-IC). $Lin^-CD34^+HLA\text{-}DR^-$ cells from the majority of ECP patients were *BCR/ABL* negative when examined either immediately following FACS selection (3 of 3 patients) or after 1–2 weeks (8 of 8 patients) or 5 weeks (10 of 13 patients) of culture. All $Lin^-CD34^+HLA\text{-}DR^-$ cells that were *BCR/ABL* mRNA negative on day 0 or after 1 week of culture remained *BCR/ABL* negative at 5 weeks of culture. In contrast to $Lin^-CD34^+HLA\text{-}DR^-$ cells selected from ECP CML marrow, $Lin^-CD34^+HLA\text{-}DR^-$ cells from >50% of AP CML patients were contaminated with *BCR/ABL* mRNA and Ph positive cells and LTC-IC. These studies demonstrate that $Lin^-CD34^+HLA\text{-}DR^-$ cells obtained from 80% of ECP CML patients (12 of 15 patients) are highly enriched in *BCR/ABL* mRNA and Ph negative cells and LTC-IC, and may thus contain benign stem cells. In contrast to normal $Lin^-CD34^+HLA\text{-}DR^-$ cells, ECP and AP CML $Lin^-CD34^+HLA\text{-}DR^-$ cells contained LTC-IC which in all but one ECP CML patients and all AP CML patients expressed the *BCR/ABL* mRNA when analyzed immediately following the FACS sort or following culture for 1 or 5 weeks in long-term cultures. These results were confirmed at the genomic level by cytogenetic analysis of secondary CFC derived from DR^+ progeny, which were Ph positive when analyzed at 1–2 and 5 weeks after culture (Figure 3).

We also examined if $CD34^+HLA\text{-}DR^-$ cells selected using clinically applicable methods have the same functional and clonal characteristics as $Lin^-CD34^+HLA\text{-}DR^-$ cells obtained using the multistep depletion method. Normal, ECP CML and AP CML BM was subjected to Ficoll-Hypaque separation, biotin-avidin column selection (CEPRATE Lab Columns, CellPro, Bothell, WA) for $CD34^+$ cells and FACS selection for $CD34^+HLA\text{-}DR^-$ cells. The number of $CD34^+HLA\text{-}DR^-$ cells

Table 1. Large scale selection by COBE spectra + Ceprate SC cell concentrator

Cell population	*Volume*	*Cells*	*% cell yield*	*%CD34⁺*	*%CD34⁺ yield*	*% CFC yield*
marrow harvest	422 ± 61(6) (mL)	18.3 ± 3.6 (6) ($\times 10^9$)				
COBE Spectra		5.6 ± 3.3 (6) ($\times 10^9$)	35 ± 8% (6)	1.4 ± 0.3% (4)		
$CD34^+$ cells		71 ± 24 (6) ($\times 10^6$)	1.4 ± 0.4% (6)	60.6 ± 7.8% (6)	52 ± 26% (4)	62 ± 19% (4)

Numbers between brackets represent number of different large scale selections performed.

Table 2. Large scale FACS selection of BCR/ABL mRNA negative $CD34^+HLA\text{-}DR^-$ cells

Cell population	*Patient # 1 (600cc)*		*Patient # 2 (210 cc)*	
	Yield	*BCR/ABL*	*Yield*	*BCR/ABL*
harvest ($\times 10^9$)	30.7		14	
BMMNC ($\times 10^9$)	6.8	+	2.1	+
$CD34^+$ (CEPRATETM) ($\times 10^6$)	98	+	38	+
$CD34^+HLA\text{-}DR^-$ ($\times 10^6$)	1.6 [1.31×105]	-	1.52 [3.01×105]	–
$CD34^+HLA\text{-}DR^+$ ($\times 10^6$)	43.8	+	12.6	+
CFC/1000 DR^- cells	26.5 [0.173×10^4]		62 [0.93×10^4]	
% LTC-IC (per 100 DR^- cells)	0.233 [0.153×10^3]	-	0.178 [0.267×10^3]	–

[x] Numbers between brackets are cells/CFC/LTC-IC per kg (numbers have been extrapolated for a full harvest of 2.5L).

that was obtained by sequential Ficoll, CD34-enrichment and FACS selection from marrow of ECP CML patients was higher than the $Lin^-CD34^+HLA\text{-}DR^-$ cell fraction obtained after using the "research" method. Likewise, the number of LTC-IC in ECP CML $CD34^+HLA\text{-}DR^-$ cells obtained following Ficoll, CD34 enrichment and FACS sort was similar to that seen in $Lin^-CD34^+HLA\text{-}DR^-$ cell populations obtained using the multistep "research" selection procedure. $CD34^+HLA\text{-}DR^-$ cells obtained by sequential ficoll, CD34 enrichment and FACS sorting from 8 of 9 ECP CML patients were *BCR/ABL* negative and LTC-IC present in these cells were *BCR/ABL* mRNA negative in 3/3 patients. Furthermore, $CD34^+HLA\text{-}DR^-$ cells examined immediately following FACS selection were also Ph negative at the genomic level (FISH negative for the *BCR/ABL* gene rearrangement in 2 of 2 patients) (Figure 3).

These studies demonstrate that a sufficiently large number of $CD34^+HLA\text{-}DR^-$ cells that are *BCR/ABL* mRNA and *BCR/ABL* DNA negative and contain "normal" numbers of LTC-IC can be selected from the steady state marrow of >80% of patients with early chronic phase CML suitable for transplantation. However, once the disease exists for a longer period of time (late chronic phase) or has features of accelerated phase disease, the number of $CD34^+HLA\text{-}DR^-$ cells that can be collected decreases, and these cells can be contaminated with *BCR/ABL* mRNA and DNA positive cells and LTC-IC in >50% of AP CML patients.

DEVELOPMENT OF A LARGE SCALE SELECTION METHOD

For clinical transplants with ex vivo selected $CD34^+HLA\text{-}DR^-$ cells, we developed the following sequential selection method: 1) ficoll-hypaque separation was replaced by a COBE Spectra mononuclear cell separation; 2) large scale $CD34^+$ enrichment using the CEPRATE SC Cell Concentrator; 3) high speed cell sorting for $CD34^+HLA\text{-}DR^-$ cells.

Two hundred to 600 cc of marrow from CML patients undergoing bone marrow harvest to serve as back-up stem cells for autologous or allogeneic unrelated transplants after informed consent was obtained using guidelines from the Human Subjects Committee at the University of Minnesota. Mononuclear cells were collected using COBE Spectra apheresis system. The total cell number present in the harvested cell populations was $18.3 \pm 3.6 \times 10^9$ cells. Following COBE™ Spectra separation the total cell number was $5.6 \pm 3.3 \times 10^9$ cells or 35 ± 8% yield (Table 1). Mononuclear cells were incubated with biotinylated anti-CD34 antibody at room temperature, washed and loaded onto the CEPRATE SC Cell Concentrator. CD34 unabsorbed cells were collected and after mechanical stirring in the column, CD34 adsorbed cells were collected. As for BMMNC or PBMNC obtained from patients with other diseases, 52 ± 26% of all $CD34^+$ cells and 61 ± 19% of CFC were recovered in the $CD34^+$ absorbed fraction which

contained on average 60.6 ± 7.8% $CD34^+$ cells.

To make clinical scale fluorescence activated cell sorting of $CD34^+HLA\text{-}DR^-$ cells from CML marrow possible, the fluidic system and software of the FACStar^{Plus} Flow Cytometry System at the University of Minnesota was modified by Becton Dickinson. This upgrade resulted in the capacity to FACS select 10,000–12,500 cells per second, resulting in >60% recovery with a >94% purity and >95% viability. $CD34^+$ cell enriched marrow of 2 patients was labeled with anti-CD34-biotin antibodies, anti-HLA-DR-FITC antibodies and Streptavidin-PE label, all of which are clinical grade. Cells were washed and sorted at 10,000–12,000 cells/min, into a $CD34^+HLA\text{-}DR^-$ and $CD34^+HLA\text{-}DR^+$ population using gates based on low horizontal and side scatter properties and on isotype matched control stains. The resulting cell yields, CFC and LTC-IC yields, as well as the results from RT-PCR analysis of freshly sorted $CD34^+HLA\text{-}DR^-$ and $CD34^+HLA\text{-}DR^+$ cells are summarized in Table 2. A calculated total number of 1.31×10^5 $CD34^+HLA\text{-}DR^-$ cells/kg and 3.01×10^5 $CD34^+HLA\text{-}DR^-$ cells/kg was collected after FACS sorting. Culture of the $CD34^+HLA\text{-}DR^-$ cells in methylcellulose progenitor culture demonstrated the presence of 26.5 and 62 CFC/1000 $CD34^+HLA\text{-}DR^-$ cells, and culture in limiting dilutions onto stromal feeders for 5 weeks demonstrated 0.233% and 0.187% LTC-IC in the $CD34^+HLA\text{-}DR^-$ cells. RT-PCR analysis of the sorted cells demonstrated that the $CD34^+HLA\text{-}DR^-$ but not the BMMNC, $CD34^+$ cells or $CD34^+HLA\text{-}DR^+$ were *BCR/ABL* mRNA negative. RT-PCR analysis of week 5 CFC demonstrated that LTC-IC were *BCR/ABL* mRNA negative.

The exact number of FACS selected $CD34^+HLA\text{-}DR^-$ cells that is required for successful, durable engraftment following autologous transplantation has not yet been determined. Autologous transplantation with partially purified $CD34^+$ cells collected from either marrow or peripheral blood has been performed in humans for the last 5 years.[35–39] These studies have demonstrated that the reinfusion of $>0.5\times10^6$ $CD34^+$ cells/kg recipient body weight results in timely (between day 10 and day 28) and durable engraftment. Recent studies in which patients with multiple myeloma underwent autotransplants with $CD34^+Thy1.1^+$ cells selected from chemotherapy/cytokine mobilized peripheral blood stem cells have indicated that reinfusion of $2\text{–}8\times10^5$ $CD34^+Thy1.1^+$ cells from PBPC results in neutrophil recovery on average at 20 days after transplant.[40] As for $CD34^+Thy1.1^+$ cells, $CD34^+HLA\text{-}DR^-$ cells are highly enriched in primitive progenitors, characterized in vitro as LTC-IC.[19] Since the majority of LTC-IC present in human $CD34^+$ cells are recovered in the $CD34^+HLA\text{-}DR^-$ cell fraction and approximately 10% of cells within the $CD34^+$ cell population is $CD34^+HLA\text{-}DR^-$ cells.[20] Thus, we believe that $1\text{–}2\times10^5$ $CD34^+HLA\text{-}DR^-$ cells/kg should be sufficient for engraftment.

These studies indicate that large scale clinical selection of $CD34^+HLA\text{-}DR^-$ cells in sufficient quantities for transplantation, which contain *BCR/ABL* mRNA

negative cells and LTC-IC, is possible. A protocol encompassing this approach, combined with ex vivo expansion of a fraction of the Ph negative progenitor pool and retroviral marking to determine the contribution of the graft to engraftment and possibly relapse after transplant, has recently obtained FDA approval and will be started at the University of Minnesota in early 1997.

PROBLEMS THAT REMAIN

1) Although ex vivo positive selection of $CD34^+HLA\text{-}DR^-$ cells can provide a graft which is significantly enriched in Ph and *BCR/ABL* mRNA negative primitive progenitors, the methods outlined above yield a sufficient number of cells in only a fraction of ECP CML patients. Thus approaches that will increase the number of Ph negative stem cells will need to be examined. We are currently examining the phenotype and absolute number of Ph negative progenitors in marrow and blood obtained after in vivo mobilization with MAC-G. If these studies demonstrate that the total number of Ph negative $CD34^+$ cells that can be collected is significantly higher than the number that can be obtained from steady state marrow and/or that Ph negative $CD34^+$ cells can be obtained from patients with more advanced disease, the in vivo mobilization approach could be merged with the ex vivo positive selection of Ph negative cells. This would then allow transplantation of a larger number of patients with grafts containing almost exclusively Ph negative stem cells.

2) The second problem that will need to be addressed is the high likelihood of persistent disease after transplant. Since allogeneic transplants for CML are associated with relapses occurring at different time points after transplant, it is clear that the preparative regimen used for transplantation (cyclophosphamide/TBI or cyclophosphamide/busulfan) does not completely eliminate the malignant clonogenic cell pool in patients with CML.[41] Resurgence of malignant hematopoiesis following autologous transplantation in CML is therefore likely not only due to incomplete removal of the malignant clone from the autologous marrow and/or peripheral blood inoculum,[42] but also from persistent disease in the host after the preparative regimen. Approaches to maintain a Ph negative state after transplant are currently being evaluated. These include, interferon-alpha treatment,[43] treatment with interleukin-2 either alone[44] or in combination with activated natural killer cells or T cells,[45,46] as is discussed elsewhere in this issue.

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REFERENCES

1. Fialkow PJ, Jacobson RJ, Papayannopoulou TH: Chronic myelocytic leukemia: Clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. *Am J Med* 63:125–130, 1977.
2. Jonas D, Lubbert M, Kawasaki ES: Clonal analysis of bcr-abl rearrangement in T lymphocytes from patients with chronic myelogenous leukemia. *Blood* 79:1017–1023, 1992.
3. Rowley JD: The Philadelphia chromosome translocation: A paradigm for understanding leukemia. *Cancer* 65:2178–2184, 1990.
4. Bartram CR, deKlein A, Hagemeijer A et al.: Translocation of the c-abl oncogene adjacent to a translocation break point in chronic myelocytic leukemia. *Nature* 1983:306:277-280.
5. Snyder DS, McGlave PB: Treatment of chronic myelogenous leukemia with bone marrow transplantation. *Hematol/Oncol Clin North Am* 4:535–557, 1990.
6. McGlave PB, Bartsch G, Anasetti C et al.: Unrelated donor bone marrow transplantation therapy for chronic myelogenous leukemia: Initial experience of the National Marrow Donor Program (NMDP). *Blood* 81:543–550, 1993.
7. Weisdorf D, Haake R, Blazar R et al.: Risk factors for acute graft-versus-host disease in histocompatible donor bone marrow transplantation. *Transplantation* 51:1197–1203, 1991.
8. Barnett MJ, Eaves CH, Phillips GL et al.: Successful autografting in chronic myeloid leukemia after maintenance of marrow in culture. *Bone Marrow Transplant* 4:345–351, 1989.
9. McGlave PB, Arthur D, Miller WJ, Lasky L, Kersey J: Autologous transplantation for CML using marrow treated ex vivo with recombinant human interferon gamma. *Bone Marrow Transplant* 6:115–120, 1990.
10. Carlo-Stella C, Mangoni L, Piovani G et al.: In vitro marrow purging in chronic myelogenous leukemia: Effect of mafosfamide and recombinant granulocyte-macrophage colony-stimulating factor. *Bone Marrow Transplant* 8:265–273, 1991.
11. Carella AM, Podesta M, Grassoni F et al.: Selective overshoot of Ph-negative blood hemopoietic cells after an intensive idarubicin-containing regimen and their repopulating capacity after reinfusion. *J Hematotherapy* 3:199–202, 1994.
13. McGlave PB, DeFabritiis P, Deisseroth A et al.: Autologous transplant therapy for chronic myelogenous leukemia prolongs survival: Results from eight transplant centers. *Lancet* 343:1486–1488, 1994.
14. Talpaz M, Kantarjian H, Liang J et al.: Percentage of Philadelphia chromosome (PH)-negative and Ph-positive cells found after autologous transplantation for chronic myelogenous leukemia depends on the percentage of diploid cells induced by conventional-dose chemotherapy before collection of autologous cells. *Blood* 85:3257–3263, 1995.
15. Simmonsson B, Olberg G, Kilander A et al.: Intensive treatment in order to minimize the Ph-positive clone in chronic myelogenous leukemia. *Stem Cells* 111(suppl 3):73–76, 1993.
16. Durant S, Taylor K, Moore D et al.: Bone marrow transplant following intensive

chemotherapy with filgrestim support and progenitor collection in advanced chronic myelogenous leukemia. *Bone Marrow Transplant* 15:S19, 1995.

17. O'Brien SG, Rule S, Spencer A et al.: Autografting in chronic phase CML using PBPCs mobilized by intermediate dose chemotherapy. *Bone Marrow Transplant* 15:S11, 1995.
18. Chalmers EA, Franklin IM, Kelsey S et al.: Mobilization of Ph-negative progenitors into the peripheral blood in chronic myelogenous leukemia (CML) using idarubicin and cytarabin. *Blood* 84 (10 suppl 1):400A, 1994.
19. Weilbaecher K, Weissman I, Blume K, Heimfeld S: Culture of phenotypically defined hematopoietic stem cells and other progenitors at limiting dilution on Dexter monolayers. *Blood* 78:945–952, 1991.
20. Verfaillie C, Blakolmer K, McGlave P: Purified primitive human hematopoietic progenitor cells with long-term in vitro repopulating capacity adhere selectively to irradiated BM stroma. *J Exp Med* 172:509–520, 1990.
21. Andrews RG, Singer JW, Bernstein ID: Human hematopoietic precursors in long term culture: Single $CD34^+$ cells that lack detectable T cell, B cell and myeloid antigens produce multiple colony forming cells when cultured with marrow stromal cells. *J Exp Med* 172:355–358, 1990.
22. Brandt J, Baird N, Lu L, Srour E, Hoffman R: Characterization of a human hematopoietic progenitor cell capable of forming blast cell containing colonies in vitro. *J Clin Invest* 82:1017–1027, 1988.
23. Baum CM, Weissman IL, Tsukamoto AS, Buckle A-M, Peault B: Isolation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci USA 89:2804–2808, 1992
24. Sutherland HJ, Eaves CJ, Eaves AC, Dragowska W, Landsdorp PM: Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. *Blood* 74:1563-1570, 1989.
25. Terstappen LWMM, Huang S, Safford M, Landsdorp PM, Loken MR: Sequential generation of hematopoietic colonies derived from single nonlineage-committed $CD34^+$ $CD38^-$ progenitor cells. *Blood* 77:1218–1227, 1991.
26. Srour EF, Leemhuis T, Brandt JE, Redmond R: Detection of T lymphocyte cells within the human $CD34^+$ $HLA\text{-}DR^-$ $CD7^-$ population of bone marrow cells. *Blood* 76:674a, 1990.
27. Srour EF, Zanjani ED, Cornetta K et al.: Persistence of human multilineage, self-renewing lymphohematopoietic stem cells in chimeric sheep. *Blood* 82:3333–3342, 1993.
28. Miller JS, Verfaillie CM, McGlave PB: The generation of natural killer cells from $CD34^+HLA\text{-}DR^-$ primitive progenitors in human long term bone marrow culture. *Blood* 80:2181,1992.
30. Verfaillie CM, Miller WJ, Boylan K, McGlave PB: Selection of benign primitive hematopoietic progenitors in chronic myelogenous leukemia on the basis of HLA-DR antigen expression. *Blood* 79:1003–1010, 1992.
31. Leemhuis T, Leibowitz D, Cox G, Silver R, Srour EF, Tricot G, Hoffman R: Identification of *BCR/ABL*-negative primitive hematopoietic progenitor cells within chronic myeloid leukemia marrow. *Blood* 81:801, 1993.
32. Kirk JA, Reems JA, Roecklein BA, Van Devanter DR, Bryant EM, Radich J, Edmands S, Lee A, Torok-Storb B: Benign marrow progenitors are enriched in the $CD34^+/HLA^-DR^{lo}$ population but not the $CD34^+/CD38^{lo}$ population of chronic myeloid leukemia: An

analysis using interphase fluorescence hybridization. *Blood* 86:737, 1995.
33. Negrin R, Weissman I: Hematopoietic stem cells in normal and malignant states. *Marrow Transplant Rev* 2:23, 1992.
34. Verfaillie CM, Bhatia R, Miller W, Mortari F, Roy V, Stiegelbauer K, Dewald G, Miller JS, McGlave PB: Benign primitive progenitors can be selected on the basis of the $CD34^+/HLA\text{-}DR^-$ phenotype in early chronic phase but not advanced phase CML. *Blood* 87:4770–4779, 1996.
35. Berenson RJ, Bensinger WI, Hill RS, Andrews RG, Garcia-Lopez J, Kalamasz DF, Still BJ, Spitzer G, Buckner CD, Bernstein ID et al.: Engraftment after infusion of $CD34^+$ marrow cells in patients with breast cancer or neuroblastoma. *Blood* 77:1717–22, 1991.
36. Andrews RG, Bryant EM, Bartelmez SH, Muirhead DY, Knitter GH, Bensinger W, Strong DM, Bernstein ID: $CD34^+$ marrow cells, devoid of T and B lymphocytes, reconstitute stable lymphopoiesis and myelopoiesis in lethally irradiated allogeneic baboons. *Blood* 80(7):1693–701, 1992.
37. Shpall EJ, Jones RB, Bearman SI, Purdy MH, Franklin WA, Heimfeld S, Berenson RJ: Transplantation of $CD34^+$ hematopoietic progenitor cells. *J Hematother* 3:145–7, 1994.
38. Dunbar CE, O'Shaughnessy JA, Cottler-Fox M et al.: Transplantation of retrovirally-marked $CD34^+$ bone marrow and peripheral blood cells in patients with multiple myeloma or breast cancer. *Blood* 1995.
39. Brugger W, Henschler R, Heimfeld S, Berenson RJ, Mertelsmann R, Kanz L: Positively selected autologous blood $CD34^+$ cells and unseparated peripheral blood progenitor cells mediate identical hematopoietic engraftment after high-dose VP16, ifosfamide, carboplatin, and epirubicin. *Blood* 1994.
40. Tricot G, Gazitt Y, Jagannath S, Vesole D, Reading C, Juttner C, Hoffman R, Barlogie B: $CD34^+Thy1^+Lin^-$ peripheral blood stem cells (PBSC) effect timely trilineage engraftment in multiple myeloma (MM). *Blood* 86 (10 suppl. 1):293a, 1995.
41. Enright H, Davies, SM, DeFor T, Shu X, Weisdorf D, Miller W, Ramsay NKC, Arthur D, Verfaillie C, Miller J, Kersey J, McGlave P: Relapse following non-T-Cell depleted allogeneic bone marrow transplant for CML: Early transplant, the use of an unrelated donor and chronic graft-versus-host disease are protective. *Blood* 88:714–720, 1996.
42. Deisseroth AB, Zhifei Z, Claxton D et al.: Genetic marking shows that Ph^+ cells present in autologous marrow of chronic myelogenous leukemia (CML) contribute to relapse after autologous bone marrow transplants in CML. *Blood* 83:3068–3076, 1994.
43. Higano CS, Raskind WH, Singer JW: Use of α–interferon for the treatment of relapse of chronic myelogenous leukemia in chronic phase after allogeneic bone marrow transplantation. *Blood* 80:1437, 1992.
44. Charak BS, Agah R, Gray D, Mazumder A: Interaction of various cytokines with interleukin 2 in the generation of killer cells from human bone marrow: Application in purging of leukemia. *Leuk Res* 15:801, 1991.
45. Mackinnon S, Hows JM, Goldman JM: Induction of in vitro graft-versus-leukemia activity following bone marrow transplantation for chronic myeloid leukemia. *Blood* 76:2037, 1990.
46. Cervantes P, McGlave PB, Verfaillie CM, Miller JS: Autologous activated natural killer cells suppress primitive chronic myelogenous leukemia progenitors in long-term culture. In Press *Blood* 1996.

CHAPTER 4
Lymphoma

BONE MARROW TRANSPLANTATION IN FOLLICULAR NON-HODGKIN'S LYMPHOMA: EUROPEAN CUP/UP TRIAL

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ABSTRACT

High-dose therapy followed by stem cell transplantation is increasingly applied in follicular non-Hodgkin's lymphoma (NHL). However, its value and the need for purging have never been proven. To assess the impact of high-dose therapy and purging, a randomized trial was initiated in patients with relapsed follicular NHL. The design of the study and the first interim results are presented. The trial is still accruing patients.

INTRODUCTION

About one-third of all patients with non-Hodgkin's lymphoma (NHL) have a follicular histology. The majority of these patients have stage III or IV disease at diagnosis and a median age of more than 50 years. Although chemotherapy can induce complete remissions, eventually almost all patients will relapse. The available survival curves do not show any evidence of a plateau suggesting cure, although the median survival may be between 4 and 10 years.[1]

There are now substantial data on patients treated with high-dose chemotherapy followed by stem cell transplantation; however, the results from these studies are hampered by insufficient numbers or inadequate follow-up.[4–11] Also, data from randomized studies are absent. Although promising, there is no evidence that treatment with high-dose therapy followed by stem cell transplantation may be beneficial for the patient.

Patients with follicular NHL frequently have bone marrow infiltration. Therefore, there are some arguments that if a high-dose therapy is considered in the treatment plan of a patient, then purging of the graft may be necessary. The first results from Gribben et al.,[12] although not obtained from a controlled randomized study, support the value of purging.

Design European (C)UP trial

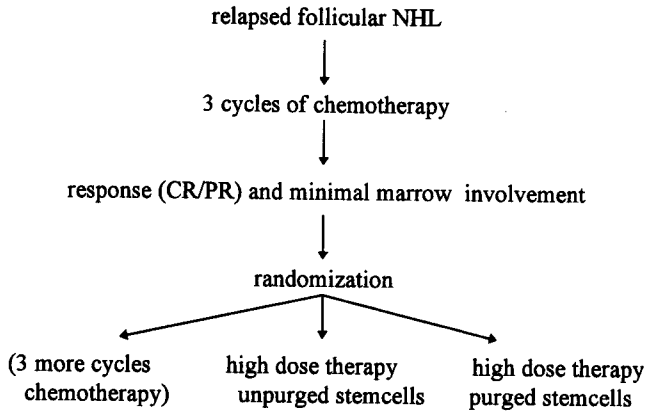

Figure 1. *Design European (CUP) trial.*

To answer the questions on efficacy of high-dose therapy and purging in patients with follicular NHL, a randomized trial was initiated comparing standard chemotherapy with high-dose therapy followed by unpurged stem cell transplantation or high-dose therapy followed by purged stem cell transplantation (CUP trial).

Study questions:

1. What is the value of high-dose therapy in patients with relapsed follicular NHL?
2. What is the value of purging in patients treated with stem cell transplantation in follicular NHL?

DESIGN OF THE STUDY

Patients are treated with three cycles of chemotherapy. The choice of chemotherapy is free, although CHOP chemotherapy is recommended. Patients are eligible for randomization if they respond after three cycles of chemotherapy. Response is defined as at least partial response according to standard criteria and minimal B-cell infiltration of the marrow (less than 20% B-lymphocytes). At the outset, patients were randomized among three arms (CUP trial), starting June 1996. Patients and doctors can also choose to have the patients randomized between the purged and unpurged high-dose arms (UP trial). (See Figure 1.)

Stem cells are collected as soon as possible after randomization, either bone marrow or peripheral blood stem cells after effective mobilization protocols.

Table 1. Patient characteristics at entry

sex	
male 60%	
female 40%	
age	
<30y	2.3%
31–40	16.1%
41–50	41.4%
51–60	34.5%
>60	5.7%
stage IV	75%
WHO 0	70.8%
LDH elevated	66%
marrow aspirate >20% B-lymphocytes	57.1%
marrow trephine positive	60.9%
B symptoms	20%
from last relapse	50% <24 months
1st relapse	68.8%

Table 2. Characteristics at randomization

CHOP	87%
CR	23.9%
PR	47.8%
<20% B lymphocytes	87%
trephine negative	72%

Table 3. Reasons for not being randomized

NR/PD	48%
died	8%
>20% B lymphocytes	10%
refusal	15%
undergoing induction	17%

Patients randomized to the purging arm will have their stem cells purged with a cocktail of anti-CD19, anti-CD20, anti-CD22, anti-CD23 and anti-CD37 plus immunomagnetic beads[13] (supported by Baxter Immunotherapy).

Patients randomized to the chemotherapy arm will be treated with three more cycles of the same chemotherapy. Patients in the stem cell transplantation arms will

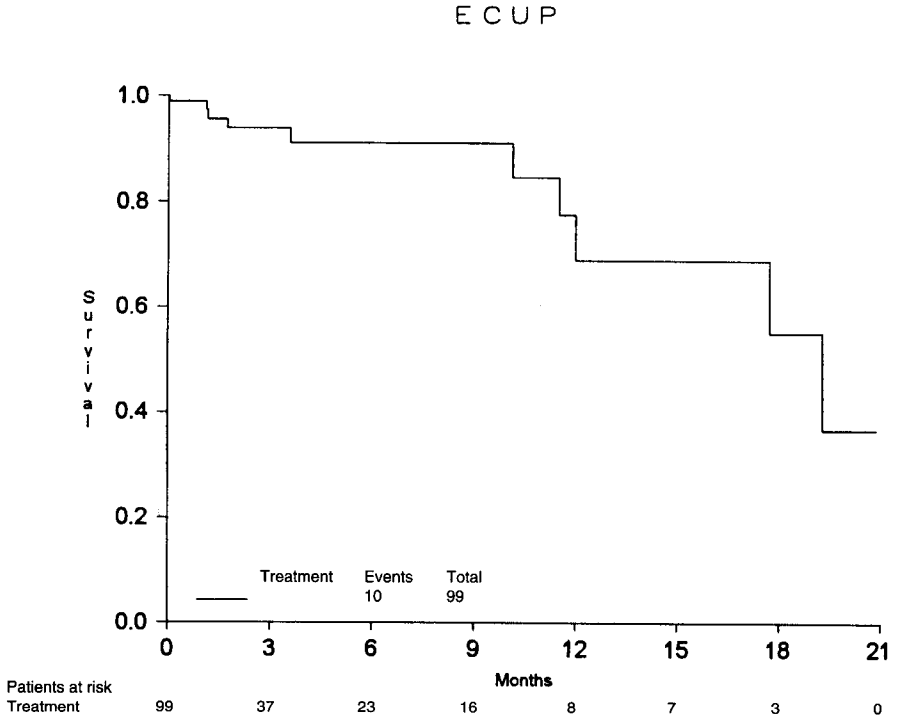

Figure 2. *Survival from entry.*

be treated with high-dose cyclophosphamide (2×60 mg/kg), followed by TBI and stem cell transplantation.

RESULTS

In June, 1996, 100 patients were entered in the study. The baseline characteristics are shown in Table 1.

Of the patients entered in the trial, 87% were treated with CHOP chemotherapy. After 3 cycles of chemotherapy, 61% of the patients had a response (see Table 2). It appeared that in the majority of patients the marrow was negative after 3 cycles of chemotherapy.

Fifty-three patients are not yet randomized. In 48% of these cases this was due to the absence of a response following the chemotherapy. Other reasons for not being randomized included death, marrow infiltration, refusal and still undergoing induction chemotherapy (see Table 3).

Figures 2 and 3 show the results of survival and disease-free survival from randomization. Because this is an ongoing randomized study, the results for the 3 specific arms are not available.

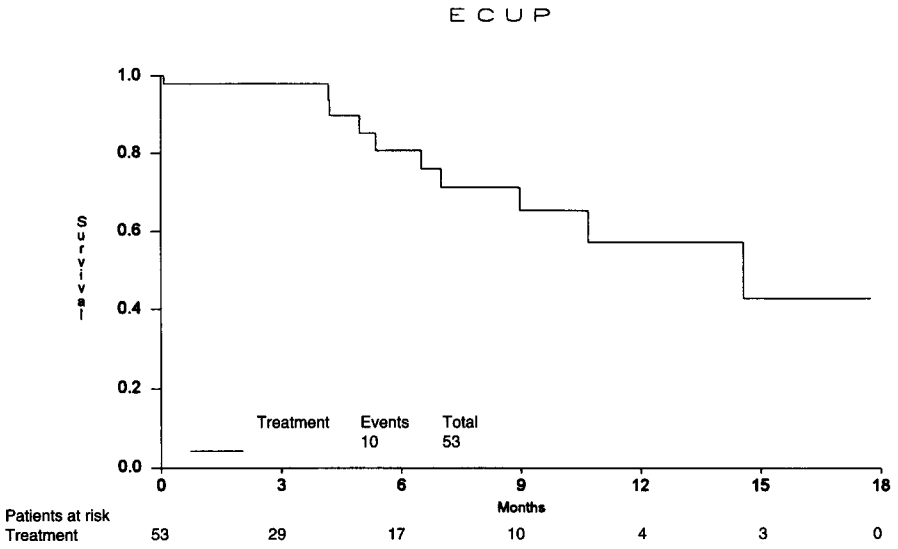

Figure 3. *Disease-free survival.*

DISCUSSION

This is the only randomized trial currently ongoing that assesses the value of high-dose therapy and purging in patients with relapsed follicular NHL. Since the trial has started to accrue patients, several centers have initiated transplantation of patients with relapsed follicular lymphoma, although data supporting this decision are not convincing. This trend has increased since the general implementation of peripheral blood stem cells has resulted in decreased morbidity and mortality. This has had a great impact on the accrual of patients in this trial.

The basic questions to be answered by this trial are still valid. Therefore, after the technical problems related to purging of peripheral blood were solved[13] in 1995, the option to use peripheral blood support was included in the trial design. In addition, we included the possibility of randomizing between the two transplant arms. This has led to increasing accrual. However, new patients are still necessary. To answer the two questions posed in this trial, about 250 patients have to be randomized.

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REFERENCES

1. Gallagher CJ, Gregory WM, Jones AE et al.: Follicular lymphoma: Prognostic factors for response and survival. *J Clin Oncol* 4:1470–1480, 1986.
2. Colombat P, Donadio D, Fouillard L et al.: Value of autologous bone marrow transplantation in follicular lymphoma: A France autogreffe retrospective study of 42 patients. *Bone Marrow Transplant* 13:157–162, 1994.
3. Vose JM, Bierman PJ, Armitage JO: High-dose chemotherapy with stem cell rescue for the treatment of follicular low grade non-Hodgkin's lymphoma. Autologous Bone Marrow Transplantation. Proceedings of the Fifth International Symposium, 479–485, 1991.
4. Schouten HC, Colombat PH, Verdonck LF et al.: Autologous bone marrow transplantation for low grade non-Hodgkin's lymphoma: The European Bone Marrow Transplant Group (EBMT) experience. *Ann Oncol* 5(S2):147–149, 1994.
5. Rohatiner A, Johnson P, Price C et al.: Myeloablative therapy with autologous bone marrow transplantation as consolidation therapy for recurrent follicular lymphoma. *J Clin Oncol* 12:1177–1185, 1994.
6. Cervantes F, Shu XO, McGlave P et al.: Autologous marrow transplantation for non-transformed low-grade non-Hodgkin's lymphoma. *Bone Marrow Transplant* 16:387–392, 1995.
7. Freedman AS, Ritz J, Neuberg D et al.: Autologous bone marrow transplantation in 69 patients with a history of low-grade B-cell non-Hodgkin's lymphoma. *Blood* 77:2524–2529, 1991.
8. Bastion Y, Brice P, Haioun C et al.: Intensive therapy with peripheral blood progenitor cell transplantation in 60 patients with poor prognosis follicular lymphoma. *Blood* 86:3257–3261, 1995.
9. Fouillard L, Gorin NC, LaPorte JP et al.: Feasibility of autologous bone marrow transplantation for early consolidation of follicular non-Hodgkin's lymphoma. *Eur J Haematol* 46:279–284, 1991.
10. Schouten HC, Bierman PJ, Vaughan WP et al.: Autologous bone marrow transplantation in follicular non-Hodgkin's lymphoma before and after histologic transformation. *Blood* 74:2579–2584, 1989.
11. Morel P, LaPorte JP, Noel MP et al.: Autologous bone marrow transplantation as consolidation therapy may prolong remission in newly diagnosed high-risk follicular lymphoma: A pilot study of 34 cases. *Leukemia* 9:576–582, 1995.
12. Gribben JG, Neuberg D, Freedman AS et al.: Detection by polymerase chain reaction of residual cells with the bcl-2 translocation is associated with increased risk of relapse after autologous bone marrow transplantation for B-cell lymphoma. *Blood* 81:3449–3457, 1993.
13. Kvalheim G, Wang MY, Pharo A et al.: Purging of tumor cells from leukapheresis products: Experimental and clinical aspects. *J Hematother* 5:427–436, 1996.

HIGH-DOSE THERAPY AND AUTOLOGOUS BONE MARROW TRANSPLANTATION IN PATIENTS WITH FOLLICULAR LYMPHOMA DURING FIRST REMISSION

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ABSTRACT

One approach that has been taken to improve the treatment of poor prognosis patients with leukemia and lymphomas is the use of high-dose therapy earlier in the course of disease. We report the results of a pilot study in previously untreated advanced stage patients with follicular lymphoma (FL) who underwent induction chemotherapy with CHOP followed by high-dose chemoradiotherapy and anti-B cell monoclonal antibody–purged autologous bone marrow transplantation (autoBMT). Eighty-three patients with previously untreated, low grade FL were enrolled on this study. After CHOP induction, only 36% achieved CR, with 77 pts undergoing bone marrow (BM) harvest. Prior to BM harvest, 70 had a known t(14;18) as determined by polymerase chain reaction (PCR), in tumor tissue, and all remained PCR positive in the BM at harvest. After autoBMT the DFS and overall survival are estimated to be 63% and 89% at 3 years, respectively, with a median follow-up of 45 months for the 43 patients who remain in CR. The patients whose BM was PCR negative after purging experienced significantly longer time to treatment failure than those whose BM remained PCR positive (p=0.0006). Continued PCR negativity in follow-up BM samples was also strongly predictive of continued CR. This study suggests that a subset of patients with advanced FL may experience prolonged clinical and molecular remissions following high-dose ablative therapy.

INTRODUCTION

The use of high-dose therapy with intent to cure patients with previously relapsed FL has had limited impact to date. In several series, at best 40% of highly selected

Table 1. Patient characteristics

total	77
female	33
male	44
age at autoBMT (years)	
<35	12
35–50	60
>50	5
histology	
follicular small cleaved	65
follicular mixed	12
stage	
III	13
IV	64
mass >10 cm	9
mass >5 cm	48
B symptoms	19
extranodal (exclusive of BM)	19
BM involvement	62

patients with good performance status and sensitive disease are alive and in unmaintained remission at 4 years.[1–5] In addition, there is considerable patient selection before autologous bone marrow transplantation (autoBMT) in these patients and only approximately 50% of patients with relapsed FL who are initially considered as candidates for autoBMT actually undergo the treatment. Therefore, at best 20% of patients with relapsed disease may actually benefit from high-dose therapy.

One strategy to improve the treatment of patients with leukemia and lymphomas with a poor prognosis is the use of high-dose consolidative therapy earlier in the course of disease. High dose therapy and autologous hematopoietic stem cell support for patients with FL in first remission may permit successful treatment for patients before the development of resistant disease following extensive conventional therapy. In this study, we report the results in patients with previously untreated advanced stage FL who, on an intent to treat basis, underwent uniform induction chemotherapy followed by high-dose chemoradiotherapy and anti-B cell monoclonal antibody–purged autoBMT. We observed that 63% of patients are disease free at 36 months after autoBMT and that 35 of 66 patients (53%) with a polymerase chain reaction (PCR)-amplifiable bcl-2/IgH rearrangement are in molecular remission at their last follow-up. Although still preliminary, these results suggest that a percentage of patients with advanced stage FL may experience prolonged remissions.

MATERIALS AND METHODS

Selection of patients and treatment protocol

Patients were eligible for this study if they were 55 years old or less; had previously untreated FL as defined by the Working Formulation (WF) including follicular small cleaved cell (WF-B) and follicular mixed small cleaved and large cell (WF-C); and had lymphoma cells that expressed the CD20 (B1) antigen as previously described. Patients had to have stage IIIB, IIIE or III with masses >10 cm, or stage IV disease. Patients with stage IV disease by virtue of minimal adenopathy (<1 cm) and < 5% marrow involvement were excluded. Additional criteria for entry included the absence of comorbid disease of the heart, kidney, lung and liver and a Karnofsky score above 80%. All patients were treated with 6–8 cycles of CHOP. At the completion of CHOP, patients in CR or with minimal disease went on to BM harvest. Minimal disease 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. Patients with 1–3 masses >2 cm after completion of CHOP could receive involved field radiotherapy of 3000 cGy. Informed consent was obtained from all patients.

Preparative therapy consisted of cyclophosphamide, 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.[6] Bone marrow was obtained, treated in vitro with anti-B cell monoclonal antibodies as previously described.[6]

Evaluation

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 for the first 2 years post-autoBMT and yearly thereafter. Complete remission (CR) was defined as the disappearance of all measurable and evaluable disease.

PCR analysis

Nested PCR amplification at the major breakpoint region (MBR) and minor cluster region (mcr) of the bcl-2/IgH rearrangement of t(14;18) were performed as previously described.[7] Analysis was performed initially on diagnostic material (lymph node biopsy, BM aspirate if histologically involved). Samples were

analyzed at the completion of induction therapy and at the time of BM harvest. Assessment was also performed after ex vivo marrow purging. Serial BM and PB samples at the time of restaging after autoBMT were also analyzed.

Statistical methods

Failure was defined as relapse of disease or death in remission. Disease-free survival (DFS) was calculated from the day of marrow transplantation (day 0) to date of failure, or to date last known alive and disease-free. Time to relapse (TTR) was calculated from the day of marrow transplantation to the date of relapse; deaths in remission were considered censored for this analysis. DFS curves and time to relapse curves were estimated by the method of Kaplan and Meier, with confidence intervals calculated using Greenwood's formula, and compared by the logrank test.[8,9] The Cox proportional hazards model was used to assess prognostic factors for time to relapse and DFS, and to build multiple regression models.

RESULTS

Patient characteristics

Eighty-three patients (median age 43) with previously untreated, advanced stage FL were eligible and registered on this study. At diagnosis, 71 of the 83 patients had follicular small cleaved cell (FSC) histology; 12 had follicular mixed small cleaved and large cell lymphoma. Between April 1988 and June 1993, 77 patients (93%) achieved a protocol eligible minimal disease state at the completion of induction and went on to autoBMT. Three patients failed to attain a protocol eligible PR, two further patients were diagnosed with second tumors (melanoma and seminoma) and one patient declined further therapy. The characteristics of the 77 patients who underwent autoBMT are detailed in Table 1.

Response to induction therapy

All patients were treated with CHOP, and 10 patients received involved field radiation therapy to residual sites that were >2 cm at the completion of chemotherapy. Only 28 patients (36%) achieved clinical CR following induction therapy. Histologic marrow involvement was seen in 36 of the patients at harvest (47%).

PCR analysis following induction

Of the 77 patients who underwent BM harvest, 70 (91%) had a PCR-amplifiable bcl-2 translocation. Six patients had no evidence of a PCR-amplifiable bcl-2/IgH rearrangement at either the mbr and mcr breakpoints in diagnostic tissue and in one other patient no premarrow purging sample was available for analysis. In keeping with our findings in previously relapsed patients who were treated with autoBMT,[10] all 70 patients continued to have PCR-detectable disease at the time of BM harvest,

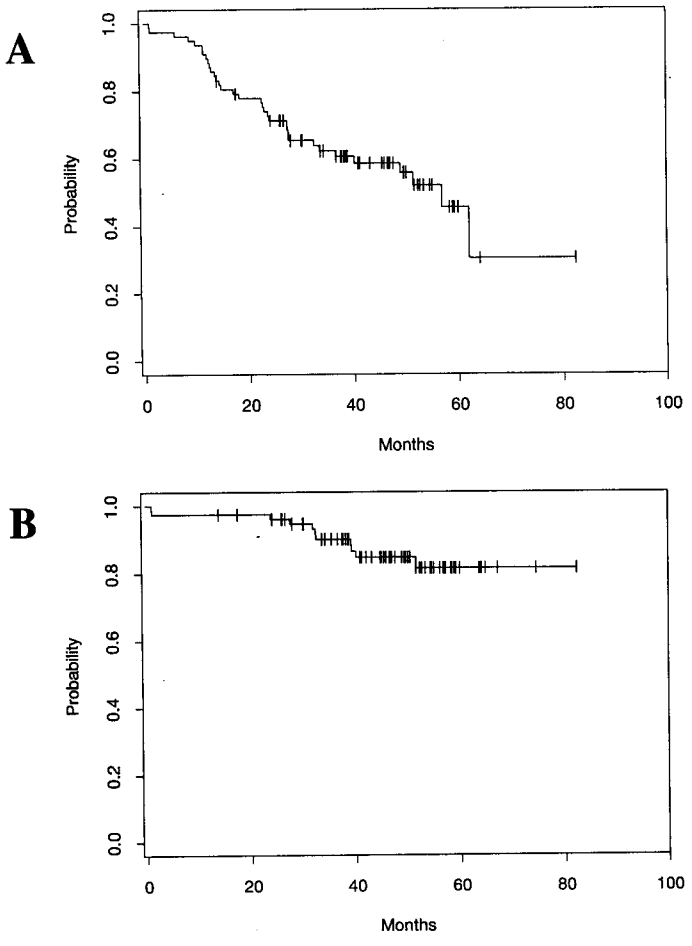


Figure 1. *Kaplan-Meier estimate of probability of DFS* **(A)** *and overall survival for 77 patients following autoBMT* **(B)**.

irrespective of the presence or absence of histologic appearance of the marrow biopsy. Following ex vivo marrow treatment, 30 patients (43%) had no PCR-detectable disease whereas 40 patients (57%) were reinfused with marrow that contained residual PCR-detectable lymphoma cells. There was no significant association between the outcome of purging and the presence of histologic evidence of disease.

Treatment outcome

There were two acute in-hospital treatment-related deaths both from diffuse alveolar hemorrhage syndrome. Four late deaths from nonlymphomatous causes were observed. Five patients have developed myelodysplasia (MDS) post autoBMT. Three patients have died without evidence of lymphoma following

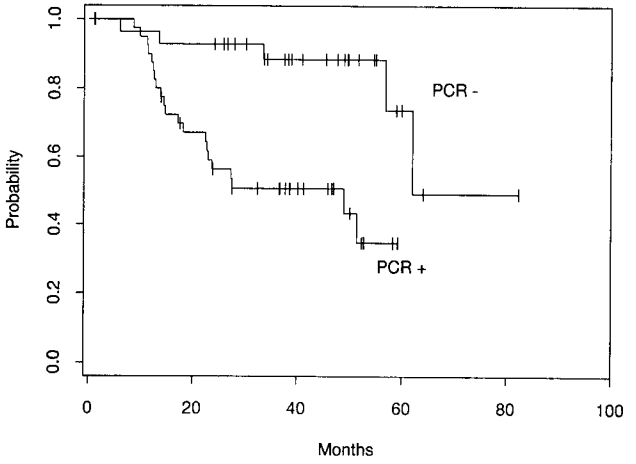


Figure 2. *Kaplan-Meier estimate of time to treatment failure following autoBMT, for 70 informative patients who were either PCR negative (PCR^-) or PCR positive (PCR^+) following ex vivo purging.*

HLA-matched sibling and matched unrelated donor allogeneic bone marrow transplants at 24, 32 and 40 months, respectively. Two additional patients remain alive: one patient relapsed at 13 months and developed MDS at 65 months; the other patient developed MDS at 18 months and remains without relapse of lymphoma at 35+ months post-autoBMT. An additional patient committed suicide at 28 months.

As of November 1, 1995, there have been 28 relapses among the remaining 71 patients. Forty-three patients remain in CCR with a median follow-up of 45 months (range 14–82 months). The Kaplan-Meier estimate of the percentage of patients alive and disease-free at 3 years is 63% (Figure 1A). The estimate of the overall survival at 3 years is 89% (Figure 1B).

Of the 28 patients who relapsed, the overwhelming majority relapsed in sites of prior disease (Table 2). Entirely new sites of disease were observed in only 6 patients, and in 4 the new sites were the only sites of relapse. Eleven of the 28 relapses involved the marrow, all of whom had a history of bone marrow infiltration, 10 at the time of harvest. Following relapse, 23 of 28 patients are alive at a median follow-up of 49 months after autoBMT.

PCR analysis following autoBMT

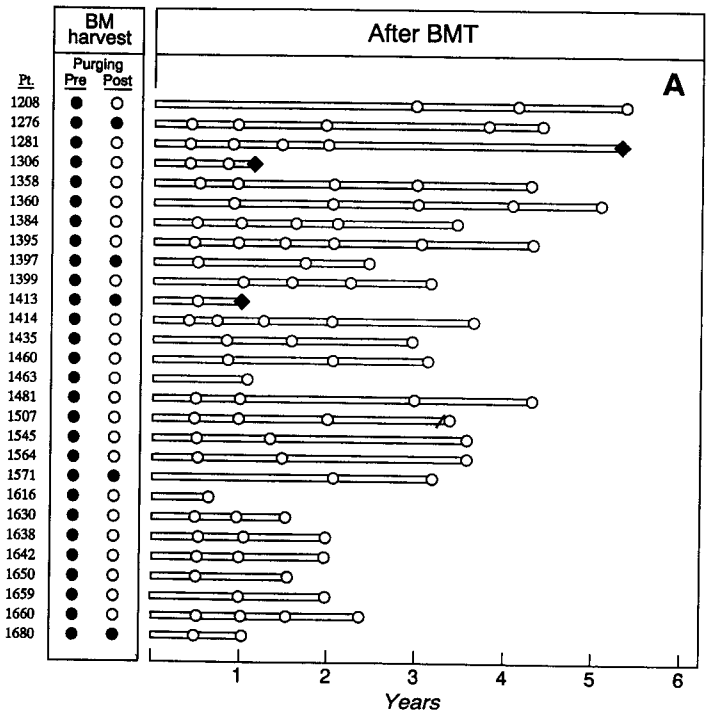
The effect of marrow purging was examined in the 70 informative patients who had a previously known bcl-2 rearrangement, all of whom had post-lysis marrow samples. Among the 30 patients who were PCR negative after purging, there have

been 5 relapses, while there have been 21 relapses among the 40 patients who were PCR positive after purging. The 3-year time to treatment failure (TTF) for the PCR negative patients is 88%, while the TTF for the patients who were PCR positive post-lysis is 51% (p=0.0006) (Figure 2).

BM samples were analyzed for assessment of minimal residual disease following autoBMT. No samples were available for analysis from four patients who had a documented PCR-amplifiable bcl-2/IgH rearrangement. Two patients died during the procedure and two patients had no follow-up BM samples sent for PCR analysis. The results obtained at the time of and following autoBMT in these patients are shown in Figure 3. In 28 patients, no BM samples analyzed had evidence of PCR-detectable lymphoma at any time point after autoBMT (Figure 3A). Of note, in only 4 of these 28 patients (14%) were PCR-detectable lymphoma cells detected after immunologic purging. Three of these patients (11%) have relapsed to date, 1 patient with lymphoma lacking a bcl-2 rearrangement. PCR-detectable minimal residual disease was detected in every BM sample obtained after autoBMT in 21 patients, 20 of whom (95%) were infused with autologous BM that contained residual PCR-detectable lymphoma (Figure 3B). Sixteen of these 21 patients (76%) have relapsed to date. The remaining 17 patients had different results obtained at different time points after autoBMT (Figure 3C). In nine of these patients, PCR-detectable lymphoma cells were detected early after autoBMT, but no PCR-detectable lymphoma cells could be detected in later samples. The time taken to convert from PCR positivity to PCR negativity varied from one year to almost three years after autoBMT. Only one of these patients has relapsed to date. In four patients (UPN #1312, 1370, 1415 and 1487) no discernible pattern could be observed. In four patients (UPN #1348, 1369, 1476 and 1496) no PCR-detectable lymphoma cells were observed early after autoBMT, but lymphoma cells were detected by PCR at later time points and persisted on subsequent sampling. The time to appearance of first detectable minimal residual disease in these patients varied from one year to four years after autoBMT.

DISCUSSION

In this report, we present the results of a study of previously untreated patients with advanced stage FL who were uniformly treated with CHOP induction followed by high-dose chemoradiotherapy and autoBMT in first remission. Within the context of studies of conventional therapy in this disease, the followup is relatively short; however, considering that the median progression-free survival in patients treated conventionally is approximately 2 years,[11] we have observed over a 2-fold increase in DFS in patients undergoing autoBMT in first remission. Considering that only 36% of the patients in the present study who underwent autoBMT were in clinical CR at the time of autoBMT, the current results are encouraging.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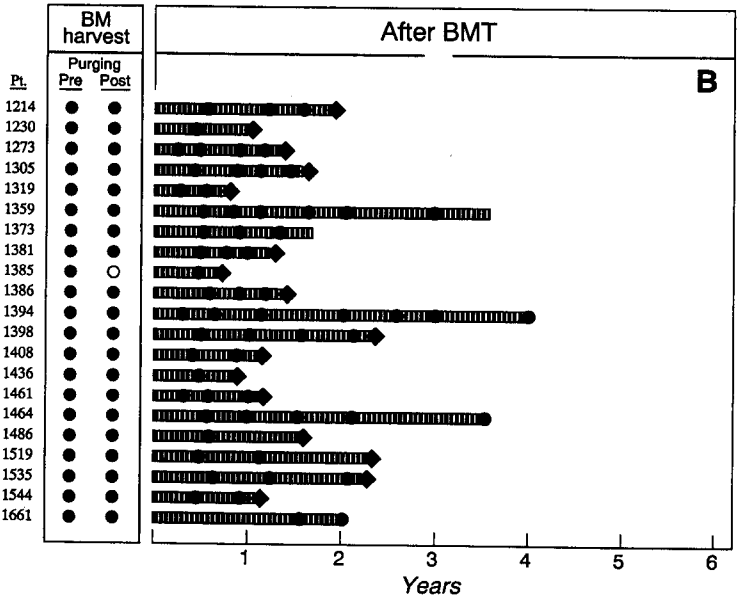
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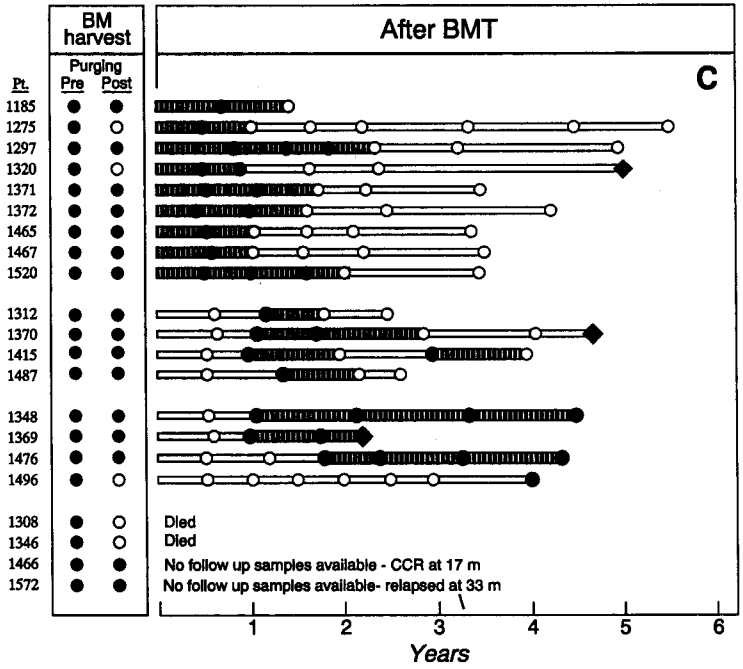

Figure 3. *PCR analysis of BM samples before and after BM purging, and after autoBMT in 70 informative patients with t(14;18).* ***(A)*** *patients in whom all post autoBMT follow-up BM samples were PCR negative;* ***(B)*** *patients in whom all post autoBMT follow-up BM samples were PCR positive; and* ***(C)*** *patients in whom post autoBMT follow-up BM samples were both PCR positive and negative.* ● *represents PCR positive results,* ○ *represents PCR negative result,* ♦ *indicates relapse.*

In FL, the evaluation of any impact on remission duration and survival will require exceedingly long follow-up. At the time of this analysis it is not clear whether there will be a plateau in the DFS curve for patients treated on this study. Therefore, endpoints other than remission duration and survival would be useful for examining the effect of autoBMT. We have previously reported that detection of minimal residual disease by PCR following autoBMT is a very useful predictor of relapse.[7,12] Analogous to our studies of patients transplanted in second or greater remission, we demonstrate here that the continued absence of PCR-detectable lymphoma cells in follow-up BM samples is a sensitive predictor of continuous remission following autoBMT in first remission. Furthermore, those patients in whom PCR-detectable disease is present early after autoBMT and becomes negative over time, appear to remain in clinical and molecular remission. Conversely, patients who were continuously PCR positive in follow-up BM specimens, or who converted from negative to positive, will almost invariably

relapse. These studies are in contrast to reports of persistent PCR-detectable lymphoma cells in a select subset of patients, with both early and advanced stage disease, who remain in continuous clinical remission following conventional treatment.[13–16] In those patients, it is unclear whether those PCR-detectable cells are capable of contributing to relapse or more likely whether with longer follow-up those patients will have clinical relapse. It appears from our studies of high-dose therapy in this specific patient population that the persistence or development of PCR-detectable lymphoma cells is highly predictive of relapse and may be a suitable surrogate endpoint for assessing the efficacy of autoBMT in a disease with a very long natural history.

A major question is whether the patients treated on this study were in fact a subgroup of patients with a poor prognosis. The International Prognostic Index for Aggressive Lymphomas has been applied to patients with FL, and risk groups of patients have been identified.[17,18] Using these criteria, the vast majority of patients with FL present with low or low-intermediate risk disease, with less than 5% of patients at high risk with a median survival of approximately 2 years. Virtually all patients in the present study were of low-intermediate risk by virtue of stage III/IV disease and the presence of extranodal disease. With conventional therapy these patients have a median survival of about 10 years.[18] By these criteria, our patients do not appear to have a very poor prognosis. One factor that suggests an unfavorable prognosis in the patients selected for this study is the CR rate of 36% following CHOP induction. This is markedly lower than the 70% CR rate to CHOP reported for advanced stage FL patients.[19,20] Although one interpretation is that 6–8 cycles of CHOP was inadequate therapy, we believe we did not select patients with exquisitely sensitive disease with a favorable prognosis. Patients who do not achieve a CR after treatment with conventional therapy have a median survival of 4 years and a DFS of 1 year.[19] Therefore, we believe that few if any of our patients would experience long-term DFS with additional treatment short of myeloablative.

If the 30 patients who are presently in a clinical and molecular CR remain so, these results would be superior to conventional treatment and autoBMT in second remission. Since following relapse only 50% of patients are candidates for autoBMT, then only 20% of relapsed patients experience long term DFS. Therefore the results in the present study appear to be encouraging and merit further exploration. The results of this study show that patients whose marrow can be purged of PCR-detectable lymphoma cells remain in clinical and molecular complete remission. Whether purging contributes to relapse or is a prognostic marker is presently unknown. However, for the subset (40%) of patients who purge PCR negative, autoBMT may be sufficient therapy and no additional therapy appears warranted. In contrast, those patients who are PCR positive after purging and/or in follow-up BM samples have an overwhelming likelihood of relapse and

merit novel approaches. Specifically, future studies using stem cell support in FL should be directed at attaining a PCR negative state through the development of more efficient purging techniques.[21] Moreover, for the patients who remain PCR positive after purging and who persist or develop PCR-detectable lymphoma in follow-up BM samples, additional treatment with antibodies, cytokines and/or vaccines are merited. We believe that high-dose myeloablative therapy is a potentially important treatment approach for advanced stage poor prognosis patients with FL. To improve the therapeutic index, future studies must be directed at decreasing toxicity and improving eradication of minimal residual disease.

REFERENCES

1. Schouten HC, Bierman PJ, Vaughan WP et al.: Autologous bone marrow transplantation in follicular non-Hodgkin's lymphoma before and after histologic transformation. *Blood* 74:2579–2584, 1989.
2. Freedman AS, Ritz J, Neuberg D et al.: Autologous bone marrow transplantation in 69 patients with a history of low grade B cell non-Hodgkin's lymphoma. *Blood* 77:2524–2529, 1991.
3. Vose J, Bierman P, Armitage J: High dose chemotherapy with stem cell rescue for the treatment of follicular low grade non-Hodgkin's lymphoma. Autologous Bone Marrow Transplantation. Proceedings of the Fifth International Symposium V:479–486, 1991.
4. Rohatiner A, Johnson P, Price C, Arnott SJ et al.: Myeloablative therapy with autologous bone marrow transplantation as consolidation therapy for recurrent follicular lymphoma. *J Clin Oncol* 12:1177–1124, 1994.
5. Colombat P, Donadio D, Fouillard L et al.: Value of autologous bone marrow transplantation in follicular lymphoma: A France Autogreffe retrospective study of 42 patients. *Bone Marrow Transplant* 13:157–162, 1994.
6. Freedman AS, Takvorian T, Anderson KC et al.: Autologous bone marrow transplantation in B-cell non-Hodgkin's lymphoma: Very low treatment-related mortality in 100 patients in sensitive relapse. *J Clin Oncol* 8:1–8, 1990.
7. Gribben J, Neuberg D, Barber M et al.: Detection of residual lymphoma cells by polymerase chain reaction in peripheral blood is significantly less predictive for relapse than detection in bone marrow. *Blood* 83 (12):3800–3807, 1994.
8. Kaplan E, Meier O: Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 53:457, 1958.
9. Mantel N: Evaluation of statistical data and two new rank order statistics arising in its consideration. *Cancer Chemothr Rep* 50:163, 1966.
10. Gribben J, Freedman A, Woo S et al.: All advanced stage non-Hodgkin's lymphomas with a polymerase chain reaction amplifiable breakpoint of bcl-2 have residual cells containing the bcl-2 rearrangement at evaluation and after treatment. *Blood* 78:3275–3280, 1991.
11. Johnson P, Rohatiner A, Whelan J et al.: Patterns of survival in patients with recurrent follicular lymphoma: A 20-year study from a single center. *J Clin Oncol* 13:140–147, 1995.
12. Gribben J, Neuberg D, Freedman A et al.: Detection by polymerase chain reaction of resid-

ual cells with the bcl-2 translocation is associated with increased risk of relapse after autologous bone marrow transplantation for B-cell lymphoma. *Blood* 81:3449–3457, 1993.

13. Price CGA, Meerabux J, Murtagh S et al.: The significance of circulating cells carrying t(14;18) in long remission from follicular lymphoma. *J Clin Oncol* 9:1527–1532, 1991.
14. Lambrechts A, Hupkes P, Dorssers L et al.: Translocation (14;18) positive cells are present in the circulation of the majority of patients with localized (stage I and II) follicular non-Hodgkin's lymphoma. *Blood* 82:2510–2516, 1993.
15. Finke J, Slanina J, Lange W et al.: Persistence of circulating t(14;18)-positive cells in long term remission after radiation therapy for localized stage follicular lymphoma. *J Clin Oncol* 11:1668–1673, 1993.
16. Berinstein NL, Jamal HH, Kuzniar B et al.: Sensitive and reproducible detection of occult disease in patients with follicular lymphoma by PCR amplification of t(14; 18) both pre- and post-treatment. *Leukemia* 7:113-9, 1993.
17. Bastion Y, Coiffier B: Is the International Prognostic Index for aggressive lymphoma patients useful for follicular lymphoma patients. *J Clin Oncol* 12:1340–1342, 1994.
18. Lopez-Guillermo A, Montserrat E, Bosch F et al.: Applicability of the International Index for aggressive lymphomas to patients with low-grade lymphoma. *J Clin Oncol* 12:1343–1348, 1994.
19. Romaguera J: Can we identify patients with low grade lymphoma for frontline ABMT. *Leuk Lymphoma* 10 (suppl):9–15, 1993.
20. Dana B, Dahlberg S, Nathwani B et al.: Long-term follow-up of patients with low-grade malignant lymphomas treated with doxorubicin-based chemotherapy or chemoimmunotherapy. *J Clin Oncol* 11:644–651, 1993.
21. Gribben J, Saporito L, Barber M et al.: Bone marrow of non-Hodgkin's lymphoma patients with a bcl-2 translocation can be purged of polymerase chain reaction-detectable lymphoma cells using monoclonal antibodies and immunomagnetic bead depletion. *Blood* 80:1083–1089, 1992.

HIGH-DOSE THERAPY FOLLOWED BY AUTOLOGOUS HEMATOPOIETIC RESCUE FOR FOLLICULAR NON-HODGKIN'S LYMPHOMA

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ABSTRACT

We have performed autologous bone marrow (autoBMT) or peripheral stem cell transplants (PSCT) on 131 patients with relapsed or refractory follicular non-Hodgkin's lymphoma (NHL). Thirty-nine patients had follicular small cleaved cell histology (FSC), 56 had follicular mixed NHL (FM), and 36 had follicular large cell NHL (FLC). There were 72 males and 59 females. Median age was 45 (range 24–67). Currently, 82 patients are alive with a median survival of 72 months. Overall survival (OS) at 5 years is projected to be 57%. Fifty-eight patients are failure-free with a median failure-free survival (FFS) of 26 months. Actuarial 5-year FFS is 39%. Patients with FLC histology had shorter OS (p=0.008) and FFS (p=0.06) compared with FSC or FM histology. No significant differences in outcome were observed between patients who received total-body irradiation compared with those who received chemotherapy alone, or between autoBMT compared with PSCT. Among PSCT patients, no significant differences in OS or FFS were observed between those who had histologic evidence of bone marrow involvement at the time of stem cell collection, and those with negative marrow. When analysis is restricted to the 95 pts with FSC and FM histology, OS and FFS at five years are projected to be 65% and 44%, respectively. Eleven patients (8%) died before day 100. Late relapses have occurred in nine patients between 2.1 and 6.2 years following transplantation. Seven patients have developed myelodysplastic syndromes between 12 and 66 (median 30) months following transplantation. Long-term FFS is possible following autoBMT or PSCT for follicular NHL. The development of second malignancies and late relapses mandate prolonged follow-up for these patients. It is not clear whether patients are cured or the natural history of disease is altered.

INTRODUCTION

High-dose therapy followed by autologous bone marrow and peripheral stem cell transplantation has become accepted therapy for patients with relapsed

intermediate-grade and high-grade non-Hodgkin's lymphoma (NHL). Prospective randomized trials have demonstrated the superiority of this approach over conventional-dose salvage chemotherapy.[1] Despite the widespread use of transplantation for patients with aggressive lymphomas, there is relatively little experience with this form of therapy for follicular lymphoma. This report will review our experience with high-dose therapy followed by autologous hematopoietic rescue for patients with follicular NHL.

PATIENTS AND METHODS

Between April, 1983, and January, 1995, 131 patients with follicular NHL received high-dose therapy followed by autologous hematopoietic rescue. With one exception, all patients had relapsed or failed to attain a complete remission after treatment with an anthracycline-containing or mitoxantrone-containing regimen. A single patient had received only pelvic irradiation prior to transplantation. Classification into follicular small cleaved cell, follicular mixed or follicular large cell histologic subtypes was performed using the counting method described by Berard et al.[2] Patients with transformed lymphomas, composite lymphomas and mixed architectural pattern were excluded from analysis.

Patients were eligible for transplantation if they had failed to achieve an initial remission or had relapsed after attaining a remission with conventional therapy. Transplant protocols were approved by the institutional review board and informed consent was obtained from all patients before transplantation.

During the early period of patient accrual, all patients were transplanted with unpurged autologous bone marrow. A bone marrow biopsy within one month of harvest was required to be morphologically free of lymphoma, although patients may have had previous bone marrow involvement. During the latter period of patient accrual, all patients with follicular small cleaved cell and follicular mixed histology were transplanted with peripheral blood stem cells, even if a bone marrow biopsy had never contained lymphoma.

Prior to April, 1991, peripheral blood stem cells were collected without chemotherapy or cytokine mobilization. Thereafter, peripheral blood stem cells were collected after mobilization with either granulocyte-macrophage colony stimulating factor, granulocyte colony stimulating factor, erythropoietin or PIXY-321, depending upon trials in place at that time.

Eighty-nine patients were treated with cyclophosphamide (60 mg/kg$\times$2) and 1200 cGy total-body irradiation (TBI) administered in six fractions. The majority of the remaining patients (n=30) received BEAC (carmustine 300 mg/m$^2\times$1, etoposide 100 mg/m$^2\times$8, cytarabine 100 mg/m$^2\times$8 and cyclophosphamide 35 mg/kg$\times$4). The remaining patients received carmustine, etoposide, cyclophosphamide and hydroxyurea (n=7), cyclophosphamide, cytarabine and TBI (n=1), cyclophos-

Table 1. Patient characteristics

median age	45	(Range 24-67)	
gender	M	72	(55%)
	F	59	(45%)
histology	FSC	39	(30%)
	FM	56	(43%)
	FLC	36	(27%)
prior chemotherapy	1	27	(21%)
	2	54	(41%)
	3	23	(18%)
	$^{3}4$	27	(21%)
regimen	with TBI	92	(70%)
	without TBI	39	(30%)
rescue	autoBMT	16	(12%)
	PSCT	115	(88%)

phamide, thiotepa and TBI (n=1), melphalan and TBI (n=1), ifosfamide, carboplatin and TBI (n=1), or cyclophosphamide, carmustine and etoposide (n=1).

Transplant outcome was analyzed with respect to overall survival and failure-free survival (FFS). Overall survival was defined as time from the date of transplant until death from any cause. FFS was defined as time from the date of transplant until relapse, disease progression or death from any cause. Survival curves were calculated according to the method of Kaplan and Meier.[3] Comparisons of these time-to-event distributions were calculated using the logrank test.

RESULTS

Between April, 1983, and January, 1995, a total of 131 patients with relapsed or refractory follicular lymphoma received autologous bone marrow or peripheral stem cell transplants at the University of Nebraska Medical Center. Thirty-nine patients had follicular small cleaved cell histology, 56 had follicular mixed histology, and 36 had follicular large cell histology. Characteristics of patients in each of these groups are displayed in Table 1.

As of July, 1996, 82 patients are alive and 58 patients remain failure-free. Overall survival is estimated to be 57% (95% confidence interval [CI], 47 to 68%) at five years, and median survival is 72 months (Figure 1). FFS is estimated to be 39% at five years (95% CI, 30 to 50%), and median FFS is 26 months (Figure 1). Overall survival was significantly worse for patients with follicular large cell histology, compared with follicular small cleaved cell and follicular mixed histology

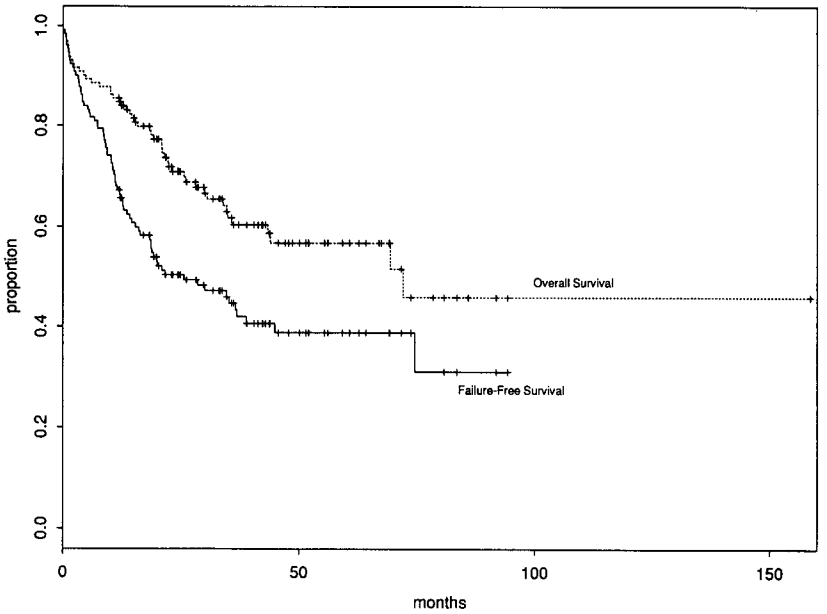

Figure 1. *Overall survival and FFS following high-dose therapy for follicular lymphoma (n=131).*

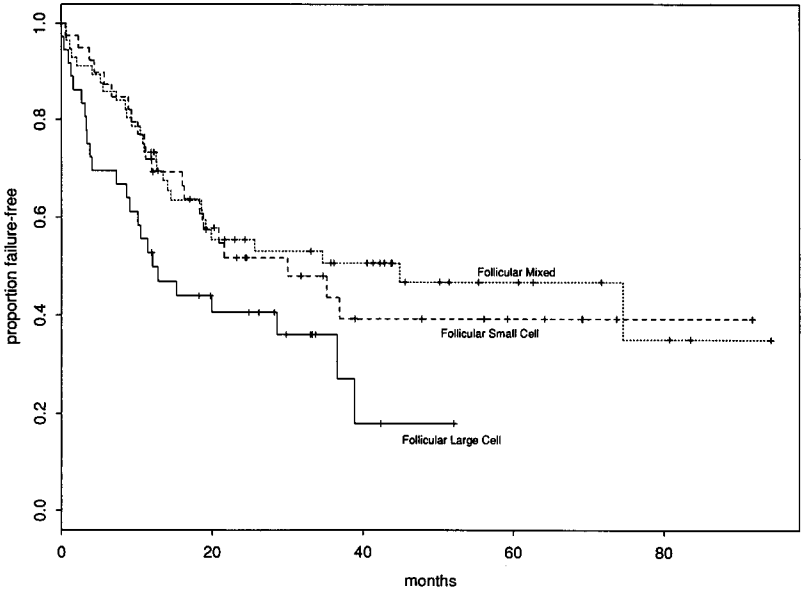

Figure 2. *FFS according to histology (p=0.06).*

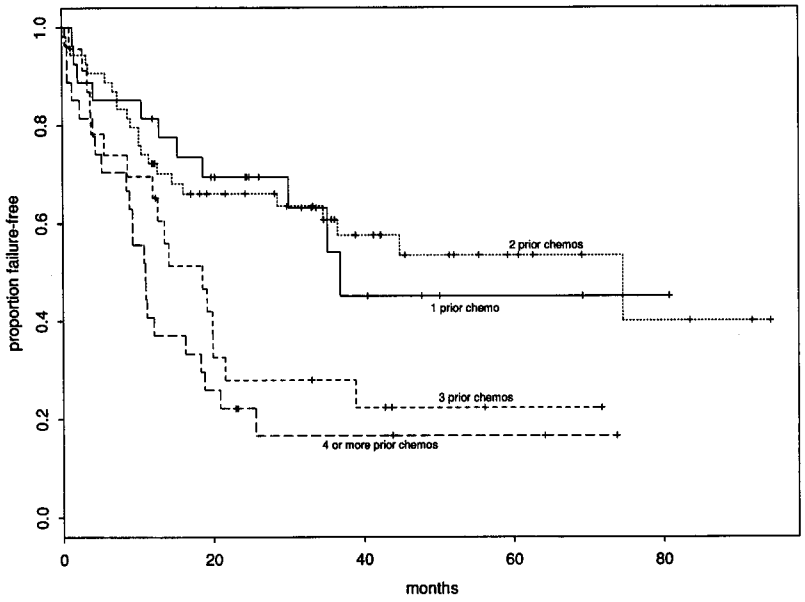

Figure 3. *FFS according to number of conventional chemotherapy regimens received prior to transplantation (p=0.0002).*

(p=0.008). Similarly, FFS was inferior for patients with follicular large cell histology (Figure 2). FFS at five years was estimated at 39% (95% CI, 25 to 61%) for patients with follicular small cleaved cell histology, 47% (95% CI, 34 to 64%) for patients with follicular mixed histology, and 18% (95% CI, 6 to 53%) for follicular large cell histology (p=0.06). Overall and FFS at five years for the 95 patients with follicular small cleaved cell and follicular mixed histology were estimated at 65% (95% CI, 55 to 77%) and 44% (95% CI, 34 to 57%), respectively.

Transplant outcome was significantly associated with the number of chemotherapy regimens patients had received prior to transplantation. Overall survival at five years was estimated at 63% (95% CI, 45 to 89%) for patients who were transplanted after receiving only one conventional chemotherapy regimen. Patients who had previously received two, three or more regimens had overall survival rates projected to be 72% (95% CI, 60 to 87%), 50% (95% CI, 31 to 82%) and 32% (95% CI, 17 to 61%), respectively. FFS at five years was estimated at 45% (95% CI, 26 to 79%) for patients transplanted after receiving only one chemotherapy regimen, 53% (95% CI 40 to 71%) after two regimens, 22% (95% CI, 10 to 50%) after three regimens, and 17% (95% CI, 7 to 41%) after four or more regimens (Figure 3, p=0.0002).

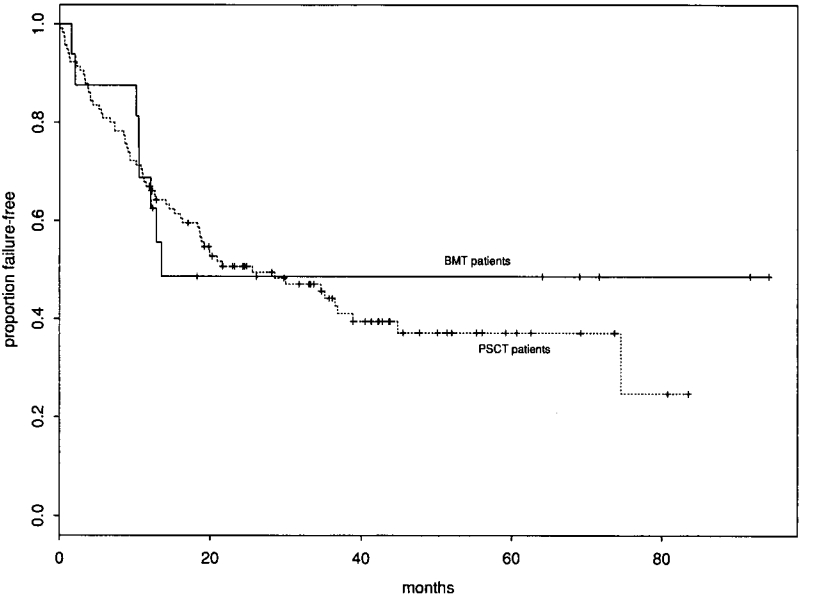

Figure 4. *FFS according to rescue source (p=0.65).*

There were 11 (8%) deaths within 100 days of transplantation. The cause of death for these patients was progressive disease (n=4), multi-organ failure (n=4), infection (n=1), central nervous system hemorrhage (n=1) and Epstein-Barr virus lymphoproliferation (n=1). Seven patients have developed a myelodysplastic syndrome between 12 and 66 months following transplantation. Four of these patients died within one year of developing myelodysplasia, while three remain alive between four and 29 months following diagnosis. One patient developed a squamous cell carcinoma of the tongue 77 months after transplantation and another patient died two months after transplantation with a disseminated Epstein-Barr virus-related lymphoproliferative disorder. In addition, nine patients have relapsed or progressed between 2.1 and 6.2 years following transplantation.

Transplant outcome was analyzed with respect to transplant regimen, rescue source and presence of bone marrow involvement at the time of peripheral stem cell collection. FFS at five years was estimated at 41% (95% CI, 31 to 55%) for patients who were transplanted with a TBI-containing regimen, compared with 33% (95% CI, 20 to 53%) for patients who received a regimen consisting only of drugs (p=0.13). FFS was estimated at 49% (95% CI, 29 to 81%) for patients transplanted with unpurged bone marrow, compared with 37% (95% CI, 28 to 49%) for patients transplanted with peripheral stem cells (Figure 4, p=0.65). The five-year FFS rate was estimated at 36% (95% CI, 24 to 54%) for the patients

transplanted with peripheral stem cells who had histologic evidence of bone marrow involvement at the time of stem cell collection. Patients with normal bone marrow at the time of peripheral stem cell collection had a FFS estimated to be 26% (95% CI, 11 to 63%, p=0.56).

DISCUSSION

Aggressive conventional chemotherapy regimens for patients with low-grade lymphomas may prolong disease-free survival compared with observation or less aggressive therapy.[4,5] Nevertheless, a continuous relapse pattern has been observed for these patients without evidence of cure or prolonged survival. These results have led to trials of high-dose therapy followed by autologous hematopoietic rescue for patients with low-grade lymphoma.

Although several reports have described the results of transplantation for low-grade lymphoma, there is relatively little experience in contrast to that of more aggressive histologic subtypes.[6–13] Like the results of conventional chemotherapy, our results and most other reports of transplantation for low-grade lymphoma show a continuous pattern of relapse without evidence that patients are cured or the natural history of disease is altered. Rohatiner et al. reported that relapse-free survival for follicular lymphoma patients transplanted in second remission was superior to historical controls, although overall survival was not statistically different.[9]

The long natural history of disease mandates prolonged follow-up after transplantation. We observed nine cases where disease progressed more than two years following transplantation. Relapses as long as nine years following transplantation for follicular low-grade lymphoma have been reported.[7] In addition, the development of secondary malignancies and myelodysplasia provide additional evidence of the need for prolonged follow-up.

Overall survival and FFS rates were significantly associated with the extent of therapy prior to transplantation. Rohatiner et al. also reported a correlation between the number of prior chemotherapy regimens and overall survival following transplantation for follicular lymphoma.[9] Similarly, Bastion et al. noted inferior overall survival and FFS in patients transplanted at a later stage of disease.[13] These observations can be interpreted to indicate that transplantation should be performed early for these patients. Alternatively, these observations may reflect the results of an intervention that is performed early in the course of a disease with a long natural history.

Results of therapy for follicular lymphoma are complicated by difficulties in reproducibly classifying these malignancies.[14] In addition, there is controversy concerning the natural history of these diseases. While some investigators feel that all follicular lymphomas have a similar natural history, there is evidence that patients with follicular large cell histology may have a natural history like that of

intermediate-grade lymphomas.[15,16] Some reports of transplantation for low-grade lymphomas have included patients with follicular large cell histology.[8,9,11,13] Differences in outcome among various series of transplantation for follicular lymphoma may be partly explained by inclusion of histologic subtypes with a spectrum of clinical behaviors.

Because of these controversies, we have attempted to evaluate outcome based upon subclassification of follicular lymphomas. We noted no differences in outcome between patients with follicular small cleaved cell and follicular mixed histology. However, patients with follicular large cell histology had a worse overall survival and FFS than other follicular lymphoma patients. Histology had no influence on transplant outcome in the series reported by Colombat et al.[8]

We found no significant differences in outcome between patients receiving TBI-containing regimens and those transplanted with regimens that contained drugs alone. Although many investigators have used TBI-containing regimens for transplantation for low-grade lymphomas, other investigators have noted equivalent results when drug-only regimens have been used.[8,10,13] The choice of preparative regimen needs close examination in light of the occurrence of myelodysplasia observed in our patients and evidence that TBI-containing regimens may contribute to this complication.[17]

The use of peripheral stem cells as a source of hematopoietic rescue has superseded the use of autologous bone marrow. We have found no difference in transplant outcome between patients transplanted with autologous peripheral stem cells as compared with unpurged autologous bone marrow. Our results using unpurged bone marrow are comparable to other series that have used purged autologous bone marrow,[6,9] or unpurged peripheral stem cells.[13] Although our data are retrospective, they demonstrate that long-term FFS is possible following transplantation with unpurged autologous bone marrow for low-grade lymphoma.

Our results have also failed to show a significantly poorer outcome in patients with histologic evidence of bone marrow involvement at the time of peripheral stem cell collection. Bastion et al. showed no difference in survival when bone marrow was involved at the time of stem cell collection.[13] Colombat et al. noted that a history of bone marrow involvement did not influence FFS.[8] Haas et al. failed to show any relapses among 22 patients transplanted with peripheral stem cells that were positive for t(14;18). Six of these patients subsequently lacked evidence of t(14;18)-containing cells in blood or bone marrow.[11] Although indirect evidence suggests that transplanted lymphoma cells may contribute to relapse,[18] the European Bone Marrow Transplant Group and others have failed to show a benefit from purging in low-grade lymphomas.[8,10,12] It is possible that transplanted lymphoma cells in blood or marrow may not be clonogenic, that insufficient numbers of tumor cells are being reinfused, or that host mechanisms may be able to eliminate malignant cells.

Our results demonstrate that long-term FFS is possible following transplantation for follicular NHL. Patients with follicular large cell histology had an inferior outcome when compared with other follicular lymphoma patients. We were unable to demonstrate that the preparative regimen or rescue source influenced outcome. Late relapses and second malignancies occurred and our data do not allow us to answer the fundamental question of whether patients are cured with this approach or whether the natural history of disease is altered. The management of low-grade lymphoma continues to be difficult since no form of therapy has conclusively been shown to be curative or to prolong survival, either at diagnosis or at relapse. Prospective randomized trials comparing conventional chemotherapy and high-dose therapy for follicular lymphoma are underway. The role of transplantation in first remission and the role of allogeneic bone marrow transplantation are being evaluated.

REFERENCES

1. Philip T, Guglielmi C, Hagenbeek et al.: Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma. *N Engl J Med* 333:1540–1545, 1995.
2. Mann RB, Berard CW: Criteria for the cytologic subclassification of follicular lymphomas: A proposed alternative method. *Hematol Oncol* 1:187–192, 1983.
3. Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 53:457–481, 1958.
4. Ezdinli EZ, Anderson JR, Melvin F et al.: Moderate versus aggressive chemotherapy of nodular lymphocytic poorly differentiated lymphoma. *J Clin Oncol* 3:769–775, 1985.
5. Young RC, Longo DL, Glatstein E et al.: The treatment of indolent lymphomas: Watchful waiting v aggressive combined modality treatment. *Semin Hematol* 25:(suppl 2)11–16, 1988.
6. Freedman AS, Ritz J, Neuberg D: Autologous bone marrow transplantation in 69 patients with a history of low-grade B-cell non-Hodgkin's lymphoma. *Blood* 77:2524–2529, 1991.
7. Fouillard L, Gorin NC, Laporte JPH et al.: Feasibility of autologous bone marrow transplantation for early consolidation of follicular non-Hodgkin's lymphoma. *Eur J Haematol* 46:279–284, 1991.
8. Colombat PH, Donadio D, Fouillard L et al.: Value of autologous bone marrow transplantation in follicular lymphoma: A France autogreffe retrospective study of 42 patients. *Bone Marrow Transplant* 13:157–162, 1994.
9. Rohatiner AZS, Johnson PWM, Price CGA et al.: Myeloablative therapy with autologous bone marrow transplantation as consolidation therapy for recurrent follicular lymphoma. *J Clin Oncol* 12:1177–1184, 1994.
10. Schouten HC, Colombat Ph, Verdonck LF et al.: Autologous bone marrow transplantation for low-grade non-Hodgkin's lymphoma: The European Bone Marrow Transplant Group experience. *Ann Oncol* 5:S147–S149, 1994 (suppl 2).
11. Haas R, Moos M, Karcher A et al.: Sequential high-dose therapy with peripheral-blood progenitor-cell support in low-grade non-Hodgkin's lymphoma. *J Clin Oncol* 12:1685–1692, 1994.

12. Cervantes F, Shu XO, McGlave PB et al.: Autologous bone marrow transplantation for non-transformed low-grade non-Hodgkin's lymphoma. *Bone Marrow Transplant* 16:387–392, 1995.
13. Bastion Y, Price P, Haioun C et al.: Intensive therapy with peripheral blood progenitor cell transplantation in 60 patients with poor-prognosis follicular lymphoma. *Blood* 86:3257–3262, 1995.
14. Metter GE, Nathwani BN, Burke JS et al.: Morphological subclassification of follicular lymphoma: Variability of diagnoses among hematopathologists, a collaborative study between the repository center and pathology panel for lymphoma clinical studies. *J Clin Oncol* 3:25–38, 1985.
15. Bartlett NL, Rizeq M, Dorfman RF et al.: Follicular large-cell lymphoma: Intermediate or low grade? *J Clin Oncol* 12:1349–1357, 1994.
16. Martin AR, Weisenburger DD, Chan WC et al.: Prognostic value of cellular proliferation and histologic grade in follicular lymphoma. *Blood* 85:3671–78, 1995.
17. Darrington D, Vose JM, Anderson JR et al.: Incidence and characterization of secondary myelodysplastic syndrome and acute myelogenous leukemia following high-dose chemoradiotherapy and autologous stem-cell transplantation for lymphoid malignancies. *J Clin Oncol* 12:2527–2534, 1994.
18. Gribben JG, Freedman AS, Neuberg D et al.: Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B-cell lymphoma. *N Engl J Med* 325:1525–33, 1991.

MYELOABLATIVE HIGH-DOSE THERAPY WITH PERIPHERAL BLOOD STEM CELL TRANSPLANTATION IN PATIENTS WITH LOW-GRADE NON-HODGKIN'S LYMPHOMA

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ABSTRACT

Between July, 1991, and March, 1996, 100 patients with low-grade non-Hodgkin's lymphoma (NHL) underwent high-dose conditioning therapy with peripheral blood stem cell (PBSC) transplantation. Fifty-two patients were males and 48 females with a median age of 46 years (range, 22–60). According to the Kiel classification, 79 patients had centroblastic-centrocytic (cb-cc) NHL, 14 patients had centrocytic (mantle cell), 6 patients had lymphocytic and one patient had marginal-zone NHL. Following high-dose cytarabine and mitoxantrone (HAM), a median of 2 leukaphereses (range, 1–7) were performed during filgrastim (RmetHuG-CSF)-supported marrow recovery. Forty-seven patients received a second cycle of HAM for further tumor reduction. At the time of autografting, 62 patients were in first complete or partial remission, whereas 38 patients were in second remission following a history of relapse or progressive disease. The high-dose conditioning therapy consisted of total body irradiation (TBI, hyperfractionated, 14.4 Gy) and cyclophosphamide (200 mg/kg). There were 5 patients who received BEAM (carmustine, etoposide, cytarabine, melphalan) because of previous radiotherapy. Following infusion of 5.95×10^6 $CD34^+$ cells/kg (range, 2.1–41.0), the median time to reach a neutrophil count of 0.5×10^9/L and a platelet count of 20.0×10^9/L was 13 and 12 days, respectively. The rate of transplantation-related mortality was 5%. Seventy-four patients are alive and in remission after a median follow-up time of 23 months (range, 4–52). Relapses were observed in 16 patients with a history of previous treatment failure and in 4 patients autografted while they were in first remission. The probability of event-free survival for patients autografted in first remission was 85% at 23

months in comparison with 70% for patients with a history of treatment failure ($p<0.01$). Myeloablative high-dose therapy with PBSC transplantation might therefore be envisaged for patients in first remission when the tumor burden is low and before drug resistance develops. Selection of $CD34^+$ cells may help to reduce the risk of reinfusing tumor cells, and the use of interferon-α is currently considered for maintenance therapy post-transplantation to prolong remission duration.

INTRODUCTION

Low-grade non-Hodgkin's lymphoma (NHL) is a heterogeneous group of lymphoproliferative diseases with an indolent clinical course in most of the cases.[1,2] The malignant cells may initially respond to cytotoxic treatment, but in patients with advanced-stage disease definite cure is rarely achieved following conventional cytotoxic chemotherapy. The probability of disease-free survival (DFS) at 5 years is around 25%,[3] while long-term remissions were observed following high-dose therapy and autologous bone marrow transplantation (autoBMT).[4–7] The curative potential of this treatment modality has been shown for patients with high-grade NHL in sensitive relapse by Philip et al.[8] With a 5-year follow-up time, 53% of the transplanted patients were alive and 46% disease-free compared with 32% and 2% in patients treated with a conventional "salvage" therapy, respectively. Peripheral blood stem cells (PBSC) are increasingly used for the support of high-dose therapy, including myeloablative conditioning regimens,[9,10] particularly because they reconstitute hematopoiesis more rapidly than does bone marrow.[11,12] Better survival rates were reported for patients with aggressive NHL when PBSC were re-infused instead of unpurged bone marrow.[13] In this regard, it might be relevant that the PBSC harvests collected post-chemotherapy contain a significantly smaller number of B-lymphoid progenitors and B-cells than bone marrow.[14,15]

We report on the efficacy of a high-dose therapy with total body irradiation (TBI) and blood stem cell transplantation in 100 patients with advanced-stage low-grade NHL.

PATIENTS AND METHODS

Patients

Since July 1991, 100 patients were enrolled in the study. Forty-eight patients were females and 52 were males. Their median age was 46 years with a range between 22 and 60 years. According to the Kiel classification, 79 patients had centroblastic-centrocytic (cb-cc) NHL, which largely corresponds to follicular lymphoma within the Working Formulation. Fourteen patients had

Table 1. Disease status at time of PBSC mobilization

	CB-CC	*MC*	*Others*	*Total*
no. of patients	79	14	7	100
first remission (%)	62	64	57	62
second remission (%)	18	22	29	19
relapse/progressive disease (%)	20	14	14	19

mantle cell NHL. In addition to histopathological examination, the latter diagnosis was confirmed by immunohistochemistry or FACS analysis to demonstrate co-expression of CD20 and CD5 on the lymphoma cells. Six patients had lymphocytic NHL, and one patient had marginal-zone NHL. The patients had a median of 6 cycles of previous cytotoxic chemotherapy (range, 0–21) and 16 had undergone previous radiotherapy. Forty-eight patients had bone marrow involvement at the time of PBSC mobilization by histopathological examination.

Sixty-two patients were included while they were in first remission, whereas 19 patients had achieved a second remission. Nineteen patients were included while they were in relapse or because of progressive disease.

All patients received high-dose cytarabine (2 g/m^2 q12 hours on day 1 and 2) and mitoxantrone (HAM) (10 mg/m^2 q24 hours on day 2 and 3) followed by a standard dose of R-metHuG-CSF (Filgrastim, Amgen, Thousand Oaks, CA) of 300 μg per day, subcutaneously.

The study was conducted under the guidelines of the Ethical Committee of the University of Heidelberg.

PBSC collection and cryopreservation procedure

PBSC collection was performed using a Fenwal CS 3000 (Baxter Deutschland GmbH, Munich, Germany) or a Spectra (Cobe Laboratories, Lakewood, CO) cell separator when a WBC >1.0×10^9/L was reached and a distinct CD34$^+$ cell population was detectable by immunofluorescence analysis. Between 10 and 20 liters of blood were processed per apheresis at flow rates between 50 and 150 mL/min. The apheresis product of 50 mL was mixed with the same volume of minimal essential medium (MEM) containing 20% dimethyl sulfoxide (DMSO). The final 100 mL cell suspension was transferred into freezing bags (Delmed, New Brunswick, NJ) and frozen to −100°C using 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.

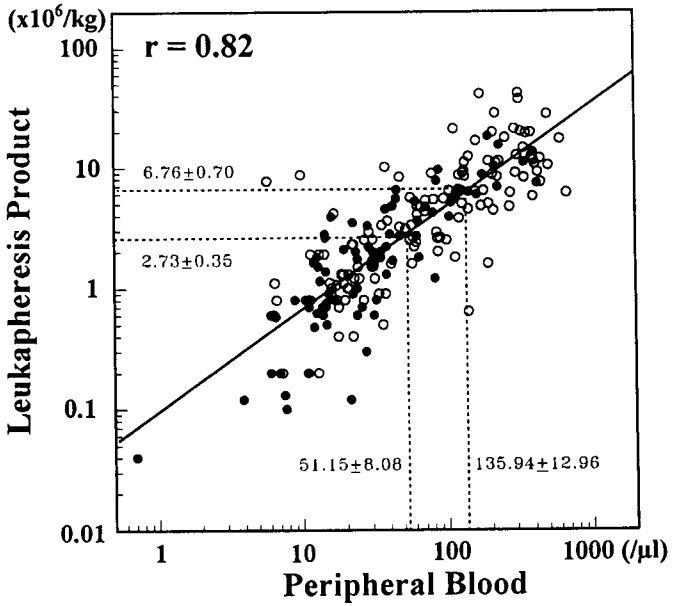

Figure 1. *Correlation between the concentration of circulating CD34⁺ cells in the peripheral blood and the yield in the respective leukapheresis product. The regression analysis is based on 223 paired samples from 100 patients with low-grade NHL mobilized during first remission (hollow circles) or during second remission (filled circles). The dotted lines and the corresponding numbers indicate the mean (±SEM) values of the grouped data, showing a 2.5-fold greater yield for patients mobilized in first remission.*

Pre-transplant conditioning regimens and intensive care post-transplant

High-dose conditioning therapy consisted of TBI (14.4 Gy, hyperfractionated, over 4 days) and cyclophosphamide (200 mg/kg, over 4 days) in 95 patients, while 5 patients received the BEAM protocol (carmustine 300 mg/m^2, etoposide 120 mg/m^2, Ara-C 800 mg/m^2 and melphalan 140 mg/m^2). The patients received prophylactic partial gut decontamination. Antibiotic combination therapy was administered for fever of greater than 38.5°C, and amphotericin-B was added for documented fungal infection or persistent fever. A platelet count of greater than 20×10^9/L was maintained by HLA-A/B matched platelet transfusions and packed red cells were given when the hemoglobin was below 8 g/dL.

Table 2. Non-hematological toxicity

days of fever >38.5°C	
median	5
range	0–18
days of IV-antibiotics	
median	13
range	0–33
red blood cell transfusions	
median	4
range	0–18
platelet transfusions	
median	5
range	1–25

Immunofluorescence staining and flow cytometry

For dual-color immunofluorescence analysis, 1×10^6 mononuclear cells or 20–50 μL of whole blood were incubated for 30 minutes at 4°C with the fluorescein (FITC)-conjugated monoclonal antibodies (mAb) HPCA-2 (CD34) and HLe-1 (CD45) and the phycoerythrin (PE)-conjugated mAb Leu-12 (CD19). Isotype identical antibodies served as control (Simultest). All antibodies were obtained from Becton Dickinson, Heidelberg, Germany. The cells were analyzed using a Becton Dickinson FACScan, as described previously.[16]

Statistical analysis

The clinical and laboratory data of the patients were analyzed according to standard statistical methods using a commercially available computer program (Statworks, Cricket Software, Philadelphia, PA). The results are given as mean ± standard error of the mean (SEM) or as median and range. Statistical significance between differences of grouped data was determined using the student's *t*-test. A significance level of $p<0.05$ was chosen. The relationship between the number of circulating $CD34^+$ cells and yield of $CD34^+$ cells/kg in the respective leukapheresis product was assessed by simple linear regression and correlation analysis. The estimate of the survival curves was computed using the Kaplan-Meier method. For comparison of the probability of disease-free and overall survival in different subgroups of patients the logrank test was used.

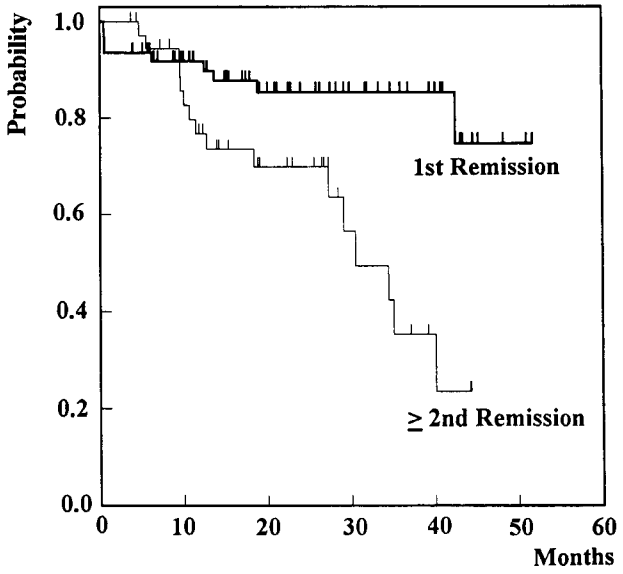

Figure 2. *Probability of event-free survival in 100 patients with low-grade NHL following high-dose conditioning therapy with peripheral blood stem cell transplantation. Patients auto-grafted in first remission (upper curve) have a better event-free survival than patients with a history of previous treatment failure (lower curve, $p<0.01$).*

RESULTS

Circulating CD34⁺ cells and PBSC collection

A close relationship was found between the number of circulating $CD34^+$ cells in the peripheral blood and the yield obtained in the respective leukapheresis product (Figure 1). The mean number of $CD34^+$ cells collected per kg body weight was approximately 2.5-fold greater in patients who were in first remission when compared with patients mobilized in second remission, relapse or during progressive disease ($6.76 \pm 0.70 \times 10^6$ $CD34^+$ cells/kg versus $2.73 \pm 0.35 \times 10^6$ $CD34^+$ cells/kg). The proportion of B-lymphoid progenitors and B cells as defined by direct immunofluorescence was extremely low. The proportion of $CD34^+/CD19^+$ cells contained in the LP products was $1.35 \pm 0.24\%$ [mean ± SEM], while the proportion of $CD19^+$ cells was $0.26 \pm 0.10\%$ [mean ± SEM]. The number of these cell subsets in the LP products was not significantly different between the two patient groups.

Autografting with G-CSF–mobilized PBSC

In 95 patients, high-dose therapy consisted of TBI (14.4 Gy, hyperfractionated over 4 days) and cyclophosphamide (200 mg/kg). Five patients with a history of previous radiotherapy received BEAM. At the time of high-dose therapy, 62 patients were in first complete or partial remission, while 38 had achieved a second complete or partial remission. Five patients died of treatment-related complications between 13 and 188 days post-transplantation. The median time to recover a neutrophil count of 0.5×10^9/L was 13 days (range, 8–34), while the median time to achieve a platelet count of 20.0×10^9/L was 12 days (range, 6–51). The reason for the delay in platelet recovery observed in 5 patients is not clear. The nonhematological toxicity encountered was moderate and mainly consisted of mucositis and neutropenic fever. The median duration of hospitalization following high-dose therapy was 18 days (range, 9–49). The parameters reflecting the treatment-related toxicity are summarized in Table 2.

Follow-up post-transplantation

Following a median time of 13 months (range, 5–42), 20 patients relapsed (17 cb-cc, 2 mantle cell NHL and 1 lymphocytic NHL). Sixteen patients in relapse had a history of previous treatment failure, and the relapses occurred at sites of previous disease, including lymph nodes, bone marrow and extranodal sites. Four relapses were observed in patients who were autografted while they were in first remission. In these patients, the sites of relapse included new localizations different from those involved at the time of initial diagnosis. One patient in complete remission developed a necrotizing fungal pneumonia with sepsis and died ten months post-transplantation.

Fifty-two patients transplanted in first remission and 22 patients autografted in second or greater remission are alive and disease-free with a median follow-up time of 23 months (range, 4–52 months). This translates into a probability of event-free survival of 85% for patients transplanted in first remission and 70% for patients with a history of previous treatment failure at the median follow-up time of 23 months (Figure 2). The difference between both patient groups is statistically significant ($p<0.01$). Dependent on the kind of previous first- and second-line cytotoxic chemotherapy the patients in relapse received a palliative treatment that consisted either of cyclophosphamide, vincristine, prednisone (COP), prednimustine with HAM (Noste), fludarabine, interferon-α or local radiotherapy. With a median observation period of 9 months (range, 3–33), 14 patients are alive after relapse, while 6 patients died due to disease progression. The probability of overall survival for patients autografted in first

remission is 91%, whereas patients with a history of previous treatment failure have a probability of overall survival of 82% at the median follow-up time of 26 months.

DISCUSSION

In this single-center study, the efficacy and tolerability of a high-dose conditioning therapy with PBSC was evaluated in a group of 100 patients with low-grade NHL. The patient population was heterogeneous with respect to the histological subtypes included, the stage and disease status at the time of entry into the study. The type of previous chemo- and radiotherapy was also different among patients. Regardless of this heterogeneity in the patients' characteristics, all patients received high-dose cytarabine and HAM with G-CSF support as consolidation or salvage therapy in an attempt to decrease the tumor load before high-dose therapy and to collect PBSC during marrow recovery. A sufficient amount of $CD34^+$ cells could be harvested during the course of 2 leukaphereses (median, 1–7). It turned out that patients mobilized in first remission obtained a 2.5-fold greater number of $CD34^+$ cells than patients with a history of previous treatment failure. This finding argues for PBSC harvesting early during the course of the disease. At the same time, the amount of B-lymphoid progenitor cells ($CD34^+/CD19^+$) and B cells in the autografts ($CD19^+$) was extremely low and not related to the disease status at the time of mobilization.

The event-free survival of 23% at the longest follow-up time for patients with a history of previous treatment failure is disappointing. The 16 relapses observed in this patient group occurred at sites of previous disease, suggesting that relapse may have developed from resistant tumor cells in vivo rather than from reinfused lymphoma cells. The group of Nadler et al.[17] and Rohatiner et al.[18] reported slightly better results for a similar group of patients with follicular NHL in chemosensitive relapse. The high-dose therapy consisted of TBI and cyclophosphamide, but purged bone marrow was used for autografting instead of PBSC. The question of whether this accounts for the difference in the clinical outcome or whether patient selection was effective cannot be answered.

To improve the DFS, the administration of interferon-α is envisaged post-transplantation for maintenance therapy. More recently, it could be shown that the probability of DFS in patients with advanced-stage low-grade NHL in first remission could be significantly prolonged following conventional cytotoxic therapy when interferon-α was given.[19–21] In addition, involved-field radiotherapy could be administered post-transplantation to sites of previous bulky disease. The majority of patients in relapse received palliative treatment, which consisted of COP, prednimustine with HAM, fludarabine, interferon-α

or local radiotherapy. Fourteen patients are alive with a median follow-up time of 9 months (range, 3–33) after relapse.

Fifty-two patients transplanted in first remission are alive and disease-free. The probability of DFS of 85% at 23 months is encouraging compared with the data reported for patients who received conventional cytotoxic chemotherapy. It should be also noted that this group of patients includes 9 patients with mantle cell NHL, which is rarely cured by conventional chemotherapy. Still, caution is warranted in interpreting the data, since the median follow-up time is still relatively short.

To reduce the risk of reinfusing tumor cells in patients with B-cell malignancies ex-vivo methods for the selection of $CD34^+$ cells are envisaged, as preliminary data suggest that $CD34^+$ cells do not contain lymphoma cells bearing the t(14;18) translocation. The impact of these ex-vivo procedures on the clinical outcome cannot be answered yet and would require gene marking studies.

In summary, a G-CSF-supported cytotoxic chemotherapy with cytarabine and HAM is an efficient anti-neoplastic treatment and permits the mobilization of PBSC in patients with low-grade NHL. PBSC-supported high-dose therapy may be particularly beneficial for patients who are in first remission following conventional cytotoxic chemotherapy.

REFERENCES

1. Rosenberg SA: The low-grade non-Hodgkin's lymphomas: Challenges and opportunities. *J Clin Oncol* 3:299–310, 1985.
2. Rohatiner AZS, Lister TA: Management of follicular lymphoma. *Curr Opin Oncol* 6:473–479, 1994.
3. Brittinger G, Bartels H, Common H et al.: Clinical and prognostic relevance of the Kiel classification of non-Hodgkin lymphomas: Results of a prospective multicenter study by the Kiel Lymphoma Study Group. *Hematol Oncol* 2:269–306, 1984.
4. Petersen FB, Appelbaum FR, Hill R et al.: Autologous transplantation for malignant lymphoma: A report of 101 cases from Seattle. *J Clin Oncol* 8:638–647, 1990.
5. Freedman AS, Takvorian T, Andersen KC et al.: Autologous bone marrow transplantation in B-cell non-Hodgkin's lymphoma: Very low treatment-related mortality in 100 patients in sensitive relapse. *J Clin Oncol* 8:784–791, 1990.
6. Freedman AS, Ritz J, Neuberg D et al.: Autologous bone marrow transplantation in 69 patients with a history of low grade B-cell non-Hodgkin's lymphoma. *Blood* 77:2524–2529, 1991.
7. Schouten HC, Bierman PJ, Baughan WP et al.: Autologous bone marrow transplantation in follicular non-Hodgkin's lymphoma before and after histologic transformation. *Blood* 74:2579–2584, 1989.
8. Philip T, Guglielmi C, Hagenbeek A et al.: Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-

Hodgkin's lymphoma. *N Engl J Med* 333:1540–1545, 1995.

9. Kessinger A, Vose JM, Bierman PJ et al.: High-dose therapy and autologous peripheral stem cell transplantation for patients with bone marrow metastases and relapsed lymphoma: An alternative to bone marrow purging. *Exp Hematol* 19:1013–1016, 1991.
10. Kessinger A, Armitage JO: The evolving role of autologous peripheral stem cell transplantation following high-dose therapy for malignancies. *Blood* 77:211–213, 1991.
11. Liberti G, Pearce R, Taghipour G et al.: Comparison of peripheral blood stem cells and autologous bone marrow transplantation for lymphoma patients: A case-controlled analysis of the EBMT registry data. *Ann Oncol* 5(suppl 2):151–153, 1994.
12. Fielding AK, Watts MJ, Goldstone AH: Peripheral blood progenitor cells versus bone marrow. *J Hematother* 3:299–304, 1994.
13. Vose JM, Anderson JR, Kessinger A et al.: High-dose chemotherapy and autologous hematopoietic stem-cell transplantation for aggressive non-Hodgkin's lymphoma. *J Clin Oncol* 11:1846–1851, 1993.
14. Haas R, Moos M, Karcher A et al.: Sequential high-dose therapy with peripheral-blood progenitor-cell support in low-grade non-Hodgkin's lymphoma. *J Clin Oncol* 12:1685–1692, 1994.
15. Haas R, Murea S, Goldschmidt H et al.: High-dose therapy with peripheral blood progenitor cell support in patients with non-Hodgkin's lymphoma. *Stem Cells* 13(suppl 3):28–35, 1995.
16. Haas R, Moehle R, Fruehauf S et al.: Patient characteristics associated with successful mobilization and autografting of peripheral blood progenitor cells in malignant lymphoma. *Blood* 83: 3787–3794, 1994.
17. Nadler LM, Takvorian T, Botnick L et al.: Anti-B1 monoclonal antibody and complement treatment in autologous bone-marrow transplantation for relapsed B-cell non-Hodgkin's lymphoma. *Lancet* 2: 427–431, 1984.
18. Rohatiner AZ, Johnson PW, Price CG et al.: Myeloablative therapy with autologous bone marrow transplantation as consolidation therapy for recurrent follicular lymphoma. *J Clin Oncol* 12: 1177–1184, 1994.
19. Solal-Celigny P, Lepage E, Brousse N et al.: Recombinant interferon alfa-2b combined with a regimen containing doxorubicin in patients with advanced follicular lymphoma. Groupe d'Etude des Lymphomes de l'Adulte. *N Engl J Med* 329: 1608–1614, 1993.
20. Smalley RV, Andersen JW, Hawkins MJ et al.: Interferon alfa combined with cytotoxic chemotherapy for patients with non-Hodgkin's lymphoma. *N Engl J Med* 327: 1336–1341, 1992.
21. Unterhalt M, Herrmann R, Nahler M et al.: Significant prolongation of disease-free survival in advanced low-grade non-Hodgkin's lymphomas (NHL) by interferon alpha maintenance. *Blood* 86(suppl 1):439, 1995, [abstr].

PERIPHERAL BLOOD STEM CELL TRANSPLANTATION IN CLL PATIENTS IN REMISSION AFTER FLUDARABINE THERAPY: A FEASIBILITY STUDY

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a clonal expansion of small lymphocytes, usually of B-cell lineage, with a low proliferative index and prolonged cell survival. It is the most common type of leukemia in adults and typically occurs in persons over 50 years old. The disease has an indolent natural history, and is incurable with conventional treatments. In most cases advanced age makes palliation of symptoms and prolongation of survival the therapeutic goals.[1,2] Currently, new agents such as fludarabine have been demonstrated to be effective as primary or salvage chemotherapy, with high percentages of transient, complete or near complete bone marrow (BM) remissions, rarely achieved with previous chemotherapy programs.[1,3,4]

These developments suggest that intensive therapy may have a role in the management of CLL. For younger patients with high risk features, who have a median survival of less than 3 years with standard treatment, the collection of autologous marrow minimally involved by malignant clone may be feasible. Recently, high-dose chemoradiotherapy with ex vivo purged autologous bone marrow transplantation (autoBMT) has been performed in patients <60 years of age at different stages of disease and preliminary results seem encouraging.[5–7] The use of peripheral blood stem cell (PBSC) transplantation in CLL has not been fully investigated, especially in patients previously treated with fludarabine.[9] In order to test the feasibility of this procedure, we performed intensification with high-dose chemotherapy followed by unmanipulated PBSC rescue in 8 young patients with CLL who had minimal residual disease after fludarabine treatment.

MATERIALS AND METHODS

Patients with B-lineage chronic lymphocytic leukemia (CLL) with minimal disease after fludarabine therapy, were eligible for the study. Eligibility also

Table 1. Disease status at autograft

Pt	*BM biopsy: pattern*	*BM biopsy: % involvement*	*CD5/CD20⁺ BM (%)*	*CD5/CD20⁺ PB ($\times 10^9$/L)*
1	int	10	9	0.259
2	int/nod	30	1.52	0.014
3	int	15	0.3	0.021
4	int	30	1.32	N.E.
5	int	10	2.64	0.439
6	int	10	1.05	0.054
7	nod	20	4.95	0.04
8	int/nod	10	0.72	0.016
median		12.5	1.42	0.04

included 60 years of age or less and stage B disease, prior fludarabine treatment, as defined by the International Workshop on CLL. Good performance status was required, with absence of concomitant disease of the lungs, liver and heart. Informed consent was obtained from all patients. Before admission all patients were evaluated by physical examination, blood-chemistry profile, complete blood count (CBC), serum protein immunoelectrophoresis, chest X-ray, chest CT scan, abdominal-pelvic ultrasound and/or computer tomography scanning. Clinical complete remission (CR) was defined as the absence of any detectable disease.

Immunophenotype analysis was performed on BM and peripheral-blood samples before and after transplantation by flow cytometry using simultaneous dual-color staining. Residual disease was determined by co-expression either of CD19 or CD20 and CD5 on B lymphocytes. The presence of more than 10% of the total lymphocyte population co-expressing CD5 and CD20 was considered positive for residual disease.

For all patients, before and after transplantation, BM samples were cryopreserved for Ig gene rearrangement analysis.

Patients were nursed in a double room, in a general hematologic ward. A central venous line was inserted in all patients before the procedure. PBSC collection was performed after high-dose cyclophosphamide (7 g/sqm) followed by subcutaneous recombinant human granulocyte colony stimulating factor (rh G-CSF) administration at 5 mcg/kg/day. Cytaphereses were performed during the hematopoietic recovery phase as soon as the leukocyte count reached at least 1×10^9/L or as soon as CD34⁺ mononuclear

cells were detectable in the peripheral blood. Aphereses of 7–10 liters were processed using appropriate blood cell separators. The required number of $CD34^+$ cells was $>2\times10^6$/kg. Collected cells were cryopreserved after obtaining samples for sterility, differentials and CFU-GM cultures. The preparative regimen was identical for all patients and consisted of BEAM (BCNU, etoposide, Ara-C, melphalan). Prophylactic allopurinol, antiemetics and hyperhydration were administered throughout the ablative chemotherapy period. Melphalan-induced stomatitis was prevented with an appealing and cheap supportive therapy: popsicle eating during melphalan infusion.[10] Within 24 hours of the completion of chemotherapy, patients received cryopreserved autologous stem cells (SC). After SC reinfusion, the patients received rhG-CSF (5 mcg/kg/day) until neutrophil recovery.

Prophylactic oral antibiotics and intravenous acyclovir (15 mg/kg/day) were given routinely during aplasia, and broad spectrum I.V. antibiotic therapy was instituted in case of fever >38°C. All blood products given after high-dose chemotherapy were irradiated. Platelet transfusions were given if the platelet count was $<10\times10^9$/L, or if clinically indicated.

PATIENT DETAILS AND RESULTS

As of August, 1996, eight CLL patients with minimal residual disease (MRD) following fludarabine therapy had undergone autologous transplantation after BEAM conditioning regimen (Table 1). Median age was 48 years (range, 43 to 56). Seven were males. Median interval from diagnosis to transplant was 41 months (range, 12 to 67). According to Binet's classification, all patients were stage B before fludarabine treatment.[2] At the time of initial evaluation for transplant, the majority of patients had residual interstitial lymphocytic infiltration on BM biopsy. The degree of residual BM involvement as assessed by percentage of intertrabecular space infiltrated varied from 10 to 30%. At the time of transplantation, 3 patients were in first clinical and immunological CR after fludarabine alone, and 5 were in second CR either after fludarabine alone (1 patient) or after fludarabine combined with Ara-C and mitoxantrone (4 patients). The median number of previous regimens was 2 (range, 1 to 5). All patients completed the planned mobilization schema with high-dose cyclophosphamide and rhG-CSF. Due to unsatisfactory PBSC collection, three patients underwent marrow collection and reinfusion (total number of nucleated cells of 1.6, 1.9 and 3.26×10^8/kg, respectively). The other 5 patients received PBSC: median number of cytaphereses was 4.5 (range, 1 to 7) and median number of $CD34^+$ cells was 3.9 (range, 1.5 to 11.3) $\times$ 10^6/kg. All patients received rhG-CSF (5 mcg/kg/day) from day +5 to neutrophil recovery. All patients engrafted;

Table 2. Recovery and follow-up

Pt	*CD34 + ($\times 10^6$/kg)*	*Day PMN >0.5*	*Day PLTS >20*	*Follow-up (months)*
1	BM	21	115	14+, A&W
2	11.3	12	15	11+, A&W
3	1.5	15	63	11+, A&W
4	3.9	9	55	8+, A&W
5	BM	24	52	4+, A&W
6	3.55	12	14	4+, A&W
7	BM	12	24	1+, A&W
8	3.9	14	31	1+, A&W
median	3.9	13	42	

median time to recovery was 13 days (range, 9 to 24) for granulocytes greater than 0.5×10^9/L, and 33 days (range, 14 to 115) for platelets greater than 20×10^9/L. Four patients developed fever with documented positive blood cultures during aplasia: two patients had staphylococcus, one had streptococcus and one had staphylococcus and streptococcus species bacteriemia. Because of prophylactic popsicle consumption, only one patient experienced severe mucositis. Late complications included two cutaneous herpes zoster infections, at 3 and 4 months after transplant.

At 6 months median follow-up (range, 1 to 14), all 8 patients are alive and well in persisting unmaintained CR (Table 2).

DISCUSSION

CLL is one of the few hematologic malignancies for which high-dose myeloablative therapy and BMT have played a limited therapeutic role. A small number of highly selected patients with CLL have been treated with allogeneic BMT.[5,7,8] Unfortunately, this disease predominantly affects elderly patients who are not eligible for allografts. Recent advances in chemotherapy with fludarabine-based regimens have produced BM remissions rarely achieved with conventional chemotherapy regimens. This offers the opportunity to collect autologous BM when it is minimally involved by the malignancy. Ex vivo treatment of harvested autologous marrow has been effectively performed to deplete malignant lymphoma cells and appears to be a logical approach for CLL.[6] An alternative approach to the problem of reinfusion of malignant lymphoid cells has been the use of peripheral blood stem cells, on the basis that these are less likely to be contaminated with tumor cells.[6,11] Although

autologous blood stem cell transplant is used increasingly in multiple myeloma and lymphoma, only 2 CLL patients have been autografted with PBSC collected after fludarabine, according to one report.[9] Delayed hematopoietic recovery was observed in both patients: one died in aplasia 3 months post-transplant and the other has persistent thrombocytopenia 12 months after the procedure. Our results show that it is possible to collect a sufficient number of PBSC in CLL patients (5 of 8 patients treated) after fludarabine treatment. Furthermore, rapid recovery and absence of serious toxicity show that intensification with PBSC support seems to be feasible in young patients with high risk CLL. No life-threatening complications were observed despite the immune deficiency associated with the underlying disease and the previous fludarabine treatment. Our results, although obtained in a heterogeneous group of patients with a short follow-up, are encouraging and justify further investigation of this therapeutic approach.

REFERENCES

1. Catovsky D and Murphy RLW: Key issues in the treatment of chronic lymphocytic leukemia (CLL). *Eur J Cancer* 31A, Nos13/14: 2146–2154, 1995.
2. Binet JL, Catovsky D, Chandra P et al.: Chronic lymphocytic leukemia: Proposal for a revised prognostic staging system. *Br J Haematol* 48: 365–367, 1981.
3. Keating MJ, Kantarjian H, O'Brien S et al.: Fludarabine: A new agent with marked cytoreductive activity in untreated chronic lymphocytic leukemia. *J Clin Oncol* 9:44, 1991.
4. Keating MJ, O'Brien S, Kantarjian H et al.: Long-term follow-up of patients with chronic lymphocytic leukemia treated with fludarabine as a single agent. *Blood* 81:1878–2884, 1993.
5. Rabinowe SN, Soiffer RJ, Gribben JG et al.: Autologous and allogenic bone marrow transplantation for poor prognosis patients with B-cell chronic lymphocytic leukemia. *Blood* 82, 4:1366–1376, 1993.
6. Khouri IF, Keating MJ, Huibert M et al.: Autologous and allogeneic bone marrow transplantation for chronic lymphocytic leukemia: Preliminary results. *J Clin Onc* 12:748–758, 1994.
7. Michallet M, Archimbaud E, Rowlings PA et al.: Hematopoietic stem cell transplant for chronic lymphocytic leukemia. Report from the EBMTG. *Bone Marrow Transplant* 17(suppl 1):March, 1996, [abstr].
8. Michallet M, Archimbaud E, Bandini G et al.: HLA-identical sibling bone marrow transplantation in younger patients with chronic lymphocytic leukemia. European Group for Blood and Marrow Transplantation and the International Bone Marrow Transplant Registry. *Ann Intern Med* 124:311–5, 1996.
9. Bastion Y, Felman P, Dumontet C et al.: Intensive radio-chemotherapy with peripheral blood stem cell transplantation in young patients with chronic lymphocytic leukemia. *Bone Marrow Transplant* 10:467–468, 1992.

10. Meloni G, Capria S, Proia A et al.: Ice pops prevent melphalan-induced stomatitis. *Lancet* 347:1691–1692, 1996.
11. Henon PR: Peripheral blood stem cell transplantations: Past, present and future. *Stem Cells* 11:154–172, 1993.

LYMPHOBLASTIC LYMPHOMA IN ADULTS: CLINICAL ASPECTS AND THERAPY

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ABSTRACT

Lymphoblastic lymphoma is a distinct subgroup of non-Hodgkin's lymphomas that the Working Formulation categorizes as a high-grade malignancy. It is characterized at diagnosis by frequent mediastinal involvement. However, bone marrow (BM) and subsequent peripheral blood involvement are common. From a histologic and cytologic point of view it is identical to acute lymphoblastic leukemia. Sequential chemotherapy has improved the prognosis of this lymphoma in terms of survival and disease-free survival (DFS). However, several negative prognostic factors present at diagnosis seem to reduce survival and DFS due to the high risk of relapse. For this reason, small groups of patients in complete remission (CR) were treated with high-dose therapy and autologous bone marrow transplantation (autoBMT) after conventional chemotherapy. An international randomized study is presently underway to determine the usefulness of autoBMT for patients responding to front-line therapy.

INTRODUCTION

Lymphoblastic lymphoma is an aggressive non-Hodgkin's lymphoma that the Working Formulation places in the group of high-grade malignancy lymphomas (Group I/WF).[1] It is more common in males and tends to affect adolescents and young adults with frequent mediastinal involvement at diagnosis.[2] Bone marrow involvement at, or immediately following diagnosis is common and leukemic evolution may frequently be the final event. Involvement of the central nervous system is not common at presentation.

Lymphoblastic lymphoma among adults is rare, making up approximately 3–4% of all cases of non-Hodgkin's lymphomas.[3] From both a histologic and cytologic point of view it is indistinguishable from acute lymphoblastic leukemia. This often leads to problems in identifying the two diseases. Clinical development of the lymphoma is usually characterized by the presence of initial adenopathy or

Table 1. Immunological characterization of 18 lymphoblastic lymphomas

Cases	*CD3*	*CD4*	*CD1a*	*CD8*	*CD71*	*CD38*	*CD2*	*HLA-DR*	*CD10*	*CD20*	*C-Ig*	*S-Ig*	*Immunotype*
1	–	–	–	–	++	+	+	+	–	–	–	–	T
2	++	++	–	++	–	++	Nd	+	+	–	–	–	T
3	–	–	–	–	+	+	–	++	++	++	++	–	pre-B
4	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
5	–	–	–	–	–	–	–	++	++	–	–	–	nonT/B
6	++	+	–	++	–	–	++	–	–	–	Nd	Nd	T
7	++	–	–	–	++	–	++	–	–	–	Nd	Nd	T
8	++	+	+	+	–	++	++	–	–	–	Nd	Nd	T
9	++	–	–	+	+	–	++	–	–	–	Nd	Nd	T
10	++	++	++	–	++	Nd	++	–	+	–	–	–	T
11	–	–	–	–	–	–	–	++	++	–	–	–	nonT/B
12	–	–	+	–	+	++	–	–	–	–	Nd	Nd	T
13	++	–	+	–	++	++	Nd	–	–	–	Nd	Nd	T
14	+	Nd	++	Nd	++	++	Nd	–	–	–	–	–	T
15	++	+	Nd	+	+	Nd	Nd	–	–	Nd	Nd	Nd	T
16	–	Nd	–	Nd	–	+	–	++	++	++	–	–	pre-B
17	++	++	–	++	+	–	–	–	–	–	Nd	Nd	T
18	–	–	–	–	–	Nd	Nd	++	++	+	–	–	pre-B

Antibody staining was scored as: –, 0 to 10%; +, 11 to 50%; ++, 51 to 100%.

Nd: not determined; Non T/B: non-T, non-B, C-Ig: cytoplasmic Ig; S-Ig: surface Ig (Santini et al.[6]).

signs of mediastinal involvement without initial clinical signs of bone marrow (BM) involvement. However, this region is nearly always compromised if diagnosis is delayed and the clinical picture is identical to that of leukemias with nodal activity. In other cases, diagnosis of BM involvement coincides with that of mediastinal and nodal activity.

Since there are no reliable criteria to identify the two illnesses, the extent of BM involvement is used as the distinguishing factor. Thus, a case characterized by less than 25% BM involvement without circulating blasts is identified as lymphoblastic lymphoma.[4] Other studies have included cases with less than 10% circulating blasts in this category of patients.[5]

There is no doubt that both lymphoma and lymphoblastic leukemia derive from precursors of the T and B lines in various stages of differentiation. Cells characterized by these lines are found in various parts of the body. Their tumoral counterparts tend to be found in the same region once there is clinical evidence of disease. Pre-B cells are mainly found in BM and so resulting neoplasms are usually leukemias. The precursor T cells originate in the BM, migrate to the thymus and are then active in secondary lymphoid organs, which include BM. So T neoplasms can be present as lymphomas or leukemias. This explains how a patient may present a clinical picture of both lymphoma and leukemia at the same time or how these may co-exist in a variety of forms during the course of illness.

IMMUNOPHENOTYPE

Even though the lymphoblastic lymphoma immunophenotype presents considerable heterogeneity,[4,5] there is no doubt that the T phenotype is dominant. Various T marker cells are present according to the stage of differentiation. The less mature cells can therefore register the transferrin receptor CD71 and an antigen often present on activated lymphocytes and plasma cells such as CD38. At the intermediary stage of differentiation thymocytes register CD1a, CD4 and CD8. This continuous differentiation is seen from the fact that cells can register both CD4 and CD8 receptors at the same time, and also are CD3 positive.

Usually lymphoblastic lymphoma cells show intermediate (cortical thymic) or mature (medullary thymic) phenotypes. However, cases do exist that register only prethymic markers.

Finally, there are a small number of cases of pre-B origin, or cases positive only for the HLA-DR and CD10 antigens as reported in Table 1 (two cases identified as non-T/non-B).[6]

Two pre-B cases which were positive for the HLA-DR, CD10 and CD20 antigens registered a small percentage of cells which were also positive for CD71 and CD38. Similar observations have been reported by others.[7,8]

Table 2. Clinical features of 45 lymphoblastic lymphomas at diagnosis (observed by the NHLCSG)

number of patients	45
sex:	males 28 females 17
median age	20 years (range 15–51)
stage:	
II bulky>15 cm	2
III	2
IV	41
mediastinal involvement	39 (86%)
mediastinal bulky disease >10 cm	20 (44%)
BM involvement	24 (53%)
spleen involvement	15 (33%)
extranodal localizations:	
pleura	16
liver	10
kidney	7
pericardium	6
CNS	3
gut/bone/skin/lung	2
muscle/pancreas	1

BM=bone marrow; CNS=central nervous system.

EPIDEMIOLOGY

While some information is available about Burkitt's lymphoma, there are very few epidemiological data for lymphoblastic lymphoma, although on the whole the latter appears to be more frequent. Lack of data is probably due to the difficulty in distinguishing between acute lymphoblastic leukemia and lymphoma. Lymphoblastic lymphoma represents approximately 5% of all non-Hodgkin's lymphomas in the United States and Europe.[9] Lymphoblastic lymphoma is generally considered to be an illness which affects children and young people, with the highest incidence between 10–20 years of age.[10] An exception to these data is reported by Nathwani in a study of 97 patients in which 50% were over 30 years of age.[11] Median age of males was 27 and of females 50 with a ratio between them of 2:1. Males showed a bimodal distribution of incidence according to age with a maximum incidence of the disease between 10–20 years of age and again in their 70s.

CLINICAL FEATURES AND STAGING

Mediastinal involvement is the most common presentation of lymphoblastic lymphoma (50–70% of cases). Lennert reports this in more than 80% of examined

Table 3. Clinical features at diagnosis of 26 lymphoblastic lymphomas undergoing autoBMT in 1st CR (treated by the NHLCSG)

number of patients	26	
sex:	males 17	females 9
median age	25 years (range 15–43)	
stage (no.):		
III	2	
IV	24	
mediastinal involvement	23	
BM involvement	11	
spleen involvement	8	
LDH > 150% maximum level	13	
extranodal localizations:		
pleura	5	
liver	4	
kidney	4	
pericardium	3	
lung	2	
gut/muscle/skin/bone	1	

cases.[10] In the 45 patients studied by our Group, the Non-Hodgkin's Lymphoma Co-operative Study Group (NHLCSG), mediastinal involvement was seen in 86% (Table 2). Pleural localization may be seen in a certain number of cases. Associated symptoms include dyspnea, dysphagia, pain and sometimes precava obstruction syndrome. Adenopathy is usually limited to the epinephral region, but numerous cases show involvement of the abdominal region and spleen, liver and kidney involvement.

Like all aggressive lymphomas, lymphoblastic lymphoma can involve any structure or organ (Table 2). Involvement of BM is common. In our patients, for whom 25% of BM involvement was considered the maximum below which lymphoblastic lymphoma was identified, over 50% presented BM involvement. Localization in the central nervous system (CNS) is relatively rare and is usually found in cases in which the illness is located in the head and neck. CNS involvement may consist of meningeal involvement, intracerebral disease, cranial nerve infiltration and paraspinal masses. CNS localization is more common in patients who show BM involvement; however, in rare cases, the two may not be connected.

Precise diagnosis is essential to therapy choice. Nodal biopsy and examination of pleural fluid when present is usually requested. BM examination is essential for staging and can be helpful in diagnosis. Mediastinal biopsy or biopsy via

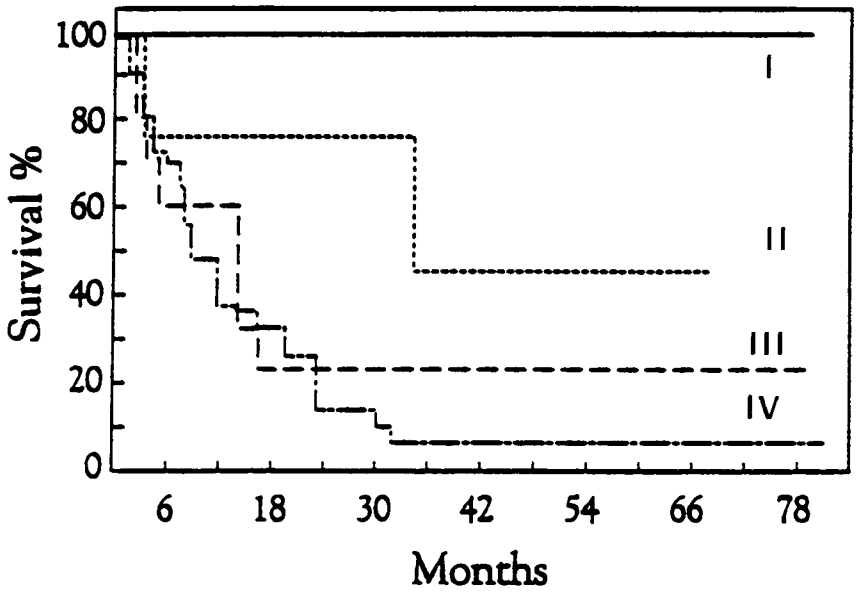

Figure 1. *Survival curves according to status of the disease ($p<0.0005$) (from Mazza et al.,[14]).*

parasternal access is necessary when the disease is limited to the mediastinum. All examinations, including radionuclide scans, are mandatory for correct staging to establish the hidden areas of illness as most adult patients affected by this lymphoma are presented at an advanced stage (Table 2).

CONVENTIONAL TREATMENT AND NEGATIVE PROGNOSTIC FACTORS

Early studies on children and adults treated with first and second generation chemotherapy regimens report extremely disappointing results with a probability of survival and disease-free survival (DFS) of around 15–30%.

The first encouraging results are to be seen with the introduction of sequential chemotherapy by Wollner et al.[12] This study used the LSA2-L2 schedule, developed from a chemotherapeutic sequence used in acute lymphoblastic leukemia. This schedule of intensive therapy including 10 drugs is made up of three phases: induction, consolidation and maintenance. It offers a 60% probability of long-term DFS for advanced stage patients. The reason for the success of this sequential and intensified therapy is not clear but it certainly introduces more drugs than standard protocols and maintains a sequence of administration that can be considered more suitable for a process that is similar in many ways to an acute lymphoblastic leukemia.

In 1983, given the good results obtained with the COMP protocol in the treatment of Burkitt's lymphoma, Anderson et al. compared it with the LSA2-L2 schedule in a randomized study of 234 young people and adolescents affected by non-Hodgkin's lymphoma.[13] Analysis of results showed how histotype of the illness and the therapeutic approach can change the outcome of patients with extensive disease. The LSA2-L2 protocol was seen to be significantly more active than the COMP in advanced stage lymphoblastic lymphoma, with a DFS of 76% compared with the 26% seen with COMP regimen (p=0.008).

A subsequent study by Coleman et al. in 1986 showed a 100% response (CR=95%; partial response=5%) in a group of 44 patients treated with two different intensive regimens, one schedule being developed from the other. With a median observation time of 26 months, results showed an overall DFS of 56% at 3 years.[5] Analysis of prognostic factors in advanced stage patients (IV according to Ann Arbor) showed which factors were likely to indicate poor DFS: LDH > 300 IU/Lt., BM or central nervous system involvement at diagnosis. Patients were divided into "low" and "high" risk groups according to whether one of these factors was present. Probability of DFS at 5 years was 94% and 19%, respectively.

Also in 1986, a study by Slater et al. reported 51 patients treated with 5 successive, individual sequential-type acute lymphoblastic leukemia protocols over a period of 15 years.[4] Patients with more than 25% BM involvement or with peripheral blasts were classified as "leukemic" lymphoblastic lymphoma. The percentage of complete remission for the whole group was 78% with an actuarial survival curve at 5 years of 45%. Survival of the 51 patients was similar to that of a group of 111 patients with acute lymphoblastic leukemia treated with similar protocols. In addition, no difference was seen between the survival of normal and "leukemic" patients. Prognostic factors associated with poor survival were: over 30 years of age, peripheral leukocytosis with white blood cells $>50\times10^9$/Lt., no CR or CR obtained more than 4 weeks after the start of therapy.

In the same year, a study by Mazza et al. of 64 patients compared a LSA2-L2 type protocol with a CHOP-type protocol.[14] Entry into remission phase showed the advantage of sequential therapy (50% compared with 38%). Factors likely to compromise survival were identified as: advanced stage, B symptoms, the presence of large tumor mass and BM involvement. The actuarial survival curve at 3 years for advanced stage patients repeatedly forecast a probability of around 20% for stage III and 10% for stage IV (Figure 1).

Today, the LSA2-L2 type sequential regimens are widely used in the treatment of lymphoblastic lymphoma. Our group has adopted a modified schedule of this type, obtaining an overall response of 79% (CR=68%; PR=11%) in the group of 45 patients reported in Table 2.

An analysis of the above shows how once the most suitable treatment has been established, the relationship between prognostic factors and outcome of

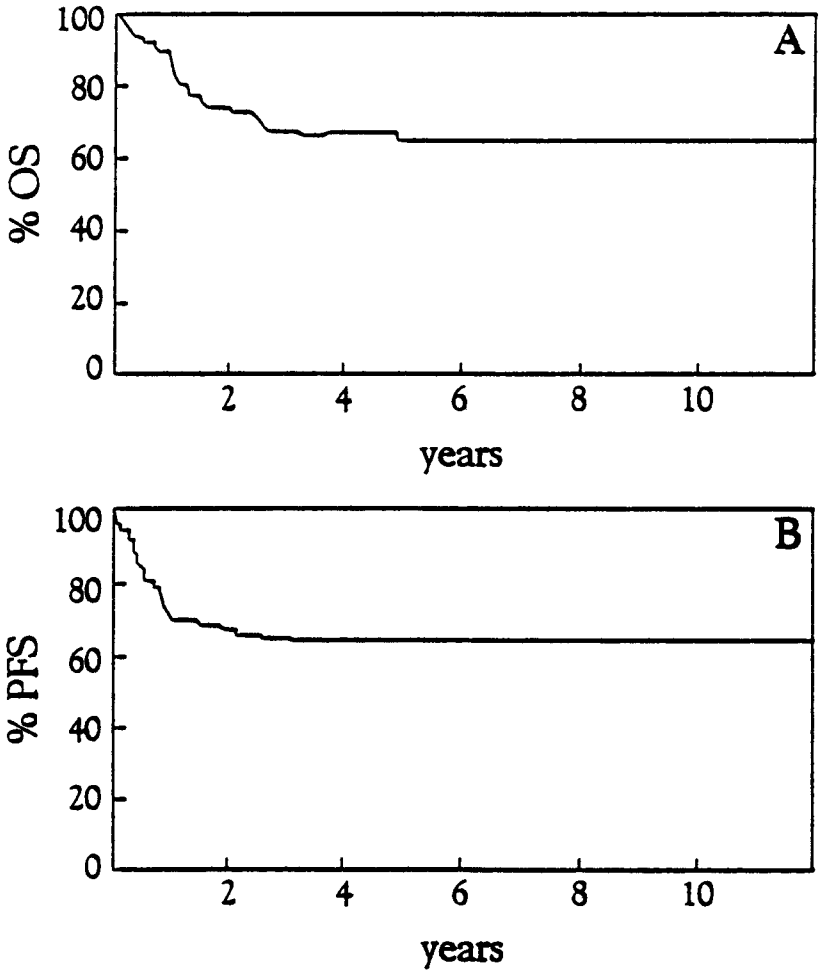

Figure 2. *Actuarial overall survival (A) and progression-free survival (B) for 105 patients undergoing autoBMT in first CR (from Sweetenham et al.[20]).*

lymphoblastic lymphoma in adults remains unpredictable. One important fact that emerges is the contrast between the strong possibility of obtaining CR and the high chance of relapse in this particular type of lymphoma. This is very evident when we analyze the data of Coleman et al. referring to advanced stage patients.[5] The same may be said of the data of Mazza et al.[14] The most important point to be noticed is that in this very aggressive disease, survival and DFS are almost identical. However, the correlation between prognostic factors on the one hand, and survival and DFS on the other, is by no means clear. This could be linked to patient groups included in the individual studies which are too small for reliable statistical

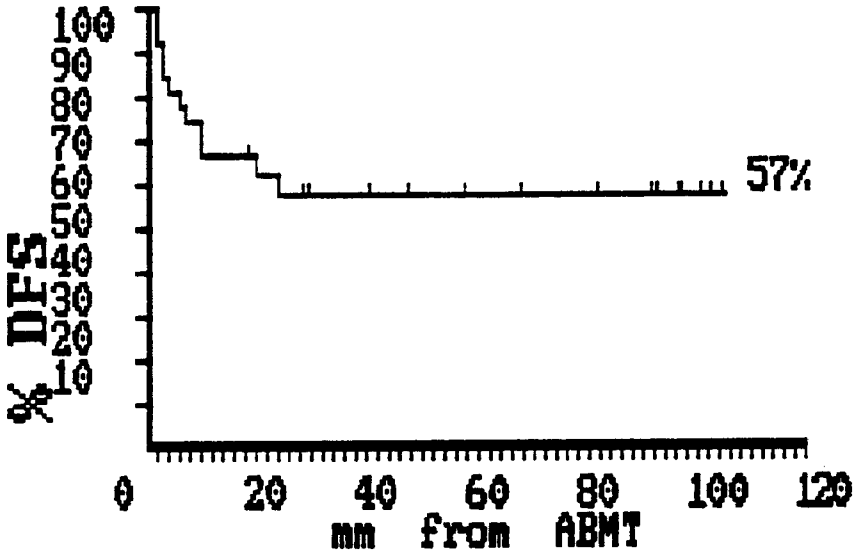

Figure 3. *Probability of DFS for 26 successive lymphoblastic lymphoma patients in first CR following an autograft (experience of the NHLCSG).*

analysis. The advanced stage of the disease certainly plays an important role in the case history of these patients. Bone marrow involvement may also be important.

In 1988, Vidzala et al. treated 76 patients under the age of 21 with a schedule of 6 drugs (A-COP) or a modified LSA-L2 schedule in a randomized comparative study.[15] Final results showed survival and DFS for the two groups to be identical. The significance of these data is represented by the fact that probability of DFS at 3 years was less than 20% in stage IV patients.

It is worth remembering the study of Morel et al. in 1992, which included 80 patients over the age of 15.[16] Of these, 66 (82%) obtained CR. The actuarial curves at three years show a probability of survival and DFS of 51% and 46%, respectively. Negative prognostic factors indicating poor possibility of complete remission were: age over 40 years, LDH more than two times normal level, and involvement of two or more extranodal sites at diagnosis. In addition, poor survival is associated with B symptoms or hemoglobin levels less than 100 g/L. Bone marrow involvement does not seem to have a negative influence on outcome. Age and LDH levels are the most important negative prognostic factors in this study.

The role of negative prognostic factors remains, therefore, controversial. These factors, though varying from one study to another, do, however, underline the fact that consistent tumor mass reduces the probability of survival and DFS.

Consideration of transplantation techniques developed over the last ten years as a valid therapeutic approach for this category of patients is therefore justified.

AUTOLOGOUS BONE MARROW TRANSPLANTATION (autoBMT)

While conventional therapy has improved the prognosis of lymphoblastic lymphoma, survival and DFS in some groups of advanced stage adult patients have been disappointing. Allogeneic or autologous transplantation may offer a valid alternative therapy for these patients. However, the rarity of this illness has meant that few specific studies have been carried out. Once CR has been obtained in lymphoblastic lymphoma the possibilities of relapse are extremely high, so most studies have looked at the role of autoBMT in first CR to increase the possibility of DFS.

In 1989, Milpied et al. reported a series of 25 patients with a median age of 23 years (range 16–43) who were treated in first CR with high doses of cyclophosphamide or melphalan and total body radiation (TBI) after a variety of sequential inductions.[17] Around 50% of patients received allogeneic and 50% autologous transplantation. The actuarial forecast at 4 years showed a probability of DFS of 69% with no difference between allogeneic and autologous transplantation.

In 1991, Santini et al. reported a group of 36 patients treated with a modified LSA2-L2 schedule.[6] Eighteen of 24 patients in CR were then treated with high-dosage cytoxan and TBI. Probability of DFS at 5 years for these patients was 74%.

In 1992, Baro et al. reported a DFS of 77% at two years in 14 patients transplanted in first CR.[18] In the same year, Verdonk et al. reported a group of nine patients in CR treated with cytoxan plus TBI.[19] Actuarial curves show a probability of DFS of 67% with a median observation time of 53 months.

Finally, in 1994, a study conducted by Sweetenham for the European Group for Bone Marrow Transplantation (EBMTG) analyzed the results of autoBMT in 214 patients affected by lymphoblastic lymphoma.[20] This study showed how patient status at transplantation is an important factor in outcome. Possibility of survival for the 105 patients treated in first CR is 63.5%. This decreases to 31% for patients transplanted in second CR and to 15% for patients with a resistant disease. In fact, progression-free survival of patients treated in first CR is 62.8%, almost identical to the probability of survival (Figure 2).

As part of these studies, the statistical relationships between prognostic factors and DFS were analyzed. However, there seem to be no factors that can indicate the outcome of transplantation in patients treated in first CR. In other words, transplantation seems able to reduce the importance of the negative factors at diagnosis which could indicate possible relapse. In our experience, the 26 patients who underwent transplantation by the end of 1992 presented a median of two

negative prognostic factors at diagnosis, which indicated a poor outcome, as well as advanced stage (Table 3). In spite of this, the statistical forecast at a little under 10 years shows a DFS probability of 57% (Figure 3).

CONCLUSIONS

In conclusion, it would seem that autologous transplantation represents an efficient weapon in the treatment of lymphoblastic lymphoma, and there is some reason to believe that it is more efficient than conventional therapy in preventing relapse in advanced stage patients in CR after conventional chemotherapy. However, many questions still remain. If on the one hand some studies on conventional treatment have given negative results, on the other there are studies that have shown overall results not so very different in terms of DFS as those reported using autoBMT. The role of negative prognostic factors in indicating possible relapse of patients treated with only conventional therapy remains very uncertain. AutoBMT offers a greater homogeneity of results. However, since entering CR is in itself a positive prognostic factor, a randomized study is needed to compare the usefulness of high dosage therapy plus autoBMT with a conventional consolidation therapy. This study is at present underway as part of the work of the EBMTG and the United Kingdom Group for the Study of Lymphomas (UKLG).

REFERENCES

1. National Cancer Institute sponsored study of classification of non-Hodgkin's lymphomas: Summary and description of a working formulation for clinical use. *Cancer* 49:2112–2135, 1982.
2. Rosen PJ, Feinstein DI, Pattingale PK et al.: Convoluted lymphocytic lymphoma in adults: A clinicopathological entity. *Ann Inter Med* 89:319–324, 1978.
3. Nathwani BN, Kim H, Rappaport H: Malignant lymphoma lymphoblastic. *Cancer* 38:964–983, 1976.
4. Slater DE, Mertelsmann R, Koziner B et al.: Lymphoblastic lymphoma in adults. *J Clin Oncol* 4:57–67, 1986.
5. Coleman CN, Picozzi VJ, Cox RS et al.: Treatment of lymphoblastic lymphoma in adults. *J Clin Oncol* 4:1628–1637, 1986.
6. Santini G, Coser P, Chisesi T et al.: Autologous bone marrow transplantation for advanced stage adult lymphoblastic lymphoma in first complete remission. Report of the Non-Hodgkin's Lymphoma Cooperative Study Group (NHLCSG). *Ann Oncol* 2(Suppl. 2):181–185, 1991.
7. Cossman J, Chaused TM, Fisher RI et al.: Diversity of immunological phenotype of lymphoblastic lymphoma. *Cancer Res* 43:4486–4490, 1983.
8. Nadler LM, Reinherz EL, Weinstein HJ et al.: Heterogeneity of T cell lymphoblastic malignancies. *Blood* 55:806–810, 1980.
9. Sandlund J, Magrath I: Lymphoblastic lymphoma. In: Magrath IT (Ed) The non-Hodgkin's

Lymphomas. Williams & Wilkins, 240–255, 1990.
10. Lennert K: Malignant lymphomas of high-grade maligancy. In: Lennert K (Ed) Malignant lymphomas. Heidelberg: Springer-Verlag, 346–469, 1978.
11. Nathwani BN, Diamond LW, Winberg CD et al.: Lymphoblastic lymphoma: A clinicopathologic study of 95 patients. *Cancer* 48:2347–2357, 1981.
12. Wollner N, Burchenal JH, Lieberman PH et al.: Non-Hodgkin's lymphoma in children: A comparative study of two modalities of therapy. *Cancer* 37:123–134, 1976.
13. Anderson JR, Wilson JF, Jenkin RDT et al.: Childhood non-Hodgkin's lymphoma: The results of a randomized therapeutic trial comparing a 4-drug regimen (COMP) with a 10-drug regimen (LSA2-L2). *Brit J Haematol* 308:559–565, 1983.
14. Mazza P, Bertini M, Macchi S et al.: Lymphoblastic lymphoma in adolescents and adults. Clinical, pathological and prognostic evaluation. *Eur J Cancer Clin Oncol* 22:1503–1510, 1986.
15. Hvizdala EV, Berard C, Callihan T et al.: Lymphoblastic lymphoma in children. A randomized trial comparing LSA2-L2 with the A-COP+ therapeutic regimen: A Pediatric Oncology Group Study. *J Clin Oncol* 6:26–33, 1988.
16. Morel P, Lepage E, Brice P et al.: Prognosis and treatment of lymphoblastic lymphoma in adults: A report on 80 patients. *J Clin Oncol* 10:1078–1085, 1992.
17. Milpied N, Ifrah N, Kuentz M et al.: Bone marrow transplantation for adult poor prognosis lymphoblastic lymphoma in first complete remission. *Br J Haematol* 73:82–87, 1989.
18. Baro J, Richard C, Sierra J et al.: Autologous bone marrow transplantation in 22 adult patients with lymphoblastic lymphoma responsive to conventional dose chemotherapy. *Bone Marrow Transpl*ant 10:33–38, 1992.
19. Verdonck LF, Dekker AW, de Gast GC et al.: Autologous bone marrow transplantation for adult poor-risk lymphoblastic lymphoma in first remission. *J Clin Oncol* 10:644–646, 1992.
20. Sweetenham JW, Liberti G, Pearce R et al.: High-dose therapy and autologous bone marrow transplantation for adult patients with lymphoblastic lymphoma: Results of the Group for Bone Marrow Transplantation. *J Clin Oncol* 12:1358–1365, 1994.

TRIALS FOR DIFFUSE, INTERMEDIATE AND HIGH GRADE NON-HODGKIN'S LYMPHOMA OF THE ITALIAN NHL COOPERATIVE STUDY GROUP (NHLCSG)

A. Porcellini and G. Santini for the NHLCSG

It has been proposed that the prognosis of patients with intermediate- and high-grade non-Hodgkin's lymphoma (NHL) would be improved by using second and third-generation chemotherapeutic agents.[1–3] In 1987, the Italian Non-Hodgkin's Cooperative Study Group (NHLCSG) started a randomized study to compare a second-generation regimen, PROMACE-MOPP, with a third-generation one, VACOP-B. Between January, 1987, and August, 1991, 221 patients with diffuse intermediate- to high-grade NHL (Working Formulation groups F, G, H and K) were randomized to receive one of the two regimens. No difference was found in complete remission (CR), disease-free survival (DFS) and OS.[4] However, when all patients were stratified for risk factors at diagnosis, according to the International Index (age, stage, PS, LDH, 2 or more extranodal involvements), it was noted that the prognosis worsened according to number of risk factors present.[4]

On the other hand, patients who achieve a partial response to first-line treatment and those who are refractory or with relapsed disease are rarely cured with conventional salvage chemotherapy.[5] Noncontrolled studies using ablative therapy with autologous bone marrow transplantation reportedly offer 3-year freedom-from-progression rates between 35 and 50% for partially responding or chemotherapy-sensitive relapsed patients.[6–9] However, the definitive role played by autologous bone marrow transplantation in the treatment of NHL is still unclear and results of randomized studies are necessary.

Based on our previous experience, in 1992 we initiated a study aimed at clarifying the role of autologous bone marrow transplantation. Patients aged 15 to 59 years, with diffuse intermediate-to high-grade NHL (Working Formulation groups F, G, H and K) stage II, III or IV were divided into three study groups: 205 patients were enrolled into studies A, B and C.

The endpoint of Study A was to clarify whether third generation chemotherapy offers satisfactory survival in patients with low tumor burden. In this study, 41 patients with stage II or III NHL with no adjunctive negative prognostic factors at diagnosis, were treated with VACOP-B.

Study B was designed with the aim of understanding whether autologous bone marrow transplantation is able to improve survival in poor prognosis patients. One hundred twenty-four NHL patients stage II and III with one or more prognostic

factors at diagnosis and stage IV, were randomized to receive either VACOP-B (and possible second line therapy in the event that CR was not attained) or VACOP-B + ABMT.

In Study C, 40 patients with the same histotype as that in the other groups but with bone marrow involved at diagnosis were included. The patients received VACOP-B until maximum response (minimum 4 weeks; in fact, patients received an average of 8 weeks). About 20–30 days after discontinuation of chemotherapy, cyclophosphamide at a dose of 7 g/m^2 followed by G-CSF (5 mcg/Kg) was administered. About 45 days after high dose therapy patients underwent transplant with PBSC rescue.

An interim analysis of our data indicates no advantage of the ABMT arm versus the conventional chemotherapy arm; however, our follow-up is too short to draw definite conclusions.

REFERENCES

1. Longo DL, De Vita VT, and Duffey PL: Superiority of Pro-MACE-Cytabom over ProMACE-MOPP in the treatment of advanced diffuse aggressive lymphoma: Results of a prospective randomized trial. *J Clinical Oncology* 925–38, 1991.
2. Shipp MA, Harrington DP, and Klatt MM: Identification of major prognostic subgroups of patients with large-cell lymphoma treated with m-BACOD or M-BACOD. *Ann Intern Med* 10:4757–67, 1986.
3. Klimo P and Connors JM: Updated clinical experience with MACOP-B. Semin in Hematol 2426–34, 1987.
4. Sertoli MR, Santini G, Chisesi T, and Porcellini A: MACOP-B versus ProMACE-MOPP in the treatment of advanced diffuse non-Hodgkin's lymphoma: Results of a prospective randomized trial by the Non-Hodgkin's Cooperative Study Group. *J Clin Oncol* 12(7):1366–74, 1994.
5. Cabanillas F, Jagannath S, Philip T, Magrath I, (eds): Management of recurrent or refractory disease. In: The Non-Hodgkin's Lymphoma. London: Edward Arnold, 1990, pp 359–72.
6. Armitage JO: Bone marrow transplantation in the treatment of patients with lymphoma. *Blood* 73:1749–58, 1989.
7. Freedman AS, Takvorian T, and Anderson KC: Autologous bone marrow transplantation in B-cell non-Hodgkin's lymphoma: Very low treatment-related mortality in 100 patients in sensitive relapse. *J Clin Onc* 8(784):791, 1990.
8. Bosly A, Coiffier B, and Gisselbrecht C: Bone marrow transplantation prolongs survival after relapse in aggressive lymphoma patients treated with the LNH-84 regimen. *J Clin Onc* 10:1615–23, 1992.
9. Philip T, Armitage JO, Spitzer G et al.: High-dose therapy and autologous bone marrow transplantation after failure of conventional chemotherapy in adults with intermediate-grade or high-grade non-Hodgkin's lymphoma. *N Engl J Med* 316:1493–1498, 1987.

AUTOLOGOUS BONE MARROW TRANSPLANTATION VERSUS SEQUENTIAL CHEMOTHERAPY IN AGGRESSIVE NON-HODGKIN'S LYMPHOMA IN FIRST COMPLETE REMISSION

Corinne Haioun

High-dose therapy with autologous stem-cell support has been proposed as a potentially curative treatment for patients with recurrent lymphoma sensitive to standard chemotherapy.[1–7] Recently, the Parma study validated the superiority of high-dose chemotherapy and autologous bone marrow transplantation (autoBMT) over standard salvage therapy for the treatment of sensitive-relapsed patients.[8]

The clinical benefits of such an approach may be increased by use in the initial phase of treatment in poor risk patients unlikely to be cured by conventional strategies.[9–12]

In a first interim analysis of lymphoma patients with unfavorable characteristics and responding to induction treatment, we found no advantage of consolidative high-dose chemotherapy followed by autologous bone marrow transplantation over sequential chemotherapy.[13] This series, however, exhibited marked prognostic heterogeneity on the basis of the international index.[14] Whether such an approach might benefit patient subgroups identified according to this index was explored.

Results of our updated analysis (541 complete remission randomized patients), which focuses on high-intermediate and high risk patients, are now in press.[15]

PATIENTS AND METHODS

Between October 1987 and February 1993, 1043 patients were enrolled in the LNH87-2 study. Eligibility criteria were age under 55 years; newly diagnosed intermediate- or high-grade non-Hodgkin's lymphoma according to the W Formulation; and at least one of the following adverse factors: ECOG status of 2 to 4, two or more extranodal sites, tumor burden of at least 10 cm, bone marrow or central nervous system involvement and Burkitt or lymphoblastic subtypes (the latter two without bone marrow or central nervous system involvement). Patients were retrospectively staged according to the age-adjusted international prognostic index.[14]

The induction treatment randomly compared four courses of two anthracycline-containing regimens given every two weeks: the LNH84 induction regimen[16] with doxorubicin 75 mg/m^2 (ACVB arm) or mitoxantrone 12 mg/m^2 (NCVB arm). The NCVB arm was stopped in December 1991, because of a significant advantage of

ACVB regimen in terms of complete response rate.[13]

Patients who achieved a complete remission were subsequently randomized between a sequential chemotherapeutic consolidation as defined in the LNH84 regimen (high-dose methotrexate, ifosfamide plus etoposide, L-asparaginase and cytarabine) or an intensive consolidation with the CBV regimen followed by autoBMT.[13]

RESULTS

Nine hundred sixteen patients were eligible for analysis with 526 in the ACVB group and 390 in the NCVB group. Their main characteristics were median age 40 years, large-cell subtypes (72%), ECOG status >2 (27%), stage III, IV (66%), number of extranodal sites >2 (24%), tumoral mass >10 cm (53%), and bone marrow involvement (24%), LDH > 1N (61%). Using the age-adjusted international index, the following distribution was observed: no risk factor, 13%; one factor, 36%; two factors, 36%; and three factors, 15%.

Sixty-eight percent of the 916 eligible patients had complete remission after induction treatment: 69% in the ACVB and 62% in the NCVB induction groups (p=0.01).

Five hundred forty-one complete-remission patients were randomized to receive the consolidation treatment of sequential chemotherapy (273) or autologous transplant (268). Main initial characteristics of the randomized patients did not differ between the two consolidative arms.

All analyses were performed on an intention-to-treat basis. The results are reported as of September 31, 1995, providing a median follow-up of 54 months.

The 5-year disease-free survival (DFS) was 52% in the sequential chemotherapy arm and 59% in the CBV arm. In the higher risk population (patients with 2–3 factors, n=236), CBV was superior to sequential chemotherapy with 5-year DFS of 59% and 39%, respectively (p=0.01) (Figure 1). The 5-year survival also differed; 65% as compared with 52% (p=0.06).

CONCLUSION

Evaluating the role of high-dose therapy for initial treatment of higher risk patients is a current challenge in lymphoma management. When this study began in 1987, the benefit of such an approach was suggested by pilot studies mentioned above.[9–12] Recently, Pettengel et al.[17] reported a retrospective comparison between standard (VAPEC-B) and intensive treatment (three cycles of ifosfamide/cytarabine, high-dose busulfan/cyclophosphamide followed by autologous blood progenitor-cell support). They concluded that high-dose therapy in high-risk (2–3 factors) patients was promising and needed to be confirmed by randomized trials.

Our prospective LNH87-2 study contributes to the resolution of this issue by

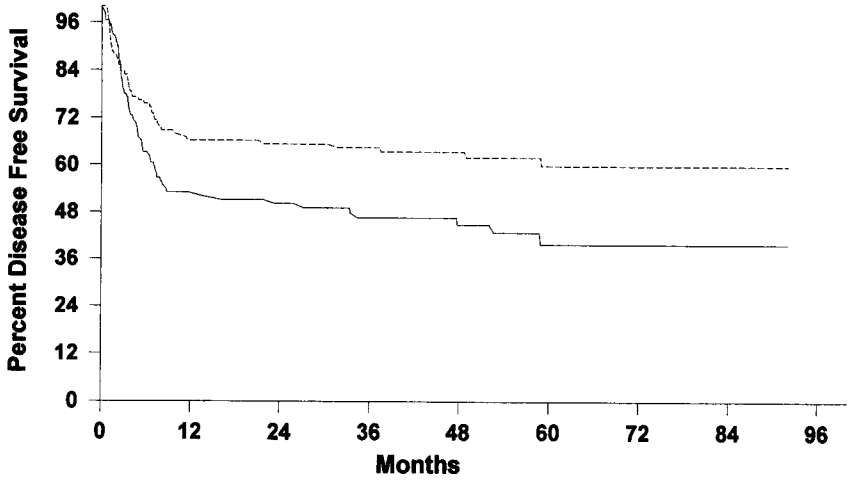

Figure 1. *Estimated DFS according to randomized consolidation procedure for high-intermediate and high risk patients. (——) Sequential chemotherapy (patients at risk, n = 111; 5-year estimate, 39%); (- - - -) AutoBMT (patients at risk, n=125; 5-year estimate, 59%). p=0.01.*

demonstrating a benefit of consolidative high-dose CBV followed by autoBMT in a large series of high-risk patients.

REFERENCES

1. Philip T, Armitage JO, Spitzer G et al.: High-dose therapy and autologous bone marrow transplantation after failure of conventional chemotherapy in adults with intermediate-grade or high-grade non-Hodgkin's lymphoma. *N Engl J Med* 316:1493–98, 1987.
2. Armitage JO: Bone marrow transplantation in the treatment of patients with lymphoma. *Blood* 73:1749–58, 1989.
3. Cabanillas F, Jagannath S, Philip T: Management of recurrent or refractory disease. In: Magrath I (ed): The Non-Hodgkin's Lymphoma. London: Edward Arnold, 1990, pp 359–372.
4. Freedman AS, Takvorian T, Anderson KC et al.: Autologous bone marrow transplantation in B-cell non-Hodgkin's lymphoma: Very low treatment-related mortality in 100 patients in sensitive relapse. *J Clin Oncol* 8:784–91, 1990.
5. Bosly A, Coiffier B, Gisselbrecht CH: Bone marrow transplantation prolongs survival after relapse in aggressive lymphoma patients treated with the LNH-84 regimen. *J Clin Oncol* 10:1615–23, 1992.
6. Peterson BA: The role of transplantation in non-Hodgkin's lymphoma. *J Clin Oncol* 12:2524–26, 1994 (editorial).
7. Vose JM: Treatment for non-Hodgkin's lymphoma in relapse. *N Engl J Med* 333:1565–66, 1995 (editorial).

8. Philip T, Guglielmi C, Hagenbeek A et al.: Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma. *N Engl J Med* 333:1540–45, 1995.
9. Gulati SC, Shank B, Black P et al.: Autologous bone marrow transplantation for patients with poor-prognosis lymphoma. *J Clin Oncol* 6:1303–13, 1988.
10. Nademanee A, Schmidt GM, O'Donnel MR et al.: High-dose chemotherapy followed by autologous bone marrow transplantation as consolidation therapy during first complete remission in adult patients with poor-risk aggressive lymphoma: A pilot study. *Blood* 80:1130–34, 1992.
11. Freedman AS, Takvorian T, Neuberg D, et al.: Autologous bone marrow transplantation in poor-prognosis intermediate-grade and high-grade B-cell non-Hodgkin's lymphoma in first remission: A pilot study. *J Clin Oncol* 11:931–36, 1993.
12. Sierra J, Conde E, Montserrat E: Autologous bone marrow transplantation for non-Hodgkin's lymphoma in first remission. *Blood* 81:1968, 1993 (letter).
13. Haioun C, Lepage E, Gisselbrecht C et al.: Comparison of autologous bone marrow transplantation with sequential chemotherapy for intermediate-grade and high grade non-Hodgkin's lymphoma in first complete remission: A study of 464 patients. *J Clin Oncol* 12:2543–51, 1994.
14. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. A predictive model for aggressive non-Hodgkin's lymphoma. *N Engl J Med* 329:987–94, 1993.
15. Haioun C, Lepage E, Gisselbrecht C et al.: Benefit of autologous bone marrow transplantation over sequential chemotherapy in poor-risk aggressive non-Hodgkin's lymphoma. Updated results of the prospective study LNH87-2. *J Clin Oncol* (in press).
16. Coiffier B, Gisselbrecht C, Herbrecht R et al.: LNH-84 regimen: A multicenter study of intensive chemotherapy in 737 patients with aggressive malignant lymphoma. *J Clin Oncol* 7:1018–26, 1989.
17. Pettengel R, Radford JA, Morgenstern GR et al.: Survival benefit from high-dose therapy with autologous blood progenitor cell transplantation in poor-prognosis non-Hodgkin's lymphoma. *J Clin Oncol* 14:586–92, 1996.

HODGKIN'S DISEASE: EVOLUTION OF THERAPIES AND STRATEGIES IMPACTING ON OUTCOME

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ABSTRACT

Improvements in chemotherapy and supportive care have enhanced the feasibility of dose-intensive treatment followed by hematopoietic stem cell transplant in relapsed Hodgkin's disease. This treatment modality is generally reserved for patients with disease responsive to standard dose therapy. Even though patients with primary refractory disease or sensitive relapse have a lower survival probability, a proportion of them do achieve long-term survival after autotransplant. In our experience, refractory patients who could be given two successive autotransplants had a disease-free survival (DFS) equivalent to that of patients with sensitive disease. The elimination of carmustine from the conditioning regimens coincided with a drop in peritransplant mortality. Prognostic factors other than previous response, which should also be taken into account before deciding for dose intensive treatment, are different from those considered for standard dose salvage therapy and include previous radiation as an adverse characteristic.

Greater knowledge of histocompatibility, improved methods of hematopoietic support, identification and use of new cytokines and combinations of cytokines, development of new chemotherapeutic regimens for dose intensive therapy, and better understanding of the actual disease processes have all led to improvements in the management of patients with hematologic neoplasia in general and Hodgkin's disease in particular.[1]

In diseases such as Hodgkin's disease in which the graft-versus-tumor effect was not considered to be important, autologous marrow transplantation was employed. For patients with marrow involvement by Hodgkin's disease or those with fibrotic inaspirable marrows, peripheral blood progenitor cells (PBPC) were demonstrably useful as a means of providing sustained hematologic reconstitution.[2] In addition, PBPC led to faster hematologic recovery after potentially myeloablative therapy.[3] While initially steady state, non-mobilized PBPC were used, it soon became apparent that larger numbers of PBPC could be mobilized with cytokines

alone or in combination with myelosuppressive chemotherapy and subsequently collected for clinical use.[4] Cytokines like GM-CSF and G-CSF were identified as being useful in accelerating neutrophil recovery after marrow transplantation and standard dose chemotherapy.[5] Newer cytokines and combinations of cytokines were shown to accelerate lineages other than neutrophils alone or to potentially effect multiple lineages.[6] The availability of better supportive care techniques has allowed for dose intensive therapy to be administered with relative facility. Newer conditioning regimens have had considerable impact on the outcome of such patients.[1,7–10] While total body irradiation was and still is used frequently as part of the conditioning pretransplant regimen in patients with hematologic malignancies, several trials demonstrated that total body irradiation was probably not as useful as part of the conditioning regimen for dose intensive chemotherapy in patients with recurrent Hodgkin's disease.[11]

High-dose chemotherapy with autologous marrow rescue was shown to be useful in achieving long-term disease-free survival (DFS) in patients with recurrent or relapsed Hodgkin's disease.[1] Regimens using cyclophosphamide, etoposide and carmustine have been used often.[8,12–14] Regimens employing higher doses of these three agents are associated with higher response rates albeit with greater peritransplant morbidity and mortality.[8]

By and large, dose intensive therapy has been noted to be effective in a subset of patients: patients with disease that is responsive to salvage therapy appear to benefit most from such treatment.[15,16] However, dose intensive chemotherapy administered as a single cycle still results in 40–70% of such patients relapsing. Moreover, patients with disease refractory to standard dose therapy, when administered initially or as salvage, are often not considered to be candidates for such therapy.

New treatment strategies for patients identified as being less likely to benefit from dose intensive regimens, i.e., those with disease resistant to initial or subsequent therapy, have been evaluated at New York Medical College and other institutions. The results of three consecutive trials in 148 patients confirm that response to previous standard-dose treatment is a predictor of survival. Figure 1 shows the survival probability of our first cohort of 67 patients, with 5 years median follow-up. But our results also indicate that a proportion of patients with resistant disease can achieve long-term survival. The DFS of refractory patients is equivalent to those whose disease is sensitive to salvage therapy if dose intensive therapy can be administered twice. It should be noted that in addition to response to previous treatment, the following initial characteristics were unfavorable: previous irradiation, initial diagnosis of Stage II disease, age over 30 and male sex.[10]

Regimens without carmustine result in long-term survival equivalent to dose intensive regimens with carmustine. In our experience, peritransplant mortality, as

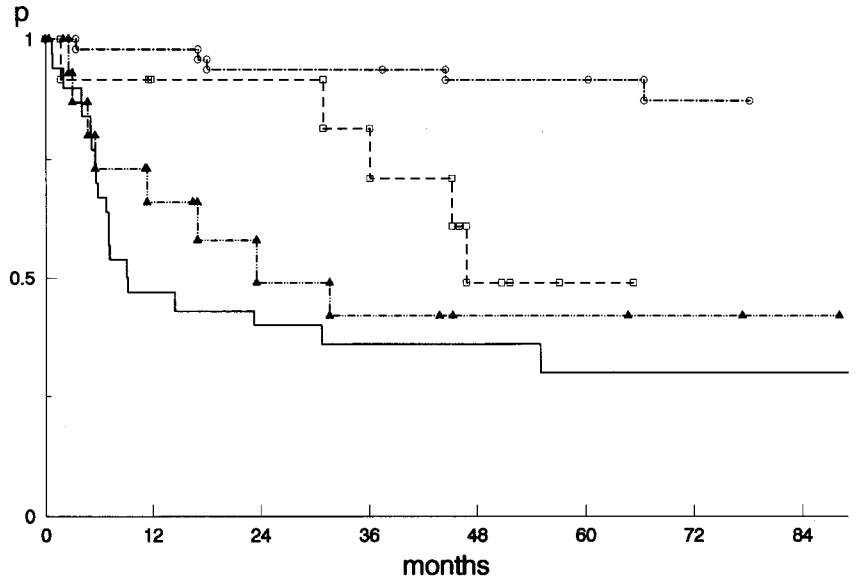

Figure 1. *Actuarial survival of 67 patients treated with the BEC (carmustine-etoposide-cyclophosphamide) + TMJ (thiotepa-mitoxantrone-carboplatin) protocol. Circles: patients with no evidence of disease at transplant; squares: patients in partial remission; triangles: primary refractory disease; solid line: patients in refractory relapse.*

well as deaths due to pulmonary or hepatic complications, were reduced when the dose of carmustine was lowered in the carmustine-etoposide-cyclophosphamide combination. Mortality was further reduced with the introduction of a regimen without carmustine. Cases of lethal or potentially lethal pulmonary toxicity dropped from 6/24 (25%) with 600 mg/m^2 of carmustine to 9/67 (13%) with 450 mg/m^2 of the drug, to 3/51 (6%) with the TMJ (thiotepa-mitoxantrone-carboplatin) combination; the respective figures for hepatic toxicity were 3/24, 8/67 and 3/51.[3]

Early treatment with dose intensive therapy may well be useful in patients at particularly high risk of relapse after standard dose therapy. The group at Genoa, in collaboration with various investigators, has been investigating the potential utility of dose intensive therapy and ABMT for such patients.[13] Entry criteria to this multinational trial include presence of extranodal disease, B symptoms, abnormal liver function tests and other unfavorable prognostic variables. These factors have been identified as being important for prognosis after standard dose therapy.

Factors that influence overall and DFS after dose intensive therapy are different from those that influence survival after standard dose therapy, also in our experience.[17–19] While investigating the utility of dose intensive therapy for patients at risk of relapse, one should consider the impact and influence of

prognostic factors that have been identified as being significant for patients undergoing dose intensive therapy.

Now that cures are not infrequent in recurrent or refractory Hodgkin's disease, the "price of cure" in terms of long-term side effects including late myelodysplasia and acute leukemia need to be considered.[20,21] Extramedullary toxicity also needs to be borne in mind.[22,23] The use of conditioning regimens without carmustine was associated with significantly reduced acute and subacute mortality. This series of paradigm shifts has made dose-intensive therapy safer to administer and allowed for less toxic regimens to be used successfully. With the knowledge thus gained, one is able to better identify factors that impact on the prognosis of patients with Hodgkin's disease after failure of initial therapy and thus provide improved care for these patients.

REFERENCES

1. Ahmed T: Autologous marrow transplantation for Hodgkin's disease: Current techniques and prospects. *Cancer Invest* 8:99–106, 1990.
2. Kessinger A, Armitage JO, Landmark JD et al.: Autologous peripheral hematopoietic stem cell transplantation restores hematopoietic function following marrow ablative therapy. *Blood* 71:723–727, 1988.
3. Ahmed T. Unpublished data.
4. Peters WP, Ross M, Vredenburgh JJ et al.: High-dose chemotherapy and autologous bone marrow support as consolidation after standard-dose adjuvant therapy for high-risk primary breast cancer. *J Clin Oncol* 11:1132–1143, 1993.
5. Gulati SC, Bennett CL: Granulocyte-macrophage colony-stimulating factor (GM-CSF) as adjunct therapy in relapsed Hodgkin's disease. *Ann Intern Med* 116:177–182, 1992.
6. Wolff SN, Ahmed T, van Besien K et al.: Simultaneous and sequential rhIL-3 (SDZ ILE 964) and rhG-CSF (Neupogen) post autologous bone marrow transplantation for lymphoma: A phase II study. *Blood* 82:287A, 1993 [abstr].
7. Jagannath S, Dicke KA, Armitage JO et al.: High-dose cyclophosphamide, carmustine, and etoposide and autologous bone marrow transplantation for relapsed Hodgkin's disease. *Ann Intern Med* 104:163–168, 1986.
8. Reece DE, Barnett MJ, Connors JM et al.: Intensive chemotherapy with cyclophosphamide, carmustine, and etoposide followed by autologous bone marrow transplantation for relapsed Hodgkin's disease. *J Clin Oncol* 9:1871–1879, 1991.
9. Chopra R, McMillan AK, Linch DC et al.: The place of high-dose BEAM therapy and autologous bone marrow transplantation in poor-risk Hodgkin's disease. A single-center eight-year study of 155 patients. *Blood* 81:1137–1145, 1993.
10. Ahmed T, Lake D, Feldman E et al.: Factors influencing prognosis after dose intensive therapy for recurrent or refractory Hodgkin's disease: Results of sequential trials. A case for treating patients with resistant disease. *Ann New York Acad Sci* 770:305–314, 1995.
11. Yahalom J, Gulati SC, Toia M et al.: Accelerated hyperfractionated total-lymphoid irradiation, high-dose chemotherapy, and autologous bone marrow transplantation for refrac-

tory and relapsing patients with Hodgkin's disease. *J Clin Oncol* 11:1062–1070, 1993.
12. Ahmed T, Ciavarella D, Feldman E et al.: High-dose, potentially myeloablative chemotherapy and autologous bone marrow transplantation for patients with advanced Hodgkin's disease. *Leukemia* 3:19–22, 1989.
13. Carella AM, Carlier P, Congiu A et al.: Autologous bone marrow transplantation as adjuvant treatment for high-risk Hodgkin's disease in first complete remission after MOPP/ABVD protocol. *Bone Marrow Transplant* 8:99–103, 1991.
14. Zulian GB, Selby P, Milan S et al.: High dose melphalan, BCNU and etoposide with autologous bone marrow transplantation for Hodgkin's disease. *Br J Cancer* 59:631–635, 1989.
15. Gulati SC, Yahalom J, Whitmarsh K et al.: Factors affecting the outcome of autologous bone marrow transplantation. *Ann Oncol* 2(suppl 1):51–55, 1991.
16. Jagannath S, Armitage JO, Dicke KA et al.: Prognostic factors for response and survival after high-dose cyclophosphamide, carmustine, and etoposide with autologous bone marrow transplantation for relapsed Hodgkin's disease. *J Clin Oncol* 7:179–185, 1989.
17. Proctor SJ, Taylor P, Donnan P et al.: A numerical prognostic index for clinical use in identification of poor-risk patients with Hodgkin's disease at diagnosis. *Eur J Cancer* 27:624–629, 1991.
18. Straus DJ, Gaynor JJ, Myers J et al.: Prognostic factors among 185 adults with newly diagnosed advanced Hodgkin's disease treated with alternating potentially non-cross-resistant chemotherapy and intermediate-dose radiation therapy. *J Clin Oncol* 8:1173–1186, 1990.
19. O'Brien ME, Milan S, Cunningham D et al.: High-dose chemotherapy and autologous bone marrow transplant in relapsed Hodgkin's disease-a pragmatic prognostic index. *Br J Cancer* 73:1272–1277, 1996.
20. Witherspoon RP, Deeg HJ, Storb R: Secondary malignancies after marrow transplantation for leukemia or aplastic anemia. *Transplant Sci* 4:33–41, 1994.
21. Coleman DN, Williams CJ, Flint A et al.: Hematologic neoplasia in patients treated for Hodgkin's disease. *N Engl J Med* 297:1249–1252, 1977.
22. Kalaycioglu M, Kavuru M, Tuason L et al.: Empiric prednisone therapy for pulmonary toxic reaction after high-dose chemotherapy containing carmustine (BCNU). *Chest* 107:482–487, 1995.
23. Wheeler C, Antin JH, Churchill WH et al.: Cyclophosphamide, carmustine, and etoposide with autologous bone marrow transplantation in refractory Hodgkin's disease and non-Hodgkin's lymphoma: A dose-finding study. *J Clin Oncol* 8:648–656, 1990.

DOSE-INTENSIVE THERAPY AND AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION: WHAT IS THE OPTIMAL TIMING?

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ABSTRACT

Although dose-intensive therapy (DIT) plus autologous hematopoietic stem cell transplantation (autoHSCT) is now an accepted part of the therapeutic armamentarium for Hodgkin's disease, a number of questions regarding proper utilization remain. Chief among these is the following question: What, in terms of disease status, is the optimal timing for employing this procedure?

We propose that for the majority of patients, the first sign of failure of conventional chemotherapy is currently optimal, with failure defined as the inability of induction chemotherapy to produce initial complete remission (CR) ("primary induction failure"), or as the first sign of relapse after chemotherapy. While our approach is supported by Phase II data, it is unlikely that the question of optimal timing will be definitively answered, or even addressed, by a randomized clinical trial. Registry data will become increasingly available but are also unlikely to be conclusive. Despite such limitations, we believe available evidence supports the time of initial failure of chemotherapy as a standard indication for the use of DIT and autoHSCT for Hodgkin's disease patients.

INTRODUCTION

By the mid-1990s, the use of DIT plus autologous hematopoietic stem cell transplantation (autoHSCT) was an accepted part of the therapeutic repertoire for patients with Hodgkin's disease. As indicated in unreviewed data from the ABMTR, roughly 403 patients were transplanted in 1994, and it is likely that a substantial number of unreported patients have been transplanted as well. These data report considerable variation in disease status at transplantation, indicating–to a degree–some uncertainty regarding the proper timing at which to intervene with autoHSCT (of course, the disease status at transplant does not always represent a specifically selected timing for the use of autoHSCT; primary induction failure is perhaps the clearest example). Finally, it should be appreciated that, for a variety of reasons, a critical evaluation of autoHSCT using randomized clinical trials to better define optimal timing vis-à-vis

disease status will not likely be performed. Table 1 lists the potential timing options for use of DIT and autoHSCT.

In order to even roughly analyze the results from the Phase II studies discussed herein, it is important to be aware of existing historical data regarding conventional therapy and the limitations thereof. In this regard, the Hodgkin's disease work of Longo et al.[1] is of particular note. In that analysis, a relatively large number of patients with primary induction failure and relapse after chemotherapy were treated with conventional salvage regimens at the National Cancer Institute. A key finding was that no primary induction failure patient experienced long-term survival with further conventional salvage regimens. In the 107 patients who relapsed after achieving a complete remission, long-term survival was 17% at 20 years. Those with an initial chemotherapy-induced remission lasting more than one year had a survival of 24%, compared with 11% in those with a shorter initial remission duration. Most failures were due to subsequent relapse or secondary malignancies—acute myelogenous leukemia occurred in 5 of 37 patients entering a second remission of Hodgkin's disease and was invariably fatal. These data highlight the problems of disease recurrence and nonrelapse mortality seen with conventional salvage therapy in this disease.

Our previous efforts to use DIT and autoHSCT in Hodgkin's disease patients concentrated on several issues, in particular the prompt identification and subsequent transplantation of patients failing chemotherapy. (These studies were performed in conjunction with Drs. Joe Connors and Paul Klimo at the British Columbia Cancer Agency, whose assistance with the study, as well as ability to organize the care of all British Columbia lymphoma patients in a centralized facility, was of critical importance to this effort.)

PATIENTS AND METHODS

Between 1985 and early 1994, a total of 107 patients with Hodgkin's disease, not in remission, were autografted in British Columbia. Also in that interval, an additional 18 patients were evaluated but not transplanted, primarily due to either the desire of the medical oncologists to treat certain patients with conventional therapy or to patient refusal; these patients have been previously reported.[2,3] Table 2 lists the clinical features of the patients autografted.

Details of protocol therapy will not be completely outlined, as they have been reported previously;[2,3] however, two cycles of conventional-dose salvage chemotherapy (usually with nitrogen mustard, vinblastine, procarbazine and prednisone [MVPP]) was utilized in most patients. It should be emphasized that MVPP was *not* used to determine chemosensitivity and thus determine eligibility for DIT and autoHSCT, but was given as a means of holding the disease under control while a transplant admission was arranged—all patients who received MVPP subsequently underwent autoHSCT. Other key points of our therapeutic

Table 1. Timing options for DIT and AHSCT in Hodgkin's disease

primary failure of induction chemotherapy
first relapse after chemotherapy
untested
sensitive after salvage chemotherapy/radiotherapy
resistant after salvage chemotherapy/radiotherapy
second remission after salvage chemotherapy/radiotherapy
second relapse after salvage chemotherapy/radiotherapy
≥ second remission/relapse

program included modifications of the original "CBV" (cyclophosphamide, BCNU [=carmustine], VP16-213 [=etoposide]) regimen, mainly with dose augmentation and schedule adjustment, but more recently with the addition of cisplatin in conventional doses. Additionally, local radiotherapy was used aggressively—usually before, but occasionally after, the transplant.

In these studies, unmanipulated marrow was used as the source of stem cells in 91 patients, mobilized blood in 13 and both sources in 3; in none of these instances was the stem cell product purified. Hematologic growth factors were utilized in the more recently transplanted patients, as per other protocols.

RESULTS BY DISEASE STATUS AT TRANSPLANT

Primary induction failure

Thirty patients who failed induction chemotherapy were autografted after DIT with CBV ± cisplatin. Twenty-eight had received both MOPP and ABVD-like regimens; such 7–8 drug regimens were the initial therapy in 22 patients. The majority had achieved a partial remission and then progressed, off therapy, during a period of observation; eight had progressed during induction chemotherapy.

Five early nonrelapse deaths were seen after autoHSCT, while recurrent disease was noted in 11. The actuarial progression-free survival (PFS) is 42% (95% confidence interval [CI] 21–61%) with a median follow-up of 4 years (range 0.2–8.2 years). No late nonrelapse events have occurred. Late relapses, known to occur particularly in nodular sclerosing disease, have occurred only rarely. These results are superior to those that would be anticipated with nontransplant therapies; however, we again emphasize that any such comparisons involve Phase II studies and are therefore not definitive.

First relapse

The 58 patients autografted in a first relapse after primary chemotherapy have now been followed a median of 5 years (range 2.75–10.3 years). These patients were

Table 2. Characteristics of 107 Hodgkin's disease patients receiving CBV ± P and AHSCT

Characteristic	*Value*
median age (range)	29 (13–53)
sex, male:female	62:45
features at diagnosis	
stage I–II	47
stage III–IV	60
"B" symptoms	58
extranodal disease	41
prior therapy	
median # drugs (range)	7 (4–12)
median # regimens (range)	1 (1–6)
radiotherapy	42
prior marrow involvement	12
disease status at protocol entry	
1st untested relapse	58
>2nd untested relapse	13
induction failure	30
resistant relapse	6
Karnofsky performance status <80%	44
"B" symptoms at protocol entry	35

entered onto the study "untested" by chemotherapy, although the majority received MVPP and/or local radiotherapy, as previously described, before DIT and autoHSCT.

The actuarial PFS has been stable at 63% since our first report of these patients in 1994.[2] Negative prognostic factors for PFS have been identified in multivariate analysis: these include first remission duration of less than 1 year, extranodal disease at relapse, and "B" symptoms at relapse. In the most recent analysis of this patient group, the PFS was 90% (95% CI, 47–99%) in patients with no adverse factors, compared with 68% (95% CI, 42–84%) in those with one factor, 51% (95% CI, 26–72%) in those with two factors and 0% in a few patients with all three factors. All patients had satisfactory "engraftment," and only 2 early nonrelapse deaths were observed. Disease progression was the primary problem, occurring in 14 patients, for a probability of 26% (95% CI, 16–40%). Four late nonrelapse deaths have occurred, including two from secondary solid tumors, one due to an automobile accident and one from interstitial lung disease. However, no cases of secondary acute myelogenous leukemia have been seen. (We speculate that the low risk of secondary hematologic malignancies is due to the "lesser" degree of

cumulative potentially leukemogenic salvage chemotherapy these patients received prior to stem cell harvest compared with those in other transplant studies.)

Similar results have been noted by others. However, relatively few studies have reported these patients alone. The series of Bierman et al.[4] is an exception and generally confirms these results.

Other timing options

Although a major emphasis of the above effort was the "early" use of DIT and autoHSCT—as soon as disease progression was evident after combination chemotherapy—a minority of patients were autografted in a more advanced disease status. For 13 patients in second or greater untested relapse, long-term PFS was approximately 25%. Nonrelapse mortality was high in these patients, presumably due at least in part to more extensive prior exposure to cytotoxic therapy and/or to the "augmented" doses of the CBV regimen used. Our only instance of secondary acute myelogenous leukemia was seen in one of these patients, who was harvested and transplanted in second relapse after heavy alkylator exposure.

Only six truly "chemorefractory" patients were treated with this regimen; none of those survived. Both nonrelapse mortality and disease progression were seen in this group. However, other workers have reported a salvage rate of approximately 30% in such patients.[5] Therefore, although better results are seen when DIT and autoHSCT is used earlier in the disease course, patients with more extensively treated disease should not routinely be excluded from this procedure.

SUMMARY AND CONCLUSIONS

Our data suggest that DIT and autoHSCT is indicated in most patients who fail induction chemotherapy, a setting in which conventional therapies are largely ineffective. In first-relapse patients, DIT and autoHSCT can produce prolonged PFS in approximately 60% of patients, with a low incidence of early nonrelapse mortality. Certain disease features present at relapse allow identification of prognostic groups for PFS. The incidence of recurrent disease despite transplantation remains the primary challenge for improving this strategy in the future.

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REFERENCES

1. Longo DL, Duffey DL, Young RC: Conventional-dose salvage combination chemotherapy in patients relapsing with Hodgkin's disease after combination chemotherapy: The low probability for cure. *J Clin Oncol* 10:210–218, 1992.
2. Reece DE, Connors JM, Spinelli JJ et al.: Intensive therapy with cyclophosphamide, carmustine, etoposide ± cisplatin, and autologous bone marrow transplantation for Hodgkin's disease in first relapse after combination chemotherapy. *Blood* 83:1193–1199, 1994.
3. Reece DE, Barnett MJ, Shepherd JD: High-dose cyclophosphamide, carmustine (BCNU), and etoposide (VP16-213) with or without cisplatin (CBV ± P) and autologous transplantation for patients with Hodgkin's disease who fail to enter a complete remission after combination chemotherapy. *Blood* 86:451–456, 1995.
4. Bierman PJ, Anderson JR, Freeman MB et al.: High-dose chemotherapy followed by autologous hematopoietic rescue for Hodgkin's disease patients following first relapse after chemotherapy. *Ann Oncol* 7(2):151–156, 1996.
5. Chopra R, McMillan AK, Linch DC et al.: The place of high-dose BEAM therapy and autologous bone marrow transplantation in poor-risk Hodgkin's disease. A single-center eight-year study of 155 patients. *Blood* 81:1137–1145, 1993.

CHAPTER 5
Myeloma

CD-34 POSITIVE PERIPHERAL BLOOD STEM CELL TRANSPLANTATION IN MULTIPLE MYELOMA

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INTRODUCTION

Multiple myeloma (MM) is characterized by the accumulation of malignant plasma cells in the bone marrow that secrete monoclonal immunoglobulin (Ig) proteins.[1] Despite the sensitivity of these cells to alkylator-based chemotherapy, median patient survival remains at 30 months with the use of standard doses of multi-agent chemotherapy.[2] In an attempt to improve upon these results, dose-intensive chemotherapy with hematopoietic support has been used to treat these patients. Despite some encouraging results in single arm trials,[3,4] many patients relapsed early or died from treatment-related toxicity. Although early results with allogeneic bone marrow transplantation (alloBMT) suggested an improvement in long-term survival,[5] a more recent update was less encouraging with high rates of both relapse and toxicity related largely to graft-versus-host disease (GVHD).[6] Because of the advanced age of most patients with MM, few patients with MM are eligible for this type of hematopoietic support; most cannot receive this therapy. As a result, autologous bone marrow transplantation (autoBMT) has been performed in a greater number of patients and has produced 3-year progression-free survival (PFS) 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.[4,7,8]

A major potential problem of autoBMT is autograft contamination. Since MM is a BM-based disease, the autograft product is most likely heavily contaminated; therefore, malignant cells are re-infused into the patient after the myeloablative chemotherapy has been completed. In order to reduce this tumor cell contamination, peripheral blood stem cell (PBSC) transplants have been performed. This should reduce the burden of tumor cells in the product and appears to speed hematological recovery, potentially reducing treatment related morbidity and mortality. However, it is clear from studies in our own laboratory and others that the peripheral blood remains contaminated by tumor cells in MM patients, although to a lesser degree.[9,10] In fact, recent studies have documented contamination of the mobilized leukapheresis product prior to reinfusion,[11–13] and have shown that the mobilization regimen may increase tumor contamination in the stem cell product. Thus, attempts to reduce this contamination have been made using a wide

variety of purging techniques, although many prior attempts have resulted in delayed engraftment without demonstrated reduction in tumor burden.[7,8]

Recent studies show that cells positively selected for CD34, an antigen expressed on early hematopoietic cells, can support recovery following myeloablative chemotherapy.[9] Since MM 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.[10] 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 MM tumor cell load.

Thus, we have attempted to ask several questions regarding the autologous product in patients undergoing high-dose therapy for MM:

1. Is there more tumor contamination in BM harvests than in leukapheresis products?
2. Does mobilization with chemotherapy and growth factors increase tumor contamination in the peripheral blood?
3. How effectively does positive selection of PBSCs with the CD34 antigen reduce tumor in PBSC products, and does this lead to a delay in engraftment?

MATERIALS AND METHODS

We initially conducted a multi-institutional pilot trial using CD34-selected PBSCs to support hematopoietic recovery following high-dose chemotherapy in the treatment of advanced MM.[18] Fifty-one patients with MM aged 34–69 years (median 52 years) were entered with a median time from diagnosis to treatment of 9 months (range 4–47 months). All patients had received prior therapy with alkylator-based (31 pts) or VAD chemotherapy (16 pts), or dexamethasone alone (4 pts), and had to show either a response or lack of progression to this treatment. Three patients had nonsecretory disease but had evidence of treatment response by bone marrow analysis. After enrollment, bone marrow was harvested as a potential source of hematopoietic support if the CD34-selected PBSC product delayed engraftment. Progenitor cells were harvested 14 days after intermediate dose cyclophosphamide (2.5 g/m^2 IV), prednisone (2 mg/kg PO QD $\times$ 4) and G-CSF (10 mg/kg SQ QD $\times$ 14). Leukaphereses were performed for 2–5 days and $CD34^+$ progenitor cells were enriched 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 anti-CD34 antibody.

In order to evaluate the tumor cell contamination in the stem cell products, we used a quantitative PCR-based assay using patient-specific immunoglobulin (Ig) gene primers. Since our previous studies have shown that the Ig heavy chain produced by the MM cells shows a high degree of somatic mutation without clonal diversity,[19] 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 from cDNA using reverse transcriptase-PCR. At least three clones were sequenced and needed to be identical to show that the sequence was from the malignant MM cell. The MM V_H sequence was then compared with all known germline sequences using a DNAsis program (Hitachi, San Bruno, CA) in order to identify both the V_H gene used and the sites of somatic mutation. Oligonucleotide primers complementary to the V_H complementarity determining region (CDR) sequences with a high degree of somatic mutation were then designed for use in the patient-specific tumor detection assay. A pair of primers (CDR1 and CDR3) for each patient should be extremely 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 used a quantitative PCR technique based on Poisson distribution analysis of positive PCR results.[20] First, DNA was extracted from the sample 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 patient's leukapheresis or bone marrow DNA was substituted for sample DNA. Six-tenths of a microgram of DNA was used in each PCR, 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.

RESULTS

The adsorbed (CD34-selected) fraction represented 0.2–2.0% (median, 0.6%) of the starting leukapheresis cell population and the CD34 purity of the collection ranged from 27 to 91% (median, 77%). A median of 7.0×10^6 adsorbed cells/kg (range: $1.6–35.3\times10^6$ cells/kg) were reinfused 1 day after preparative conditioning with busulfan (0.875 mg/kg q6h × 16 doses) and cyclophosphamide (60 mg/kg q.d. × 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 (3×10^6 IU/m^2) TIW and decadron (20 mg/m^2 q.d. $\times$ 4 every four weeks) beginning 100 days after transplantation and continued for one year or until there was evidence of progressive disease.

Since a BM back-up was required in this trial, it was possible to determine the relative amount of tumor in the BM harvest compared with the unselected PBSC product.[11] In 13 patients analyzed, the percentage of tumor cells contaminating the BM harvest (median 0.74% with a range of 0.12–6.98%) was higher (median 238-fold; range 18 to >34,900-fold) than in the PBSC specimens (median 0.0024% with a range of <0.0002–0.352%). However, because of the increased total number of cells required for PBSC support compared to BM transplant in these patients, the increase in tumor cell contamination in the total PBSC autograft compared to the BM harvest was less marked (BM:PBSC total tumor contamination ratios ranging from 0.9 to >4,500; median, 14).

Next, we determined the amount of tumor contaminating the PBSC product before and after CD34 selection. Prior to CD34 selection, tumor cells were detectable in the leukapheresis product in 11 of 18 patients analyzed. In the 11 patients with detectable tumor, the percentage and total number of tumor cells/kg in the unselected product varied from 0.0006–0.23% and 3660–2,139,000 tumor cells/kg, respectively. By contrast, tumor contamination of the CD34-selected autologous product was detectable in only three patients (0.0005%, 0.0030% and 0.0190%). In the 11 patients with detectable tumor in the unselected product, CD34 selection reduced the total number of tumor cells in the final product by >2.7 to >4.5 logs and by 2.8, 3.0 and 3.2 logs in the three patients with detectable tumor in the CD34-selected product.

Despite this marked reduction in tumor contamination, engraftment was rapid with a median time to both ANC > 500/mm^3 (range, 11–16 days) and untransfused platelets >20,000/mm^3 of 12 days (range 9 to >52 days), 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 2×10^6 CD34 cells/kg, below which platelet engraftment was prolonged and incomplete.

Seven patients could not be evaluated because of early death or nonsecretory disease, while the remaining patients all showed either a complete (6 pts), partial (35 patients) or minimal response (3 patients). Nineteen patients have relapsed after a median follow-up of 27 months (range 2–36 months). Twenty two patients (43%) remain alive in continued remission without evidence of disease progression. The 3-year actuarial progression-free and overall survival for these patients is 34 and 55%, respectively.

Because of these results, a large multi-center phase III trial has been undertaken involving 133 patients, and has recently finished accrual. This trial compares both tumor contamination in the stem cell product and engraftment in patients undergoing either CD34-selected or unselected PBSC transplantation for advanced multiple myeloma. Precise measurement of tumor burden using the PCR assay in stem cell products, peripheral blood or bone marrow before and following transplant is being done in our laboratory. Although overall results from this trial are not yet available,

we were able to analyze the amount of tumor in the peripheral blood before mobilization chemotherapy and during leukapheresis in 16 patients to date. Peripheral blood was collected on the day of mobilization chemotherapy (day 0) and on the first day of leukapheresis. The mononuclear cells were separated by density gradient centrifugation. All stem cell collections were completed on each patient within 24 hours of the latter blood sample except in three cases (within 48–72 hours). There was a 0.2 log decrease (range 0.8 logs increase–2.39 logs decrease) in the percentage contamination in the peripheral blood mononuclear cell (PBMC) fraction on the first day of leukapheresis compared with day 0 (before the mobilization regimen). No tumor was detected in the granulocyte layer. Thus, the total number of tumor cells/mm^3 was determined by multiplying the percentage of tumor in the PBMCs by the total number of PBMCs/mm^3. Overall, this showed the amount of tumor in the peripheral blood before mobilization and during leukapheresis to be the same (median 0.0 logs; range 1.0 logs increase–2.0 logs decrease) comparing the first day of leukapheresis with day 0. Whether individual increases or decreases in tumor burden during mobilization predict overall outcome remains to be determined.

DISCUSSION

These results demonstrate several important clinical findings for multiple myeloma patients undergoing high-dose therapy with autologous support. Overall, unselected PBSC products contain approximately 14-fold fewer tumor cells than bone marrow harvests. Second, a mobilization regimen employing chemotherapy and G-CSF does not significantly increase tumor in the peripheral blood. Third, CD34-selection of PBSCs greatly reduces tumor in the stem cell product, and remains an effective form of purified hematopoietic support for patients with multiple myeloma undergoing myeloablative chemotherapy. Other attempts to reduce tumor contamination in stem cell products from MM patients have used flow-sorting with multiple surface markers to identify a tumor-free stem cell population,[22] but clinical results with use of these purified products are not yet available. Whether a reduction in tumor contamination in the autologous stem cell product using any of these techniques improves overall survival remains to be answered.

REFERENCES

1. Vescio RA, Lichtenstein A, Berenson J: Myeloma, macroglobulinemia and heavy chain disease. In: Haskell CM (ed): Cancer Treatment (ed 4). Philadelphia: WB Saunders, 1995, pp 1094–1116.
2. Hansen OP, Galton DAG: Classification and prognostic variables in myelomatosis. *Scand J Haematol* 35:10, 1985.
3. Harousseau JL, Milpied N, Laporte JP et al.: Double-intensive therapy in high-risk multiple myeloma. *Blood* 79:2827–2833, 1991.
4. Jagannath S, Barlogie B, Dicke K et al.: Autologous bone marrow transplantation in mul-

tiple myeloma: Identification of prognostic factors. *Blood* 76:1860–1866, 1990.
5. Gahrton G, Tura S, Ljungman P et al.: for the European Group for Bone Marrow Transplantation: Allogeneic bone marrow transplantation in multiple myeloma. *N Eng J Med* 325:1267, 1991.
6. Gahrton G, Tura S, Ljungman P et al.: Prognostic factors in allogeneic bone marrow transplantation for multiple myeloma. *J Clin Onco* 13:1312–1322, 1995.
7. Anderson KC, Barut BA, Ritz J et al.: Monoclonal antibody-purged autologous bone marrow transplantation therapy for multiple myeloma. *Blood* 77:712, 1991.
8. Gobbi M, Cavo M, Tazzari PL et al.: Autologous bone marrow transplantation with immunotoxin-purged marrow for advanced multiple myeloma. *Eur J Haematol* 43 (Suppl 1):176, 1989.
9. Berenson RJ, Andrews RG, Bensinger WI et al.: Engraftment after infusion of $CD34^+$ marrow cells in breast cancer or neuroblastoma. *Blood* 77:1717, 1991.
10. Vescio RA, Hong CH, Cao J et al.: The hematopoietic stem cell antigen, CD34, is not expressed on the malignant cells in multiple myeloma. *Blood* 84:3283–3290, 1994.
11. Vescio RA, Han EJ, Schiller GJ et al.: Quantitative comparison of multiple myeloma tumor contamination to bone marrow harvest and leukapheresis autografts. *Bone Marrow Transplant* 15:16–22, 1996.
12. Lemoli M, Fortuna A, Motta MR, et al.: Concomitant mobilization of plasma cells and hematopoietic progenitors into peripheral blood of multiple myeloma patients: positive selection and transplantation of enriched $CD34^+$ cells to remove circulating tumor cells. *Blood* 87:1625–1634, 1996.
13. Gazitt Y, Tian E, Barlogie B, et al.: Differential mobilization of myeloma cells and normal hematopoietic stem cells in multiple myeloma after treatment with cyclophosphamide and granulocyte-macrophage colony-stimulating factor. *Blood* 87:805–811, 1996.
14. Anderson KC, Barut BA, Ritz J, et al.: Monoclonal antibody-purged autologous bone marrow transplantation therapy for multiple myeloma. *Blood* 77:712, 1991.
15. Gobbi M, Cavo M, Tazzari PL, et al.: Autologous bone marrow transplantation with immunotoxin-purged marrow for advanced multiple myeloma. *Eur J Haematol* 43 (Suppl 1):176, 1989.
16. Berenson RJ, Andrews RG, Bensinger WI, et al.: Engraftment after infusion of $CD34^+$ marrow cells in breast cancer or neuroblastoma. *Blood* 77:1717, 1991.
17. Vescio RA, Hong CH, Cao J, et al.: The hematopoietic stem cell antigen, CD34, is not expressed on the malignant cells in multiple myeloma. *Blood* 84:3283–3290, 1994.
18. Schiller G, Vescio R, Freytes C, et al.: Transplantation of $CD34^+$ peripheral blood progenitor cells after high-dose chemotherapy for patients with advanced multiple myeloma. *Blood* 86:390–397, 1995.
19. Vescio RA, Hong CH, Cao J, et al.: Somatic mutation of the heavy chain variable region in multiple myeloma is unaccompanied by either intraclonal diversity or clonal progression. *Blood* 82 (Suppl 1):259a, 1993.
20. Molesh DA, Hall JM: Quantitative analysis of $CD34^+$ stem cells using RT-PCR on whole cells. *Cold Spring Laboratory Press* 3:278, 1994.
21. Vescio RA, Han EJ, Schiller GJ, et al.: Quantitative comparison of multiple myeloma tumor contamination to bone marrow harvest and leukapheresis autografts. *Bone Marrow Transplantation* 15:16–22, 1996.
22. Gazitt Y, Reading CL, Hoffman R, et al.: Purified $CD34^+Lyn^-Thy^+$ stem cells do not contain clonal myeloma cells. *Blood* 86:381–389, 1995.

HIGHLY PURIFIED STEM CELL TRANSPLANTS FOR MULTIPLE MYELOMA

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Multiple myeloma is a debilitating hematologic malignancy that is characterized by painful bone destruction, recurrent infections and renal failure affecting patients predominantly over the age of 50 years.[1] Chemotherapy at standard doses produces good palliation with significant tumor cytoreduction in half of the patients, and extension of median life expectancy to three years; but seldom is the response complete (<5%) and it is probably never curative.[2,3] Several studies in the eighties showed that escalation of doses of alkylating agents results in higher complete responses.[4,5] More recently, our "Total Therapy" approach with double autotransplants as well as the randomized clinical trial comparing standard chemotherapy with high-dose therapy for newly diagnosed myeloma patients have shown convincingly that high-dose therapy induces higher complete remission (CR) rates and indeed prolongs remission duration and survival.[6,7] Feasibility of high-dose therapy for patients up to 70 years and for those with renal impairment has clearly made this therapy within reach of the majority of myeloma patients.[6,8]

Bone marrow (BM) involvement with plasma cells is a hallmark of this disease.[9] Given the fact that myeloma patients seldom achieve a CR with standard therapy, tumor cell contamination of marrow harvest is expected even in CR, if more sensitive tools to detect minimal residual disease are used. In our program, marrow contamination up to 30% plasma cells has been allowed at the time of the harvest, especially in relapsed or refractory patients. No adverse clinical outcome could be shown in either remission duration or overall survival that could be attributed to reinfusion of tumor cells,[7,10] indicating that the amount of tumor cells remaining in the body after high-dose chemotherapy is considerable and higher than the amount of tumor cells infused with the BM. It is impossible to evaluate the role of tumor cell contamination of the stem cell graft in disease relapse. However, gene marking studies of autologous BM by Brenner and others have clearly indicated that re-infused tumor cells do cause relapse.[11]

Among patients with less than a year of prior therapy, blood stem cells collected after high dose cyclophosphamide and GM-CSF provided significantly faster granulocyte and platelet recoveries to critical levels than BM autografts (Figure 1). As a result, the treatment-related mortality has declined from 10–15% range to ≤2%.[12] It was presumed initially that peripheral blood stem cells would perhaps

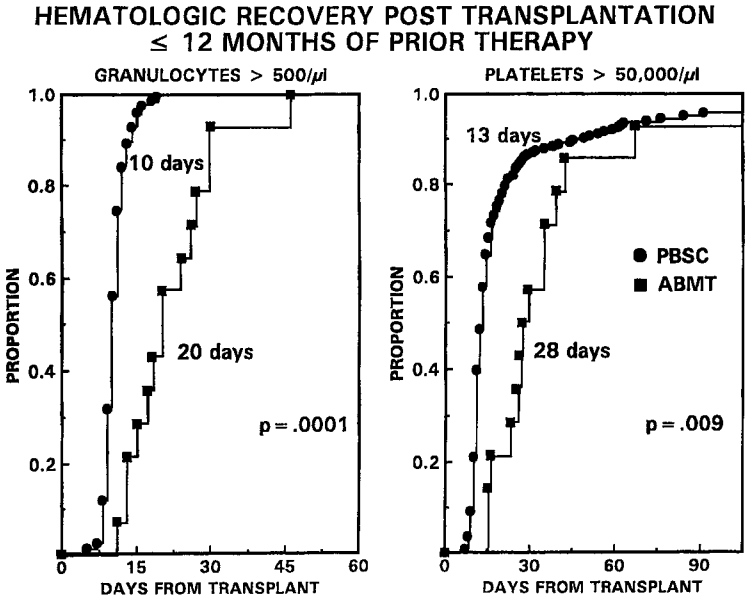

Figure 1. *Rapid granulocyte and platelet recovery after peripheral blood stem cell rescue compared with autologous marrow transplant in patients with less than one year of prior chemotherapy.*

have less tumor cell contamination. By applying PCR for CDRIII region of the immunoglobulin heavy chain, Billadeau showed that the peripheral blood of newly diagnosed myeloma patients contain 0.001 to 1.0% circulating tumor cells, significantly lower than in the marrow.[13] However, there are indications that these circulating tumor cells may actually harbor phenotypically more immature cells ($CD19^+$) that include drug-resistant clonotypic cells that may underlie relapse,[14] and did have a higher proliferation rate.[15]

While in steady-state the number of circulating clonotypic may be very low, several groups have shown that the process of stem cell mobilization with chemotherapy and/or growth factors also results in tumor cell mobilization.[15,16] Gazitt et al showed that there was differential mobilization of stem cells and tumor cells; $CD34^+$ cells were seen in higher frequency during the early phase of recovery from cytopenia when WBC had reached 500/μL while plasma cells were mobilized in greater numbers later when WBC had already reached (5000/μL.[15] However, Lemoli et al noted no differential mobilization of stem cells and tumor cells.[16] In some patients these mobilized tumor cells have a high labeling index. Thus, depletion of these tumor cells from the stem cell product prior to transplantation could potentially prolong the remission duration especially in good risk patients

who have attained a CR following the transplant.

$CD34^+/Thy1^+/Lineage\text{-}ve$ cells are a subset of $CD34^+$ cells that contain all the pluripotent hematopoietic stem cells. These cells are capable of multilineage differentiation with extensive self renewal capacity.[17] Following high-dose cyclophosphamide at 6 g/m^2 and GM-CSF $CD34^+/Thy1^+/Lin\text{-}ve$ cells are mobilized in the peripheral blood with the same kinetics as $CD34^+$ cells though they represent typically 10–50% of the CD34 population and may be present more transiently than the $CD34^+$ cells. In preclinical studies, stem cell purification ($CD34^+/Thy1^+/Lin\text{-}ve$) led to an overall enrichment by about 50-fold in all 10 patients studied with purity reaching up to 90%. Quantitative PCR amplification of patient-specific CDRIII DNA sequences showed depletion of clonal B cells by 2.7 to 7.3 logs, with the highest log reduction noted in the samples initially containing the most tumor cells.[18]

As a substantial number of patients do not achieve a CR after an autotransplant, it is unlikely that in vitro manipulation of stem cell product alone will result in long-term disease control in a substantial proportion of myeloma patients. Preliminary results to date indicate that there is not a substantial difference in the relapse pattern of the disease following tumor cell purging.[16,19,20] Immune recovery, especially T cell function recovery, is delayed following such highly selected stem cell transplants because all of the mature T cells are removed. This could potentially result in early relapse in those patients who did not achieve a CR following the selected stem cell transplant. Post-transplant immune modulation such as idiotype vaccine or dendritic cell vaccine to stimulate cell mediated cytotoxicity against tumor may be required for ultimate cure.[21,22]

REFERENCES

1. Kyle RA: Multiple Myeloma: Review of 869 cases. *Mayo Clin Proc* 50:29–40, 1975.
2. Alexanian R, Dimopoulos M: The treatment of multiple myeloma. *N Engl J Med* 330:484–489, 1994.
3. Barlogie B, Epstein J, Selvanayagam P et al.: Plasma cell Myeloma—New biological insights and advances in therapy. *Blood* 73:865–879, 1989.
4. McElwain T, Powles R: High-dose intravenous melphalan for plasma-cell leukemia and myeloma. *Lancet* 1:822–824, 1983.
5. Barlogie B, Hall R, Zander A et al.: High-dose melphalan with autologous bone marrow transplantation for multiple myeloma. *Blood* 67:1298–301, 1986.
6. Jagannath S, Tricot G, Vesole D et al.: Total therapy with tandem transplants for 231 newly diagnosed patients with multiple myeloma. *Blood* 88:685a, 1996.
7. Attal M, Harousseau JL, Stoppa AM et al.: A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Francais du Myelome. *N Engl J Med* 335:91–97, 1996.
8. Jagannath S, Barlogie B, Vesole D et al.: Autotransplants can be performed safely in mul-

tiple myeloma patients with renal insufficiency. *Blood* 86:809a, 1995.
9. Kyle RA: Diagnosis and management of multiple myeloma and related disorders. *Prog Hematol* 14:257–282, 1986.
10. Jagannath S, Barlogie B, Dicke K et al.: Autologous bone marrow transplantation in multiple myeloma: identification of prognostic factors. *Blood* 76:1860–1866, 1990.
11. Brenner MK, Rill DR, Moen RC et al.: Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. *Lancet* 341:85–86, 1993.
12. Jagannath S, Vesole DH, Glenn L et al.: Low-risk intensive therapy for multiple myeloma with combined autologous bone marrow and blood stem cell support. *Blood* 80:1666–1672, 1992.
13. Billadeau D, Quam L, Thomas W et al.: Detection and quantitation of malignant cells in the peripheral blood of multiple myeloma patients. *Blood* 80:1818–1824, 1992.
14. Bergsagel PL, Smith AM, Szczepek A et al.: In multiple myeloma, clonotypic B lymphocytes are detectable among $CD19^+$ peripheral blood cells expressing CD38, CD56, and monotypic Ig light chain [published erratum appears in *Blood* 1995 Jun 1; 85(11):3365]. *Blood* 85:436–447, 1995.
15. Gazitt Y, Tian E, Barlogie B et al.: Differential mobilization of myeloma cells and normal hematopoietic stem cells in multiple myeloma after treatment with cyclophosphamide and granulocyte-macrophage colony-stimulating factor. *Blood* 87:805–811, 1996.
16. Lemoli RM, Fortuna A, Motta MR et al.: Concomitant mobilization of plasma cells and hematopoietic progenitors into peripheral blood of multiple myeloma patients: Positive selection and transplantation of enriched $CD34^+$ cells to remove circulating tumor cells. *Blood* 87:1625–1634, 1996.
17. Murray L, Chen B, Galy A et al.: Enrichment of human hematopoietic stem cell activity in the $CD34^+Thy\text{-}1^+Lin^-$ subpopulation from mobilized peripheral blood. *Blood* 85:368–378, 1995.
18. Gazitt Y, Reading CC, Hoffman R et al.: Purified $CD34^+$ Lin^- Thy^+ stem cells do not contain clonal myeloma cells. *Blood* 86:381–389, 1995.
19. Anderson KC, Andersen J, Soiffer R et al.: Monoclonal antibody-purged bone marrow transplantation therapy for multiple myeloma. *Blood* 82:2568–2576, 1993.
20. Schiller G, Vescio R, Freytes C et al.: Transplantation of $CD34^+$ peripheral blood progenitor cells after high-dose chemotherapy for patients with advanced multiple myeloma. *Blood* 86:390–397, 1995.
21. Kwak LW, Taub DD, Duffey PL et al.: Transfer of myeloma idiotype-specific immunity from an actively immunised marrow donor. *Lancet* 345:1016–1020, 1995.
22. Hsu FJ, Benike C, Fagnoni F et al.: Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* 2:52–58, 1996.

GRAFT VERSUS MYELOMA EFFECT: RISKS AND BENEFITS

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INTRODUCTION

Little progress has been made during the last 30 years in extending the overall survival of patients with multiple myeloma using standard therapy. No cures have been observed and fewer than 5% of patients live longer than 10 years. Recent studies indicate an improved CR rate, event-free survival and overall survival in patients who receive high-dose chemo/radiotherapy with autologous stem cells.[1,2] Nevertheless, relapses after autologous stem cell transplants are frequent and it is still unclear whether any patient can be cured with this approach.[3] One of the problems with autologous stem cell transplants is the contamination of the grafts with tumor cells. It has recently been shown that CD34 selected or $CD34^+$ Lin^- Thy_1^+ stem cells do contain significantly fewer clonal myeloma cells.[4,5] However, the major reason for relapse is not contamination of the graft, but persistent disease in the patient. Although further intensification with tandem autotransplants seems to further increase the CR rate and event-free survival,[2] it is very likely that chemo/radiotherapy-resistant myeloma cells persist in a large proportion of patients post-transplantation. A different treatment approach is required to cure this disease.

The advantage of an allogeneic transplant is that not only is a tumor-free graft is provided, but also a graft-versus-myeloma (GVM) effect can be exploited. We and others have recently shown that a GVM effect exists.[6,7] However, allogeneic transplantation in multiple myeloma is associated with an exceedingly high transplant-related mortality. In a recently published study of the European Group for Blood and Marrow Transplantation, autologous stem cell transplantation was compared with allotransplantation.[8] In that study, the transplant-related mortality of the allogeneic group was 41% versus 13% for the autologous stem cell transplant group. This higher transplant-related mortality of allogeneic transplantation was not compensated for by a lower rate of relapse and disease progression. The risk of relapse in the autologous transplant group was 70% at 48 months compared with 50% in the alloBMT group. However, in patients alive at one year post-transplantation, a significantly better progression-free survival was seen for allotransplant patients when compared with autologous stem cell transplants. This suggests that allogeneic

transplantation provides a more durable response and that cures may be obtained at a higher frequency with this intervention.

Graft-versus-host disease (GVHD) is caused by the recognition of histocompatibility antigens by donor lymphocytes and is probably triggered by a "cytokine storm." Evidence for the existence of a graft-versus-leukemia and a graft-versus-lymphoma effect in humans has mainly been indirect. Patients with acute and/or chronic GVHD have a lower relapse rate than those with no or minimal GVHD.[9] Direct experimental evidence of a graft-versus-leukemia effect in humans was first reported by Kolb and subsequently confirmed by others.[10–12] The highest frequency (75%) of durable complete remissions with donor lymphocyte infusions after relapse post-allotransplants has been seen in chronic myelogenous leukemia (CML). This compares with 29% in AML and 0% in acute lymphocytic leukemia (ALL) according to the EBMT data.[13] However, infusion of donor lymphocytes has a substantial complication rate. GVHD grades 2–4 is seen in 41% of patients, myelosuppression in 36% and death in 12%, due to pancytopenia in 4%, GVHD in 6% and infections in 2%.[13]

Four cases of myeloma will be presented in which a GVM effect was seen with donor cell infusions only, while in one patient a GVM effect was seen only after high-dose chemotherapy with infusion of donor stem cells and lymphocytes.

CASE ONE

A 40-year-old female with IgG kappa stage IIA, presented with bone marrow (BM) plasmacytosis of 70% at diagnosis and more than 2% plasmablasts. M-protein was 3.3 g/dL. Cytogenetics were normal. She received one course of VMCP and two courses of dexamethasone pulsing. Her disease was progressive with a BM plasmacytosis of 75% and M-protein of 5.8 g/dL. She then received an autotransplant with melphalan 100 mg/m^2 and peripheral blood stem cell support. She had a transient decline in M-protein from 6.2 to 2.5 g/dL with a subsequent rapid increase to 4.3 g/dL. Because of her young age, she then received a matched unrelated donor transplant after conditioning with hyperfractionated total body irradiation of 1375 cGy, thiotepa 10 mg/kg and cyclophosphamide 120 mg/kg combined with antithymocyte globulin at 120 mg/kg. T cells were removed from the marrow graft by a two-step soybean lectin agglutination and sheep red blood cell rosetting procedure. Post-transplantation the myeloma protein decreased from 4.3 to 1.7 g/dL on day +21 with a subsequent increase to 3.4 g/dL on day +67 (Figure 1). BM plasmacytosis on day +67 was 90%. On day +116, she received fresh donor peripheral blood cells from 100 mL of donor blood. Fourteen days after infusion she developed a skin rash. Skin biopsy was diagnostic for GVHD grade IV. She also had mild diarrhea up to a maximum of 1100 mL/day, while liver functions were only mildly elevated. She was started on a combination of methylprednisolone and

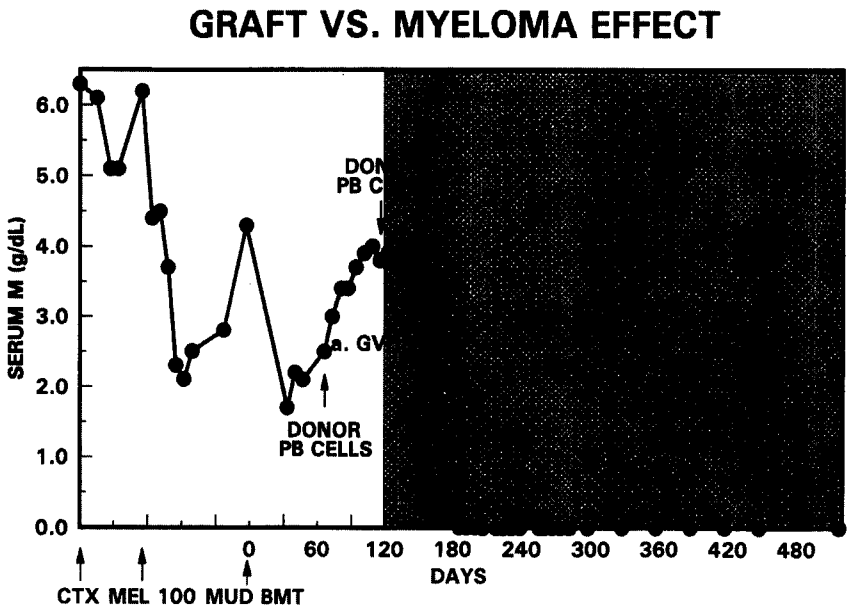

Figure 1.

cyclosporine with slow improvement of the acute GVHD. Forty-two days after infusion of the peripheral donor cells, no evidence of myeloma was present by immunofixation electrophoresis of blood and urine nor by BM biopsy. DNA fingerprinting indicated the presence only of donor cells. She subsequently developed signs of chronic GVHD about 360 days after the T cell–depleted transplant requiring prednisone and cyclosporine. Around day 480, a small serum M-protein of 0.5 g/dL was detected, which remained stale during the next three months. Unfortunately, the patient developed an invasive aspergillus infection of the lung while on prednisone and cyclosporine requiring ventilatory support and ultimately succumbed to this complication.

CASE TWO

A 42-year-old male with kappa light chain disease, stage IIIB, had Bence-Jones proteinuria of 7 g/24 hours, creatinine 3 mg/dL, a complex cytogenetic karyotype including -13 and BM plasmacytosis of 70% at diagnosis. He was treated with 3 cycles of VAD to a moderate response. He then received high-dose cyclophosphamide 6 g/m^2 for stem cell mobilization. A first autotransplant was performed with melphalan 200 mg/m^2 resulting in a decrease in urine M-protein to 200 mg/24 hrs. A subsequent

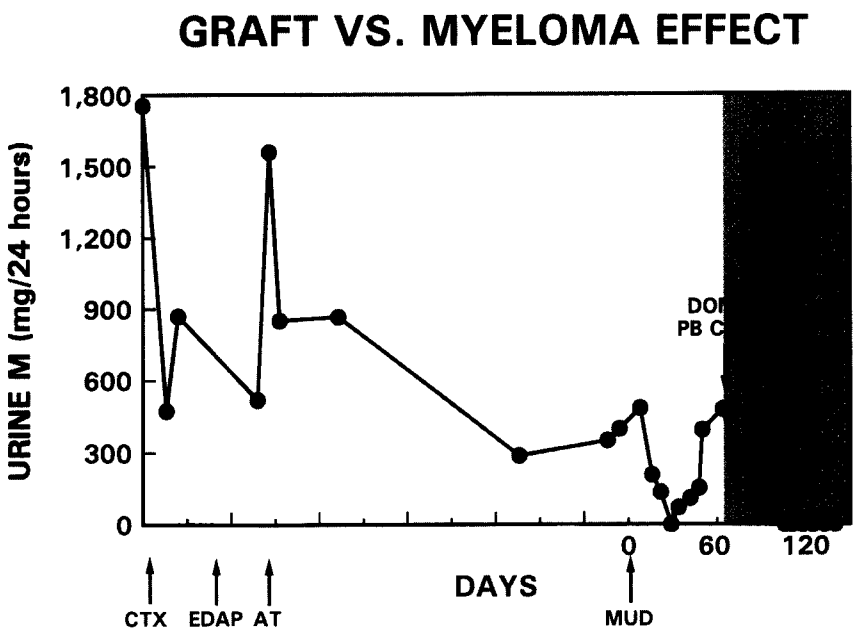

Figure 2.

rise in M-protein was seen with an increase in BM plasmacytosis. He subsequently received a matched unrelated donor transplant after conditioning identical to the regimen given in case one. Post-transplantation, the urine M disappeared completely, but reappeared (450 mg/24 hours) by day 45 (Figure 2). BM plasmacytosis was 30%. He then received donor peripheral blood stem cells with complete disappearance of urine M-protein and marrow plasmacytosis. His course was complicated by acute, and subsequently, chronic GVHD with mainly liver manifestations. Multiple BM biopsies performed subsequently remained negative for myeloma and urine M-protein remained 0. Eleven months after his matched unrelated donor transplant while on immunosuppressive therapy, the patient developed invasive pulmonary aspergillosis and died from this complication.

CASE THREE

A 34-year-old female with kappa light chain disease, stage IIIA, presented with BM plasmacytosis of 23%, Bence-Jones proteinuria of 11.6 g/24 hours and a complex cytogenetic karyotype including –13. She received three courses of VAD and had some decrease in urine M-protein to 3 grams/24 hours, but no decrease in BM plasmacytosis. She then received high-dose cyclophosphamide for stem cell mobilization followed by an autologous transplant with melphalan 200 mg/m^2. No

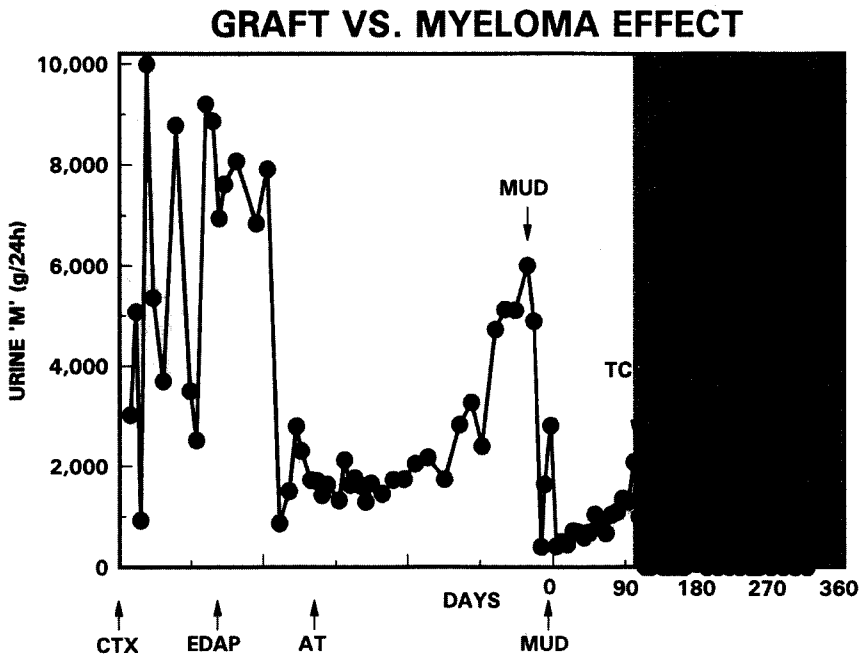

Figure 3.

further reduction was seen in urine M-protein or in BM plasmacytosis after the autologous transplant and 3 months later the patient clearly had progressive disease with an increase in urine M-protein to 6 g/24 hours and BM plasmacytosis of 50%. She then received a matched unrelated donor transplant with the same conditioning as patients one and two. About 90 days after transplantation, she had an increase of urine M-protein from trace on day 30 to 2 g/24 hours and BM plasmacytosis from 10 to 40% (Figure 3). She then received donor T cells with complete resolution of all disease. After T cell infusion, she developed acute, and subsequently, chronic GVHD requiring prednisone and cyclosporine. Eleven months after the matched unrelated donor transplant, she developed invasive pulmonary aspergillosis while on immunosuppressive therapy and died of this complication.

CASE FOUR

A 30-year-old male with IgA kappa stage IIIA at diagnosis had immunoglobulin of 7.3 and IgG of 4.5 g/dL and Bence-Jones proteinuria of 167 mg/24 hours and a BM plasmacytosis of 80%. He had a complex chromosomal karyotype including t(13;20). He received three courses of VAD with no effect on IgA protein. BM plasmacytosis was 35%. He then received high-dose cyclophosphamide for stem cell mobilization and an autotransplant with melphalan 200 mg/m^2. Serum M-protein

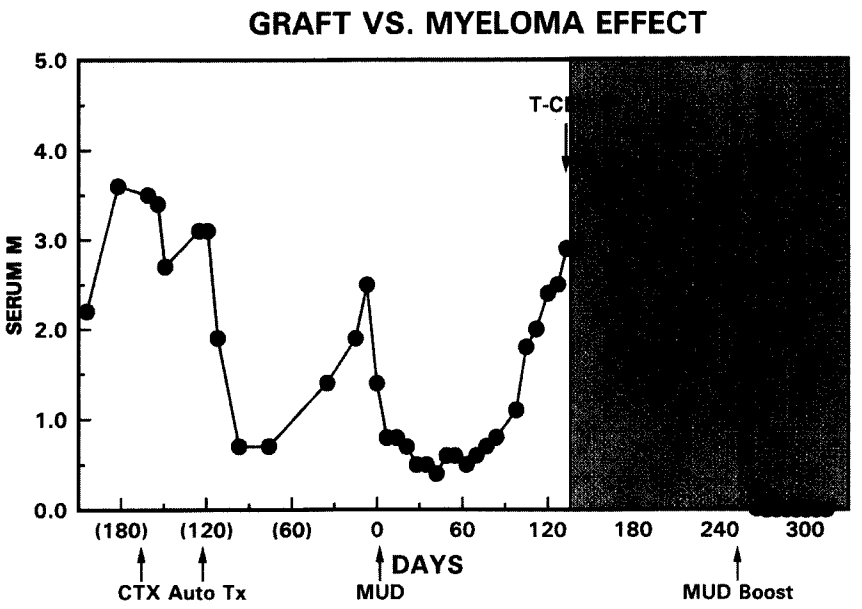

Figure 4.

decreased to 0.9 mg/dL. However, two months later, serum M-protein progressively increased to 2.4 g/dL. He then proceeded with a matched unrelated donor transplant again with the same conditioning regimen as the prior patients. After transplantation, serum M-protein decreased to 0.4 g/dL, but beyond day 60 progressively rose to a 3.9 g/dL by day +125 (Figure 4). After infusion of donor T cells, a decrease was seen in M-protein from 3.9 g/dL to 1.4 g/dL, but subsequently increased again to 3.6 g/dL. The patient never developed GVHD. It was then decided to treat him with melphalan 200 mg/m^2 and a non-T cell depleted allograft. Thirty days after transplant there was no evidence of disease as measured by immunofixation of the serum and BM biopsy. However, he subsequently developed acute GVHD, multi-organ failure and VOD of the liver and died 52 days after transplant.

These cases clearly illustrate that a GVM effect can be seen after allogeneic transplantation. All four patients had a complete resolution of their disease. In the first three patients, the only manipulation performed was the infusion of donor cells. Therefore, the effect can be attributed only to a GVM effect. In the fourth patient, infusion of allo donor cells resulted in a temporary response, but no GVHD. In contrast, the first three cases all had acute and chronic GVHD. In the fourth patient, we reasoned that a lack of GVM effect was due to lack of activation of donor cells and that a cytokine storm might be necessary to activate donor T cells. A single dose of melphalan 200 mg/m^2 was given to this patient. This patient had been completely

refractory to melphalan 200 mg/m^2 and an autotransplant. It is therefore very unlikely that the complete resolution of the myeloma was effected by melphalan. However, after melphalan, the patient developed acute GVHD requiring treatment with prednisone and cyclosporine. The appearance of GVHD was associated with the complete disappearance of all evidence of myeloma.

It is an amazing experience to see patients who are obviously completely refractory to high-dose chemotherapy and radiotherapy, subsequently attain complete remissions just by the infusion of donor lymphocytes. The graft-versus-tumor effect seen in multiple myeloma is probably equally potent as that observed in chronic myeloid leukemia (CML) transplants. However, our findings are somewhat different from those reported with CML. Whereas control of CML seems to require large quantities of peripheral blood mononuclear cells, a complete response in our patients was seen after infusion of only 10^6/kg CD3 cells. However, it is unknown whether this might be due to the fact that our patients had unrelated donor transplants. It may be that unrelated donor transplants require fewer T cells to elicit a graft-versus-tumor effect compared with sibling transplants. While the best results in CML are seen in patients in stable chronic phase with almost no effect seen in patients relapsing in blast crisis, our patients achieved a response upon administration of donor cells in fulminant and aggressive relapse. Finally, whereas in CML, there is a very slow disappearance of disease as measured by cytogenetics and molecular methods, in our patients a GVM effect was seen very early on. It is unknown whether a lasting GVM effect can be seen without the presence of acute GVHD and its major complications, as evidenced in our patients. Three of our patients died more than 11 months after transplantation due to invasive aspergillosis. Because of the high complication rate of acute and chronic GVHD, we have now instituted a new protocol, whereby donor T cells are transfected with a suicide gene, the viral thymidine kinase gene, that makes the lymphocytes susceptible to killing by ganciclovir. As soon as evidence of acute GVHD is observed, administration of ganciclovir should then eliminate all the infused donor T cells. Murine leukemia data have shown that the antileukemic effect of an allograft is attained within 6 to 7 days and precedes the onset of severe GVHD.[14] Although the same cells may cause a GVM effect and GVHD, it is possible that the two entities are separable in time.

It is likely that future progress in cancer therapy will depend on the judicious and safe manipulation of the immune system rather than on further intensification of cytotoxic agents.

REFERENCES

1. Attal, Harousseau J, Stoppa A et al.: A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. *N Engl J Med* 335:91–97, 1996.

2. Barlogie B, Jagannath S, Vesole D et al.: Superiority of tandem autologous transplantation over standard therapy for previously untreated multiple myeloma. *Blood* (in press), 1996.
3. Tricot G, Jagannath S, Vesole D et al.: Hematopoietic stem cells transplants for multiple myeloma. *Leuk Lymphoma*, 22:25–36, 1996.
4. Schiller G, Vescio R, Freytes C et al.: Transplantation of $CD34^+$ peripheral blood progenitor cells after high-dose chemotherapy for patients with advanced multiple myeloma. *Blood* 86:390–397, 1995.
5. Gazitt Y, Reading C, Hoffman R et al.: Purified $CD34^+$ Lin^- Thy^+ stem cells do not contain clonal myeloma cells. *Blood* 86:1–9, 1995.
6. Tricot G, Vesole D, Jagannath S et al.: Graft-versus-myeloma effect: Proof of principle. *Blood* 87:1196–1198, 1996.
7. Verdonck L, Lokhorst H, Dekker A et al.: Graft-versus-myeloma effect in two cases. *Lancet* 347:800–801, 1996.
8. Bjorkstrand B, Ljungman P, Svensson H et al.: Allogeneic bone marrow transplantation versus autologous stem cell transplantation in multiple myeloma: A retrospective case-matched study from the European Group for blood and marrow transplantation. *Blood* 88:4711–4718, 1996.
9. Weiden P, Sullivan K, Flournoy N et al.: Antileukemic effect of chronic graft-versus-host disease. *New Engl J Med* 301:1529, 1981.
10. Kolb H, Mittermuller J, Clemm C et al.: Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood* 76:2462, 1990.
11. Drobyski W, Keever C, Roth M et al.: Salvage immunotherapy using donor leukocyte infusions as treatment for relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation: Efficacy and toxicity of a defined T-cell dose. *Blood* 82:2310, 1993.
12. Porter D, Roth M, McGarigle C et al.: Induction of graft-versus-host disease as immunotherapy for relapsed chronic myeloid leukemia. *New Engl J Med* 330:100, 1994.
13. Kolb H-J, Schattenberg A, Goldman JM et al.: Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood* 86:2041–2050, 1995.
14. Boranic M, Tonkovic I et al.: Time pattern of the antileukemic effect of graft-versus-host reaction. *Cancer Res* 31:1140, 1971.

AUTOTRANSPLANTS FOR MULTIPLE MYELOMA—10 YEARS LATER

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INTRODUCTION

Three decades of standard dose chemotherapy trials research in multiple myeloma (MM) have confirmed that, unfortunately, combination chemotherapy is not superior to the "gold standard" melphalan-prednisone (MP) (Figure 1).[1] In reviewing the details of these trials, the lack of progress is perhaps not surprising considering that relatively low doses of individual agents with mild anti-tumor activity were combined so that, by design, the overall myelosuppressive activity was almost comparable in order to avoid excessive morbidity and mortality in typically immunosuppressed elderly patients. Dose escalation was first tested with the VAD regimen incorporating high doses of dexamethasone, resulting in unexpectedly high response rates in MP-refractory disease.[2] The late Tim McElwain and colleagues from the Royal Marsden Hospital were the first to observe true complete remissions (CR) in about a dozen patients with high risk newly diagnosed or advanced and refractory MM following unsupported high dose melphalan at a dose of 140 mg/m^2 (MEL 140).[3] This observation prompted our team to introduce autologous transplantation in the early 1980s, initially in support of MEL 140[4] and, subsequently, of truly myeloablative therapy with MEL 140 plus total body irradiation (TBI, 850 cGy), using autologous bone marrow that could contain up to 30% plasma cells.[5] The underlying hypothesis was that the reinfusion of typically hypoproliferative tumor cells with typically low in vitro clonogenic potential would not contribute significantly to disease recurrence. In this report, we update these early results and report on tandem autotransplants introduced in late 1989 as a means of further increasing tumor cytoreduction in an effort to increase CR rates and extend event-free survival (EFS) and overall survival (OS). The tandem autotransplant approach utilized, from the outset, peripheral blood stem cells (PBSC) collected after high dose cyclophosphamide (HDCTX) initially with GM-CSF and, in later years, with G-CSF.[6] All PBSCs were collected before the first autotransplant and, using the mobilization regimens mentioned, typically provided more rapid engraftment than autologous bone marrow transplants (autoBMT), thus reducing transplant-related mortality to well under 5%.

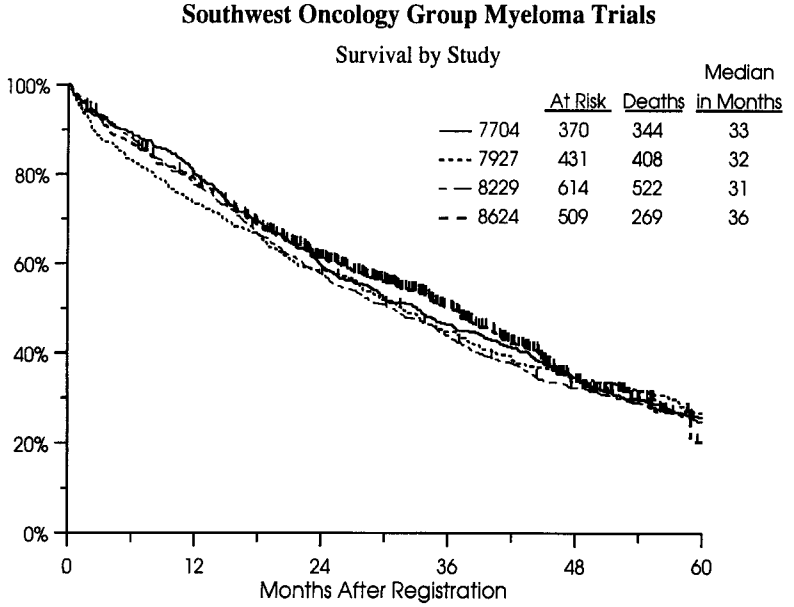

Figure 1.

UPDATE ON ABMT WITH MEL 140 OR THIOTEPA 750 MG/M^2 + TBI 850 CGY

An update of the 55 patients treated with TBI 850 cGy and MEL 140 or, when intravenous melphalan was temporarily unavailable, with thiotepa 750 mg/m^2 (THIO 750) revealed 9-year EFS/OS rates of 10/20% in the case of MEL 140 + TBI and 0/0% with THIO 750 +TBI (Table 1).[7] The superiority of MEL 140 + TBI could not be explained by more favorable prognostic features, thus supporting, despite the relatively small number of patients in the THIO 750 + TBI group, our notion that MEL has superior antitumor activity in MM. Included in this table are results of trials with MEL 100 ± GM-CSF without hemopoietic stem cell support, conducted exclusively in refractory MM.[8,9] Examination of prognostic factors for all 102 subjects revealed superior EFS and OS in the case of low beta-2-microglobulin (B2M, ≤2.5 mg/L) and younger age ≤50 years). A comparison of the 63 patients with low B2M and/or age < 50 yrs with the remaining 39 older subjects with B2M > 2.5 mg/L revealed that the former group had a lower mortality (≤60 days) (8% versus 26%, p=0.01) but comparable CR rates (16% versus 10%, p=0.4) and enjoyed superior EFS/OS (15/36 months versus 3/5 months, p=0.0001/0.0001).

Table 1. TBI + ABMT and MEL 100 + GM-CSF for refractory/advanced myeloma—long-term follow-up

Treatment	*N*	*%ED*	*%CR*	*EFS*	*OS*
MEL 140-TBI	37	16	24	15	34
THIO 750-TBI	18	0	11	7	23
MEL 100	23	22	0	3	5
MEL 100 + GM-CSF	24	17	4	6	22
	p	0.2	0.1	0.008	0.03

TANDEM AUTOTRANSPLANTS FOR MM—EXPERIENCE WITH 470 PATIENTS

This group comprises 180 patients undergoing our "Total Therapy" program for newly diagnosed patients[10] and 290 referred to this center after having received prior treatment elsewhere.[11] Overall patient characteristics are summarized in Table 2, with approximately one-half over age 50, having stage III at diagnosis, B2M >2.5 mg/L, CRP >0.4 mg/dL and > 12 months of prior therapy prior to transplant. The transplant regimen consisted of melphalan 200 mg/m^2 (MEL 200) with the first autotransplant and was repeated with the second autotransplant in case ≥75% tumor mass reduction (PR, including normal marrow aspirate and

Table 2. Tandem autotransplant for myeloma patient characteristics (N=470)

Parameter	*Total n=470*	*"Total Therapy" n=180*	*Prior therapy n=290*	*p*
age >50 years	54*	47	58	.02
stage III at diagnosis	48	50	47	.5
Ig Isotype G	56	59	54	.3
A	18	19	17	.5
other	26	22	29	.06
B2M >2.5 mg/L pretransplant	44	46	42	.4
CRP >0.4 mg/dL pretransplant	49	42	53	.02
<50% tumor cytoreduction prior to transplant	38	30	43	.006
>12 months of prior therapy	54	0	72	.0001
>3 lines of prior therapy	14	0	23	.0001
Mel 200→Mel 200	42	61	31	.0001
Mel 200→other/auto	28	16	36	.0001
Mel 200→other/allo	7	7	6	.6
Mel 200→no second transplant	23	16	27	.003

**percent*

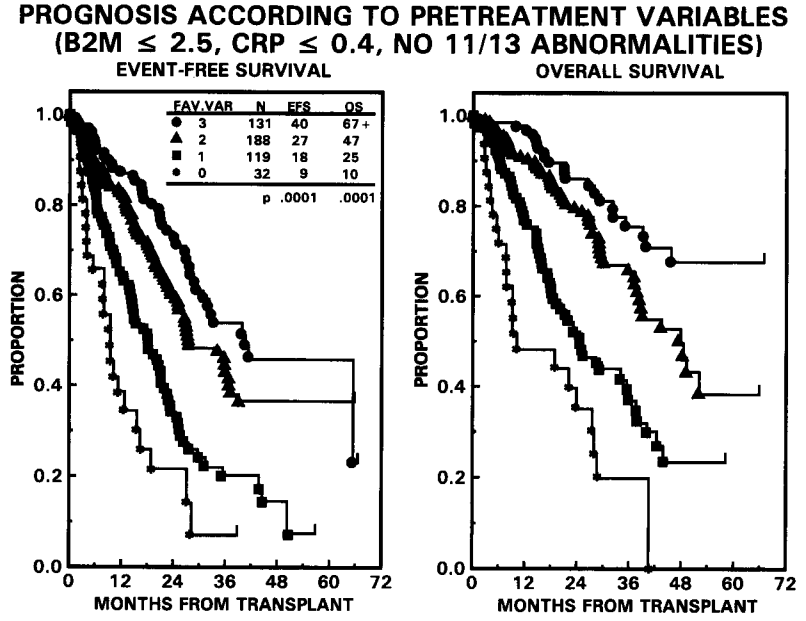

Figure 2.

biopsy) was sustained; otherwise, patients received MEL 140 + TBI (850–1125 cGy) or MEL 200 + high dose cyclophosphamide (HDCTX) 4.8 g/m^2. Nearly 80% of patients completed 2 transplants. The transplant-related mortality at 12 months was 7%, 36% achieved CR, and median durations of EFS/OS were 26/41 months, with 5 year projections of 30/40%, respectively. Among the double transplant recipients, CR increased from 24% after one to 44% after 2 transplants including 17% previously in PR and 3% with <PR status after the first transplant.

A prognostic factor analysis revealed significantly superior EFS and OS durations in the case of low B2M and low CRP serum levels prior to first transplant, sensitive rather than refractory MM (defined by ≥50% tumor mass reduction with standard therapy prior to transplant), the absence of abnormalities of chromosomes 11 and 13, serum creatinine ≤2 mg/dL, ≤12 months of prior therapy, Durie-Salmon stage < III and non-IgA isotype. On the basis of the 3 dominant favorable variables identified on multivariate regression analysis (B2M ≤ 2.5 mg/L, CRP ≤ 0.4 mg/dL, no abnormalities of chromosomes 11/13), 4 distinct risk categories could be defined (Figure 2). Patients with ≥ 2 favorable variables enjoyed distinctly superior EFS and OS durations (319 patients) as opposed to the 151 remaining patients with <2 favorable parameters; a particularly poor outcome was noted among the 32 patients lacking any of these favorable features with EFS/OS durations of 9/10 months. Thus, we were able to identify, among a large cohort of uniformly treated patients, a

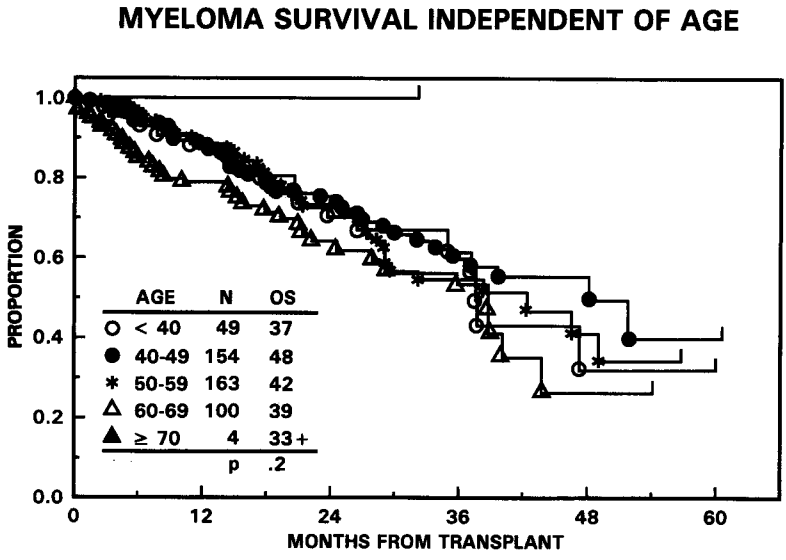

Figure 3.

particularly high risk group in whom novel potentially hazardous treatment approaches would appear justified. In order to appreciate the impact of obtaining CR and of the timely application of 2 transplants within 6 months, a 6-month landmark analysis was performed among the 440 patients surviving this time interval. Both CR and timely application of 2 transplants emerged as significant variables in addition to the pretransplant parameters already mentioned, supporting our hypothesis that "more is better"; i.e., greater tumor cytoreduction, effected by a second cycle of high dose therapy and reflected in a higher biochemically and hematologically defined CR rate, was associated with superior prognosis. In clinical practice, disease- and patient-related variables cannot be modified, whereas the timing and type of therapeutic intervention are flexible. EFS and OS durations were significantly longer among the 176 patients who received a "timely double transplant (TDT)," i.e., first transplant within 12 months of initiation of standard therapy and second transplant within 6 months of the first transplant with median EFS/OS durations of 44/66+ months compared to 23/40 months for the remaining 264 patients (p=0.0001/0.0003, respectively).

Of considerable clinical importance is our observation that impaired renal function did not affect melphalan pharmacokinetics,[12] so that we have performed autotransplants in 23 patients with serum creatinine levels >2mg/dL including 7 patients on hemodialysis. Compared to 46 patients with serum creatinine levels ≤2 mg/dL, who were matched for CRP, Ig isotype, duration of prior therapy and

sensitivity to standard therapy. The renal failure group had an early mortality of 0% versus 2% among the remaining patients, and engraftment kinetics were identical with median times to granulocytes >500/μL of 11/11 days (p=0.3) and to platelets exceeding 50,000/μL of 16/22 days (p=0.4). Thus MEL 200 as one of the most active regimens for MM no longer needs to be withheld in patients with renal failure that is usually a result of high tumor burden with hypercalcemia and excessive Bence Jones proteinuria; in fact, improvement in renal function (creatinine ≤1.2 mg/dL) as a result of PBSC-supported MEL 200 was observed among 43% of the 23 patients treated.

Another critical variable in MM therapy pertains to patients' frequently advanced age, which has been one of the main obstacles to investigating dose intensification in this disease. Examination of survival after first autotransplant revealed no difference as a function of age by decade (Figure 3).[13] In fact, we have now treated 23 patients over the age of 70 including one at age 82, employing MEL 200 in 11 and MEL 140 in 12 patients ("minitransplant"). Two patients died within 60 days, CR was achieved in 21%, and median EFS/OS durations are 9/23 months, reflecting these patients' overall less favorable pretransplant prognostic features rather than their advanced age.

SALVAGE THERAPY FOR POST-TRANSPLANT RELAPSE

An important issue concerns the question of "life after relapse post-transplant" posed by patients and referring physicians alike.[14] We therefore examined the outcome of 196 patients who had relapsed, including 91 after a scheduled tandem transplant. The median time from relapse post-transplantation to salvage therapy was 9 months (range 3–44 months). Standard dose therapy was employed in 147 and a further transplant in 49 patients; eventually, 63 of 105 relapsing after a single transplant and 19 of 91 relapsing after a tandem transplant received an additional transplant regimen. Ten percent died within 2 months, 8% achieved CR, and post-salvage EFS/OS were 8/14 months, respectively On multivariate regression analysis, EFS and OS were longer with primary salvage transplant and low pre-salvage B2M ≤ 2.5 mg/L. As summarized in Table 3, 60 day post-transplant mortality was 0%, 9% and 13% (p=0.1), respectively, among the 25 patients exhibiting both favorable features as opposed to the 76 with one unfavorable parameter and 95 patients lacking both favorable factors; the corresponding CR rates were 32%, 7% and 3%, respectively (p=0.0001); and median EFS/OS durations were 64+/64+ months, 9/15 months, and 5/9 months (p=0.0001/.0001, respectively). In addition, those with only one prior transplant had longer EFS, whereas OS was longer when relapse had occurred ≥12 months after transplant. When examined separately among the 105 patients with only one prior transplant and the 91 with relapse after two transplants, differences in prognostic factors

Table 3. Clinical outcome according to low pre-salvage B2M and primary salvage transplant

B2M ≤ 2.5 presalvage *Primary salvage TX*	*N*	*%ED*	*p*	*%CR*	*p*	*EFS*	*p*	*OS*	*p*
both	25	0		32		64+		64+	
			0.04		0.001		0.0001		0.005
either/or	76	9		7		9		15	
			0.7		0.3		0.004		0.0001
none	95	13		3		5		9	
	P	0.1		0.0001		0.0001		0.0001	

associated with EFS and OS were noted: in case of relapse after only one transplant, low B2M and primary salvage transplant both favored prolonged EFS (64+ versus 9 months, p=0.0001) and OS (64+ versus 11 months, p=0.0001); by contrast, with relapse following a tandem transplant, late relapse >12 months after transplant rather than an additional transplant emerged as a second key variable in addition to low pre-salvage B2M (EFS, 8 versus 5 months, p=0.05; OS, 15 versus 9 months, p=0.0003). Collectively, these data indicated that post-transplant relapse should not be considered as ultimate treatment failure. Those patients with only one prior transplant definitely benefit from timely administration of further high dose therapy (in line with our current practice of a scheduled tandem transplant within 6 months); thus, in case only a single autotransplant is planned, sufficient PBSC should be obtained before first transplant for an eventual subsequent transplant procedure. Finally, selected patients relapsing after a tandem transplant procedure deserve further therapeutic consideration, especially when B2M is low and relapse occurred more than one year after the last transplant.

SUMMARY AND CONCLUSION

Autotransplants, especially with mobilized PBSC providing rapid hemopoietic recovery, have become an indispensable armamentarium in the management of patients afflicted with MM and can be conducted with a high degree of safety even in high risk patients presenting with renal failure and advanced age. Not unexpectedly, the best results are obtained when this approach is applied early during the illness with superior results when implemented within the first 12 months from initiation of therapy. Formal comparisons of autotransplant-supported high-dose therapy and standard-dose chemotherapy are now available. Thus, the French Myeloma Intergroup Trial (IFM 90) has recently concluded that the 100 patients randomized to MEL 140 + TBI 800 cGy enjoyed higher CR rates (22%

versus 5%, p=0.001) and superior 5-year EFS (28% versus 10%, p=0.01) and OS (52% versus 12%, p=0.03) than the 100 patients receiving VMCP/VBAP standard chemotherapy.[15] Our Total Therapy experience, using remission induction with mutually noncross resistant VAD, HDCTX and EDAP followed by tandem autotransplants and interferon maintenance, has yielded superior outcome when evaluated in a pair mate analysis vis-à-vis standard SWOG trials.[10] Thus, on an intent-to-treat basis, the 124 Total Therapy patients with a minimum follow-up of 24 months had a CR rate of 25% after one and 40% after two transplants, median EFS was 49 months and 5-year OS expectation was 61%; by comparison, the 124 pair mates receiving standard therapy had median durations of EFS/OS of 20/46 months (p=0.0001/0.003). The apparently greater tumor cytoreduction effected by a tandem versus a single transplant has prompted the current IFM randomized trial of one versus two transplants, also comparing PBSC versus ABMT. Investigators in the United States and Canada are participating in a North American Intergroup Trial (SWOG 9321) that compares MEL 140 + TBI 1200 cGy with PBSC support versus VBMCP standard chemotherapy in a randomized fashion, regardless of response to induction with VAD $\times$ 4 and HDCTX 4.5 g/m^2; all those achieving at least PR status ($\geq$75% tumor cytoreduction) are then randomized to interferon maintenance versus observation, in order to determine the role of this controversial maintenance strategy after autotransplantation. The mandatory PBSC collection also on the standard treatment arm and the plan for salvage transplantation upon relapse on VBMCP assure that all patients are candidates for myeloablative therapy, an important ethical issue in light of improved prognosis with high dose therapy suggested by several phase II autotransplant trials[10] and, more recently, by the IFM randomized trial.[15] SWOG 9321 will answer the question of optimal timing of myeloablative therapy, i.e., early following remission induction versus salvage after failure on standard VBMCP.

Studies in progress in our program address, among good risk patients (favorable cytogenetics, $\leq$12 months of prior therapy, non-IgA isotype), the role of CD34 selection[16,17] in order to reduce the contribution to relapse from tumor cell reinfusion in the process of autografting. In the poor risk category, we are evaluating post-double transplant chemotherapy with our DCEP regimen that combines high dose dexamethasone and 96-hour continuous infusions of cyclophosphamide, etoposide and cisplatin, administered every 3 months $\times$ 4. With the notion of a profound graft-versus-myeloma (GVM) effect in the process of allotransplants[18] that are, however, associated with a high one-year transplant-related mortality of 30–40%, a trial is underway at our Center that utilizes T cell depletion of allografts to avoid GVHD. Following hemopoietic engraftment, a small dose of thymidine kinase gene-transduced donor mononuclear cells is infused to exert the therapeutically beneficial GVM effect; in case GVHD ensues, ganciclovir will induce donor T cell suicide.[19] Based on pilot trials in acute

leukemia by Nagler et al.,[20] we are also evaluating the safety and efficacy of haploidentical donor mononuclear cell transfusion together with a second autograft as a means of exerting a GVM effect during the cytokine storm elicited in the process of autotransplantation. Rejection of donor mononuclear cells is expected to ensue within 3–4 weeks so that the risk of severe GVHD is hopefully minimized. In collaboration with the National Cancer Institute, idiotype vaccination is being explored in the context of both autologous and allogeneic transplantation.[21]

While research in both autologous and allogeneic transplantation continues, new agent evaluation has to be pursued along with immunological approaches involving gene therapy to either enhance tumor immunogenicity (vaccination with GM-CSF-transduced tumor cells) or augment host immunity (idiotype vaccination;[3,20] infusion of autologous dendritic cells exposed ex vivo to paraprotein for better tumor antigen presentation to cytoxic T lymphocytes).

REFERENCES

1. Jagannath S, Vesole DH, Tricot G, Crowley J, Salmon S, Barlogie B: Hemopoietic stem cell transplants for multiple myeloma. *Oncol* 11:89, 1994.
2. Barlogie B, Smith L, Alexanian R: Effective treatment of advanced multiple myeloma refractory to alkylating agents. *NEJM* 310:1353, 1984.
3. McElwain TJ, Powles RJ: High dose intravenous melphalan for plasma-cell leukemia and myeloma. *Lancet* 2:822, 1983.
4. Barlogie B, Hall R, Zander A, Dicke K, Alexanian R: High-dose melphalan with autologous bone marrow transplantation for multiple myeloma. *Blood* 67:1298, 1986.
5. Barlogie B, Alexanian R, Dicke KA, Zagars G, Spitzer, Jagannath S, Horowitz L: High-dose chemoradiotherapy and autologous bone marrow transplantation for resistant multiple myeloma. *Blood* 70:869, 1987.
6. Jagannath S, Vesole D, Glenn L, Crowley J, Barlogie B: Low-risk intensive therapy for multiple myeloma with combined autologous bone marrow and blood stem cell support. *Blood* 89:1666, 1992.
7. Jagannath S, Barlogie B, Dicke K, Alexanian R, Zagars G, Cheson B, LeMaistre FC, Smallwood L, Pruitt K, Dixon DO: Autologous bone marrow transplantation in multiple myeloma: Identification of prognostic factors. *Blood* 76:1860, 1990.
8. Barlogie B, Alexanian R, Smallwood L, Cheson B, Dixon D, Dicke K, Cabannilas F: Prognostic factors with high-dose melphalan for refractory multiple myeloma. *Blood* 72:2015–2019, 1988.
9. Barlogie B, Jagannath S, Dixon DO, Cheson B, Smallwood L, Hendrickson A, Purvis JD, Bonnem E, Alexanian R: High-dose melphalan and GM-CSF for refractory multiple myeloma. *Blood* 76:677, 1990.
10. Barlogie B, Crowley J, Jagannath S, Vesole D, Tricot G, Jacobson J, Salmon S: Superior outcome after early autotransplantation (AT) with "Total Therapy" (TT) compared to standard SWOG treatment (ST) for multiple myeloma (MM). *Blood,* 86(suppl 1 abstr):207a, 1995.

11. Vesole D, Tricot G, Jagannath S, Desikan KR, Siegel D, Bracy D, Miller L, Cheson B, Crowley J, Barlogie B: Autotransplants in multiple myeloma: What have we learned? *Blood* 88:838 1996.
12. Tricot G, Alberts DS, Johnson CS, Roe DJ, Dorr RT, Vesole DH, Jagannath S, Meyers R, Barlogie B: Safety of autotransplants with high dose melphalan in renal failure: A pharmacokinetic and toxicity study. *Clin Can Rsch* 2:947, 1996.
13 Vesole D, Jagannath S, Tricot G, Siegel D, Desikan KR, Vaught L, Barlogie B: Efficacy and safety of high dose therapy with autotransplantation (AT) for multiple myeloma (MM) in patients over age 60. *Blood* 86(suppl 1 abstr):206a, 1995.
14. Tricot G, Jagannath S, Vesole D, Crowley J, Barlogie B: Relapse of multiple myeloma after autologous transplantation: Survival after salvage therapy. *BMT* 16:7, 1995.
15 Attal M, Harousseau JL, Stoppa AM, Sotto JJ, Fuzibet JG, Rossi JF, Casassus P, Maisonneuve H, Facon T, Ifrah N, Payen C, Bataille R: A prospective randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. *New Engl J Med* 335:91, 1996.
16. Vescio RA, Hong CH, Cao J, Kim A, Schiller GJ, Lichtenstein AK, Berenson RJ, Berenson JR: The hematopoietic stem cell antigen, CD34, is not expressed on the malignant cells in multiple myeloma. *Blood* 84:3283, 1994.
17 Gazitt Y, Reading C, Hoffman R, Lee HL, Wickrema A, Vesole D, Jagannath S, Condino J, Lee B, Barlogie B, Tricot G: Purified $CD34^+/THY\text{-}1^+/LIN^-$ stem cells do not contain clonal myeloma cells. *Blood* 86:381, 1995.
18. Tricot G, Vesole DH, Jagannath S, Hilton J, Munshi N, Barlogie B: Graft-versus-myeloma effect: Proof of principle. *Blood* 87:1196, 1996.
19. Munshi NC, Govindarajan R, Drake R, Ding LM, Iyer R, Saylors R, Kornbluth J, Marcus S, Chiang Y, Ennist D, Kwak L, Reynolds C, Tricot G, Barlogie B: Thymidine kinase (TK) gene transduced human lymphocytes can be highly purified, remain fully functional and are killed efficiently with gancylovir. *Blood* (in press).
20. Nagler A, Ackerstein A, Or R, Drakos P, Kapelushnik Y, Mehta J, Naparstek E, Slavin S: Adoptive immunotherapy with mismatched allogeneic peripheral blood lymphocytes (PBL) following autologous bone marrow transplantation (ABMT). *Exp Hematol* 20:705, 1992.
21. Kwak LW, Taub DD, Duffey PL, Bensinger WI, Bryant EM, Reynolds CW, Longo DL: Transfer of myeloma idiotype-specific immunity from an actively immunized marrow donor. *Lancet* 245:1016, 1995.

HIGH-DOSE THERAPY WITH PERIPHERAL BLOOD PROGENITOR CELL TRANSPLANTATION IN MULTIPLE MYELOMA

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ABSTRACT

The objective of our study was to evaluate the efficacy and toxicity of a high-dose therapy with peripheral blood progenitor (PBPC) transplantation in patients with multiple myeloma (MM). Between June, 1992, and May, 1996, 100 patients (67 males/33 females) with a median age of 51 years (range 30–65) were transplanted at our center. Patients had been pretreated until best response to conventional chemotherapy. PBPCs were collected during granulocyte colony-stimulating factor (G-CSF) enhanced leukocyte recovery following treatment with high-dose cyclophosphamide (HD-CY, 88 patients) or dose-escalated ifosfamide/mitoxantrone (12 patients). Fifty patients were treated with total body irradiation (TBI) + melphalan 140 mg/m^2 while 50 patients received melphalan 200 mg/m^2. Following PBPC autografting, the median time to reach platelets $\geq 20 \times 10^9$/L and neutrophils $\geq 0.5 \times 10^9$/L was 11 and 14 days with no difference between the treatment groups. In the TBI group, significantly longer periods of total parenteral nutrition were required due to severe mucositis, representing higher nonhematological toxicity. Two patients from the TBI group died from transplant-related complications. Following high-dose treatment, remission state improved in 43 out of 98 patients. No statistically significant advantage with respect to reaching CR or PR was observed with TBI + HD-melphalan compared with the treatment with HD-melphalan alone. The optimal conditioning regimen, with particular regard to the inclusion or omission of TBI, should be prospectively investigated.

INTRODUCTION

Multiple myeloma (MM) is a B cell disorder with a median patient survival of 3 years using conventional chemotherapy.[1] Most randomized trials have failed to show an improvement in survival with combination chemotherapy over melphalan with or without prednisone.[2,3] A promising treatment approach for multiple myeloma is based

Table 1. Patient characteristics (n=100)

	TBI + 140mg/m² *melphalan* *n=50*	*200mg/m²* *melphalan* *n=50*	*p=*
age (years)	50 (30–60)*	54 (40–65)*	0.0070
paraprotein			
G/A/BJ/D	29/13/7/1	26/8/12/2	0.6029
asecretory	1	2	
males/females	32/18	35/15	0.5205
stage I/II/III	3/11/36	0/10/40	0.1717
stage B	6 patients	12 patients	0.4356
no. of previous therapy cycles	7 (3–28)*	8 (3–25)*	0.8312
previous radiation	18/50	24/50	0.3111
β-microglobulin ≥6g/L[a]	4 patients	3 patients	0.9999
β-microglobulin 2.5g/L[a]	13 patients	15 patients	0.8240
hypercalcemia[a]	8 patients	10 patients	0.7953

**Median (range)*
[a]at diagnosis

on reports of high complete remission rates after the administration of dose-escalated alkylating agents, which was piloted by T.J. McElwain in the early 1980s using dose-escalated melphalan.[4] Hematopoietic progenitor cell transplantation allows a further increase in the dose of cytotoxic drugs and the addition of total-body irradiation (TBI). The randomized IFM 90 study showed a significantly longer event-free survival (EFS) and overall survival (OS) in patients treated with high-dose melphalan and TBI followed by autologous bone marrow transplantation.[5] We present our single center experience of 100 patients with multiple myeloma who were included in a high-dose treatment program with peripheral blood progenitor (PBPC) transplantation.

PATIENTS AND METHODS

From June, 1992, to May, 1996, 100 patients with multiple myeloma were enrolled into this study. Inclusion criteria were high-risk MM, defined as stage III at diagnosis according to the Salmon-Durie classification[6] as well as progressive stage II disease or symptomatic stage I disease, and an age >18 and < 65 years. Sixty-seven patients were male and 33 patients female with a median age of 51 years (range 30–65). Patient characteristics are shown in Table 1. Patients had been pretreated with conventional chemotherapy (VAD or M2-protocol) until the paraprotein level reached a plateau or complete remission was achieved. The median number of previous chemotherapy cycles was 7 (range 3–28) and 42 out of the 100 patients had a history of previous irradiation. Most patients had advanced disease (76 patients with stage III), 3 patients with symptoms of the disease were in stage I and were included on their own request.

Complete and partial remission were defined according to the EBMT criteria.[7] For PR, a 50% reduction of the M-component in serum or 75% reduction of urine paraprotein are required. CR requires normal serum protein electrophoresis or proteinuria <0.25 g/L and less than 5% plasma cells in the bone marrow (BM) but a negative immunofixation is not required for CR.

PBPC mobilization

To mobilize PBPC and to reduce the tumor load before melphalan-containing high-dose therapy, 88 patients received high-dose cyclophosphamide (HD-CY, 14 patients with 4 g/m^2, 74 patients with 7 g/m^2 CY) or in case of pre-existing heart disease or amyloidosis with ifosfamide/mitoxantrone (12 patients). Twenty-four hours post-chemotherapy, Filgrastim (R-metHuG-CSF, 300 μg/ day, Amgen-Roche, München, Germany) was commenced to increase the number of circulating hematopoietic progenitor cells during leukocyte recovery. Administration of G-CSF was continued until PBPC harvesting was completed. Measurements of circulating CD34$^+$ cells were commenced when the WBC reached a level of greater than 1.0×10^9/L. PBPC collection was initiated when a distinct population of CD34$^+$ cells (CD34$^+$ > 10 cells/μL) could be detected.

Collection of PBPCs and cryopreservation procedure

Harvesting was performed with a Fenwal CS 3000 (Baxter Deutschland, München, Germany) or with a Cobe Spectra (Cobe, Lakewood, CO). For each leukapheresis, 10–20 L blood was processed at a flow rate of 50 to 150 m¬/min using large bore central venous catheters as previously described.[8] The apheresis product of a 50 mL cell suspension was mixed with the same volume of minimal essential medium containing 20% dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany). The final 100 mL cell suspension was transferred into freezing bags (Delmed, New Brunswick, NJ) and frozen to −100°C using a computer-controlled cryopreservation device (Cryoson BV-6; Cryoson Deutschland, Germany). The frozen cells were transferred into liquid-phase nitrogen and stored at −196°C. The PBPC mobilization and collection was considered successful if $\geq2.0\times10^6$ CD34$^+$ cells/kg BW were harvested.

Clonogenic assay for hematopoietic progenitor cells

The concentration of hematopoietic progenitor cells in each single leukapheresis product and in the peripheral blood was assessed using a semisolid clonogenic culture assay (Terry Fox Laboratories, Vancouver, Canada) as previously described.[9]

Immunofluorescence staining and flow cytometry

For immunofluorescence analysis, 20 μL of whole blood or 1×10^6 mononuclear cells were incubated for 30 minutes at 4°C with the fluorescein isothiocyanate

Table 2. Toxicity following high-dose therapy with PBPCT

	TBI + 140mg/m² *melphalan* *(n=50)*	*200mg/m2* *melphalan* *(n=50)*	*p=*
days with fever >38.5°C*	4 (1–11)	1 (0–9)	<0.0001
days with antibiotic treatment*	12 (6–27)	6 (0–41)	<0.0001
days with antimycotic treatment*	0 (0–15)	0 (0–0)	0.0003
days of total parenteral nutrition*	11 (2–22)	0 (0–36)	<0.0001
no. of platelet transfusions*	4 (1–16)	1 (0–50)	<0.0001
no. of erythrocyte transfusions*	2.5 (0–10)	2 (0–8)	0.0495
days in hospital*	18 (12–58)	17 (12–39)	0.2085

**Median (range)*

(FITC)-conjugated monoclonal antibody (moAb) HPCA-2 (anti-CD34, Becton Dickinson, Heidelberg, Germany) and analyzed using a Becton Dickinson FACScan as previously described.[9]

Pretransplantation conditioning regimen and intensive care post-transplantation

In 50 patients, the pretransplantation conditioning therapy consisted of TBI (14.4 Gy, hyperfractionated over 4 days) and 140 mg/m² melphalan or in 50 patients of 200 mg/m² melphalan alone because of previous spinal radiotherapy, refusal of TBI or reduced pulmonary diffusion capacity. Following high-dose conditioning therapy, PBPCs were reinfused without additional BM or growth factor support. Patients received prophylactic bowel decontamination, and antibiotic combination therapy was administered for fever >38.5°C while amphotericin-B was added in case of documented fungal infection or persistent fever. A platelet count greater than 20×10^9/L was maintained by HLA-A/-B-matched platelet transfusions, and packed red blood cells were given when the hemoglobin level was less than 8 g/dL.

Table 3. Remission status in the course of treatment (98 patients)

	After conventional treatment *TBI/MP*	*After mobilization therapy* *TBI/MP*	*After PBPCT* *TBI/MP*
CR	7/4	12/4	24/16
PR	37/35	36/40	24/33
MR	6/11	2/6	0/1
p=	0.5287	0.0560	0.1527

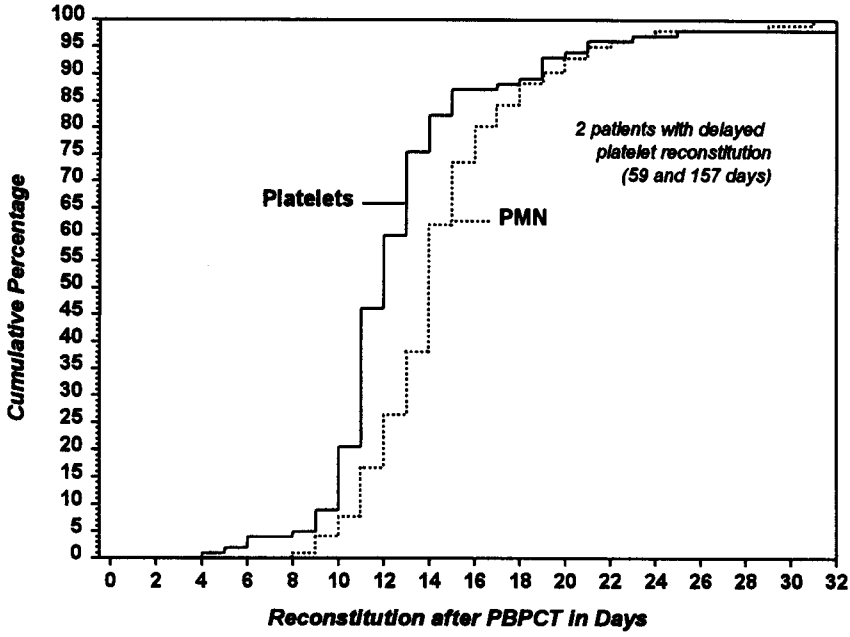

Figure 1. *Reconstitution of neutrophils and platelets after PBPCT of 100 patients with multiple myeloma.*

Statistical analysis

The clinical and laboratory data from the patients were evaluated according to standard statistical methods using a commercially available computer program (Statistica for Windows, Stat Soft, Tulsa, OK).

RESULTS

PBPC collection and autografting with G-CSF-mobilized PBPC

PBPC were harvested with a median of 3 leukaphereses (range 1–10) resulting in 3.3×10^6 $CD34^+$ cells/kg BW (median; range 1.5×10^6–58.3×10^6 $CD34^+$ cells/kg BW). Fifty patients received TBI followed by 140 mg/m^2 melphalan while 50 patients were treated with 200 mg/m^2 melphalan alone.

Autografts contained between 1.5×10^6 and 29.0×10^6 $CD34^+$ cells/kg BW (median 3.3) and between 4.0 and 181.4×10^4 CFU-C/kg BW (median 15.45). Only one patient was autografted with less than 2.0×10^6 $CD34^+$ cells/kg BW. A neutrophil count of 0.5×10^9/L was reached after a median of 14 days with a range from 8 to 31 days. Platelets recovered rapidly reaching an increasing count of more

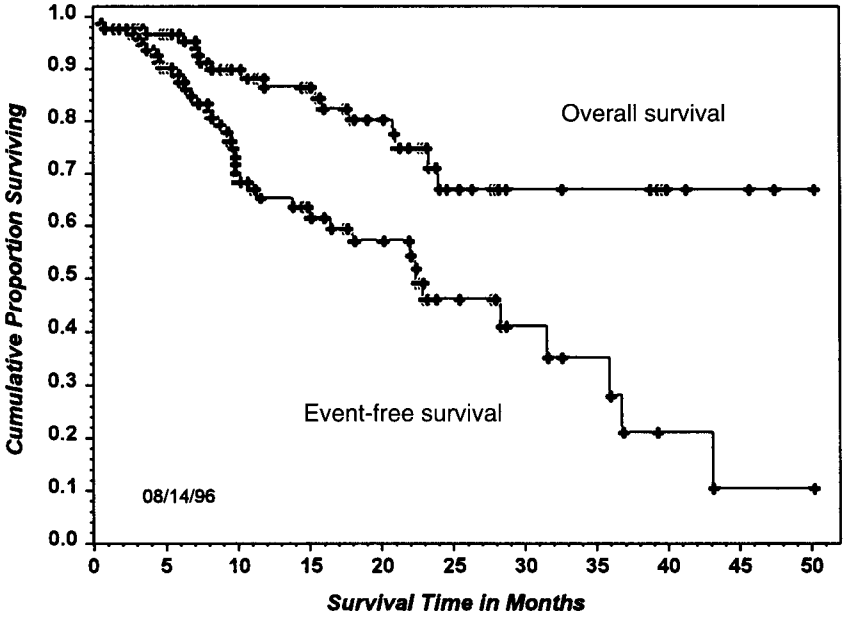

Figure 2. *Overall and event-free survival after PBPCT of 100 patients with multiple myeloma.*

than 20×10^9/L between 4 and 157 days (median 11) after transplantation without platelet transfusion. Hematological reconstitution is shown as cumulative frequency in Figure 1. Nonhematological toxicity is shown in Table 2.

Two transplantation-related deaths were observed in the TBI group (1 patient with severe mucositis and sepsis with multi-organ failure at day 23, a second patient with cardiac failure 13 days post-transplantation).

Follow-up post-transplantation

Following sequential high-dose therapy, the remission state improved in 43 of 98 patients (24 patients from the TBI group and 19 patients from the melphalan group, Table 3). Fifty-eight patients are alive in remission after a median follow-up time of 16.0 months (range 3-51), while 40 patients relapsed between 2 and 43 months after transplantation. Ten patients died of relapsed disease, 4 patients of infectious complications after disease progression, one patient of CMV infection and one of pneumonia following allogeneic transplantation.

The EFS is 50% 22 months post-transplantation and the OS is 78% after 36 months (Figure 2). A plateau in EFS is not reached after PBPCT. In our patient

group, the main factors predicting relapse were elevated beta-2 microglobulin, stage III according to the Salmon-Durie classification, hypercalcemia and IgA subtype.

DISCUSSION

In patients with multiple myeloma, high-dose therapies have been shown to improve remission rates compared with conventional treatment. The final analysis of the French multicenter trial IFM 90 showed a significantly longer EFS and OS following high-dose treatment.[5] Similar results were presented last year by Barlogie et al. at the ASH meeting[10]. The data from the Royal Marsden group[11] show that high-dose melphalan is the most active agent in the treatment of MM. Data concerning the role of TBI in the high-dose concept are only available for a small patient group. In a retrospective analysis of 85 patients, the remission state in chemosensitive patients was improved with TBI plus HD-melphalan compared to HD-melphalan alone.[12] In refractory patients, the toxicity of TBI-containing treatment is up to 20%.[13] No randomized study comparing TBI plus chemotherapy with HD-melphalan has yet been carried out.[14]

Our results from 100 patients show no advantage of the TBI-containing high-dose therapy in reaching PR or CR over the treatment with HD-melphalan without TBI. On the other hand, treatment-related toxicity was significantly increased in the TBI group, including the two toxic deaths documented. Prospective randomized studies are required to address the question of the optimal high-dose regimen before transplantation of autologous PBPC in patients with multiple myeloma.

ACKNOWLEDGMENTS

The authors thank the nursing staff of the Bone Marrow Transplantation Unit for their outstanding care of these patients; Margit Pförsich, Kirsten Flentje, Evi Holdermann and Magdalena Volk for their excellent technical assistance. We are indebted to Prof. Meuer (Institute for Immunology and Serology at the University of Heidelberg) and his colleagues for providing the packed erythrocyte and HLA-matched platelet transfusions. We gratefully acknowledge the proofreading of the manuscript by Dr. David Maclachlan, MRCGP.

REFERENCES

1. Alexanian R, Dimopoulos M: The treatment of multiple myeloma. *N Engl J Med* 330:484–489, 1994.
2. Samson D: Principles of chemotherapy and radiotherapy. In: Gahrton M, Durie BGM (eds) Multiple Myeloma. London-Sydney-Auckland: Arnold, 1996, pp 108–129.
3. Gregory WM, Richards MA, Malpas JS: Combination therapy versus melphalan and prednisolone in the treatment of multiple myeloma: An overview of published trials. *J Clin*

Oncol 10:334–342, 1992.

4. McElwain TJ, Powles RL: High dose intravenous melphalan for plasma cell leukemia and myeloma. *Lancet* 2:822–824, 1983.
5. Attal M, Harousseau JL, Stoppa et al.: High dose therapy in multiple myeloma: Final analysis of a prospective randomized study of the "Intergroup Francais Du Myelome" (IFM 90). *New Engl J Med* 335, 91–97, 1996.
6. Durie B, Salmon S: A clinical staging system for multiple myeloma. *Cancer* 36:842–852, 1975.
7. Attal M: A randomized study testing the effect of bone marrow/peripheral blood stem cell transplantation or conventional chemotherapy, with or without alpha interferon for aggressive myeloma. EORTC Leukemia Cooperative Group, EBMT Myeloma Study Group, French Myeloma Study Groups (GEM-POF), 1993.
8. Murea S, Goldschmidt H, Hahn U et al.: Successful collection and transplantation of peripheral blood stem cells in cancer patients using large-volume leukapheresis. *J Clin Apher,* in press.
9. Hohaus S, Goldschmidt H, Ehrhardt R et al.: Successful autografting following myeloablative conditioning therapy with blood stem cells mobilized by chemotherapy plus rhG-CSF. *Exp Hematol* 21: 508–514, 1993.
10. Barlogie B, Crowley J, Jagannath S et al.: Superior outcome after early autotransplantation (AT) with "Total Therapy" (TT) compared to standard SWOG treatment (ST) for multiple myeloma (MM). *Blood* 86: 207a, [abstr], 1995.
11. Cunningham D, Paz-Ares L, Milan S et al.: High-dose melphalan and autologous bone marrow transplantation as consolidation in previously untreated myeloma. *J Clin Oncol* 12: 759–763, 1994.
12. Jagannath S, Barlogie B: Autologous bone marrow transplantation for multiple myeloma. *Hematol Oncol Clin North Am* 6:451–462, 1992.
13. Vesole DH, Barlogie B, Jagannath S et al.: High-dose therapy for refractory multiple myeloma: Improved prognosis with better supportive care and double transplants. *Blood* 84:950–956, 1994.
14. Mohrbacher A, Anderson KC: Bone marrow transplantation in multiple myeloma. In: Malpas JS, Bergsagel DE, Kyle RA (eds) Myeloma. Biology and Management. Oxford: Oxford University Press, 1995, pp 322–352.

CHAPTER 6
Breast Cancer

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

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The treatment of Stage II-B (>9+ nodes), III-A and III-B breast cancer with conventional-dose chemotherapy, surgery, and irradiation has resulted in improved relapse-free survival (RFS) 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 noncross-resistant combinations of chemotherapy, and the use of combinations of chemotherapy and P170 glycoprotein-blocking drugs to overcome drug resistance. A four-year 74% RFS has been reported for Stage II-B (>9+ nodes) 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 noncross-resistant combination of drugs following treatment of Stage II-B (>9+ nodes), 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 cis-platinum combination (McCFUD). In patients who had inflammatory breast cancer, hyperthermia was administered in combination with radiotherapy. The noncross-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 (>9+ nodes), III-A or III-B breast cancer were entered on study between January of 1986 and January

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%)	

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; they then started on McCFUD chemotherapy. Three courses of McCFUD were administered with a 28-day treatment cycle. If patients had T_4 breast lesions or a diagnosis of inflammatory breast cancer, they received hyperthermia in combination with irradiation. Patient characteristics and tumor prognostic factors are shown in Table 1. The chemotherapy doses and schedules are outlined in Tables 2 and 3.

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 × 6

Table 3. Advanced primary breast cancer: 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 hour, 6 hours after methotrexate	X				
cis-platinum 60 mg/M^2 IV		X			
leucovorin 15 mg p.o. q.6h (8 doses)		X	X		
Cytoxan 300 mg/M^2 C.I. × 3 days			X	X	X

Cycle repeated every 28 days × 3

RESULTS

After a median follow-up period of 81 months, Kaplan-Meier projection of RFS is 62% at 7 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-A group superior to the results in the III-B and II-B subsets. The patients who were ER-positive appeared to continue to relapse later than the ER-negative patients. When patients were ER/PR-positive, they had received concomitant hormone therapy with chemotherapy and irradiation. This usually consisted of tamoxifen for a period of 5 years; occasionally Lupron or oophorectomy was used in combination with tamoxifen in the premenopausal patients. The combined RFS curve for the three stages are shown in Figure 1. Figure 2 shows the projected overall combined survival for the three subsets. Table 4 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.

DISCUSSION

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

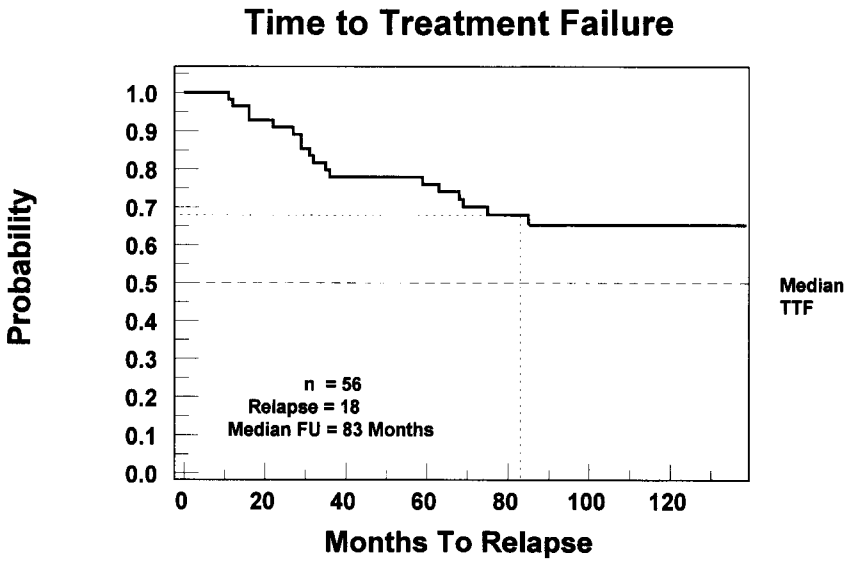

Figure 1. *LABC time to treatment failure.*

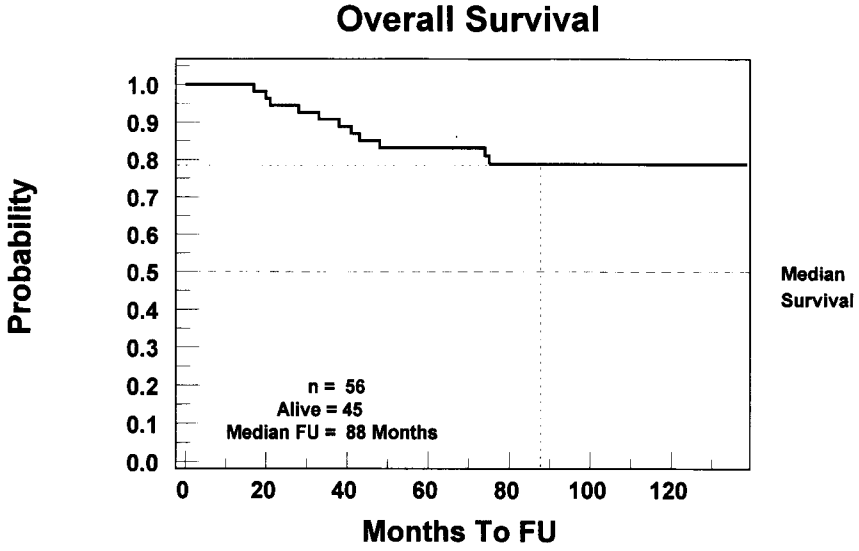

Figure 2. *LABC overall survival.*

The program described appears to have very reasonable efficacy. It can be administered in the outpatient setting and with the advent of granulocyte colony-stimulating factor (G-CSF), can be done with very little risk of infection. Because

Table 4. Relapse

number of patients:		18
sites and frequency of recurrence:		
	brain	4 (29%)
	bone	8 (43%)
	liver	3 (14%)
	lung	1 (7%)
	chest wall	1 (7%)
	meningeal	1 (7%)

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 RFS 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 routine follow-up of patients with MRI of the brain.

A 76% overall survival of this patient population at 7 years, suggests relapses can be successfully treated and that marrow function remains sufficiently intact to pursue aggressive systemic chemotherapy.

REFERENCES

1. Buzdar AU, Smith TL, Blumenschein GR et al.: Breast cancer adjuvant therapy trials of M.D. Anderson Hospital: Results of two studies. In: Jones SE and Salmon SE (eds). Adjuvant Therapy of Cancer IV. Orlando: Grune & Stratton, Inc., 1984, pp 217–225.
2. Peters WP, Ross M, Vredenburgh JJ et al.: High-dose chemotherapy and autologous bone marrow support as consolidation after standard-dose adjuvant therapy for high-risk primary breast cancer. *J Clin Oncol* 11:1132–1143, 1993.
3. Blumenschein GR, DiStefano A, Gomez-Yeyille JE: Response to initial combination chemotherapy as a predictor of response to CMF cisplatinum (McCFUD) combination therapy for metastatic breast cancer. *Proc Am Assoc Cancer Res* 28:199 [abstr #789], 1987.
4. Blumenschein GR, DiStefano A, Firstenberg BA et al.: Response of measurable metastatic breast cancer to combination cyclophosphamide, doxorubicin, VP16 (CaVe). *Proc Am Assoc Cancer Res* 29:196 [abstr #780], 1988.

HIGH-DOSE CHEMOTHERAPY IN BREAST CANCER IN EUROPE IN 1996: SURVEY CONDUCTED BY THE EUROPEAN GROUP FOR BLOOD AND MARROW TRANSPLANTATION (EBMT)

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ABSTRACT

The number of patients treated with high-dose chemotherapy (HDCT) and hematopoietic support has been expanding all over Europe. In 1994, 845 breast cancer patients received HDCT, whereas in 1995 the figure was 1354 (9 allo including two twins transplants). At least thirteen major adjuvant studies are running through the continent, eleven of them being phase III randomized trials. The most common conventional arm is FEC (with doses of Epidoxorubicin ranging from 75 mg/sqm to 120 mg/sqm), but two Institutions (Milan and the Anglo-Celtic cooperative trial) prefer to adopt the sequence anthracycline/CMF. The minimum number of positive lymph nodes for a patient to be included in a trial varies from ≥4 (Milan) to ≥10 (Germany). The preferred HDCT regimen is STAMP V (with modifications in the Carboplatin dose). Two recent European reports have shown that in phase II trials, there is an event-free survival (EFS) at 5 years of 56% (Milan ≥10 nodes ASCO '95) or at 4 years of 70% (Spain ≥10 nodes ASCO '96). In 1999 the first results will be available for the ongoing trials. For metastatic disease there are half a dozen studies planned or ongoing, three being randomized. On behalf of the EBMT we set up a survey among 70 European centers treating breast cancer patients. The main results were: PBPCT is preferred by >90%; mobilization is obtained by means of chemotherapy and growth factors by 92%, growth factors alone 3%. Collection time is chosen by means of: $CD34^+$ cells alone (59%), WBC and $CD34^+$ (9%), WBC (18%) and fixed dates (11%). The number of $CD34^+$ cells for a single autograft: from <3 to $>8\times10^6$/Kg. Despite the expansion of HDCT in Europe, some aspects are not extensively studied at present: the role of contamination of tumor cells and cost-benefit analyses. It must be remembered that in many European countries there are no insurance policies as in the United States, leading, for the moment, to an easier and simpler accrual of patients, even for metastatic disease.

Table 1. Major ongoing adjuvant trials (randomized) in Europe for high-risk breast cancer

Group arm	*No. nodes arm*	*Standard*	*High-dose pts 08/96*	*Support*	*Accrual*
France/EBMT	≥8	FEC	CAM	PBPC	120
Milan NCI	≥4	EPI→CMF	HDS	PBPC	270
Netherlands	≥4	FEC	FEC + STAMP V	PBPC	380
Netherlands	apex +ve	FEC	FEC + STAMP V	PBPC	95 (closed)
United Kingdom	≥6	FEC	FEC + STAMP V	PBPC	70
Anglo-Celtic	≥4	ADM→CMF	ADM→STAMP V	PBPC	145
Scandinavian	≥4* or > 6	FEC	FEC + STAMP V	PBPC	100
Hamburg	≥10	EC→CMF	EC +CTM	PBPC	70
Tübingen	≥10	FEC→CMF	VIP-E+ ICE	PBPC	new
Düsseldorf	≥10	FEC→CMF	EC→ECT	PBPC	new

CAM: cyclophosphamide, L-PAM, mitoxantrone. HDS: high-dose sequential chemotherapy. STAMP V: carboplatin, cyclophosphamide, thiotepa. CTM: cyclophosphamide, thiotepa, mitoxantrone. VIP: etoposide, ifosfamide, carboplatin. ECT: epirubicin, cyclophosphamide, thiotepa.
**depending on biological features (S-phase, etc.).*

OVERVIEW

The use of high-dose chemotherapy (HDCT) is expanding in Europe for adult as well as pediatric solid tumors. The main efforts are directed towards two cancer types: breast cancer and neuroblastoma. In the EBMT Registry, as of March 1996, 1075 and 1070 cases were registered, respectively. Not all groups devoted to HDCT in solid tumors report their cases to the Registry (nearly 40%). An EBMT survey conducted by Professor Gratwohl from Basel in 1996 indicates that in 1993, 400 patients with breast carcinoma in any stage were transplanted; in 1994 the figure was 845 and in 1995, the figure was 1354 with a yearly increase of 100% and 60%, respectively.[1]

The reason for this expansion may be found in some good phase II study results from the United States and Europe, mostly in the adjuvant setting.[2,3] Moreover, some enthusiasm is found among physicians who had not been involved in the field of HDCT in solid tumors until recently. The policy of autotransplantation in Europe is much less restricted compared to the North American policy (i.e., less insurance coverage difficulties, if any; an easier method of trials approval and so on). At present at least forty trials are ongoing or planned in Europe, the vast majority in the adjuvant setting; of the thirteen major adjuvant trials ten are randomized. In Table 1 the main characteristics together with patients' accrual are shown. A difference in terms of number of positive axillary nodes between Europe and the United States considered for

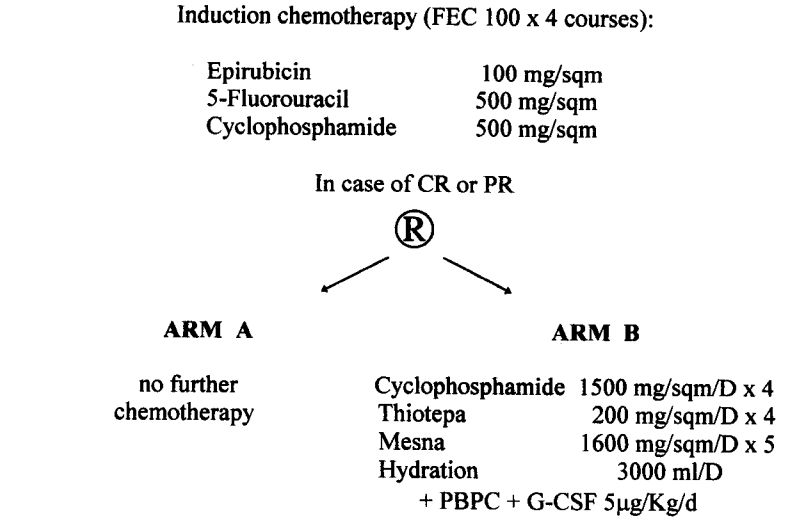

Figure 1. *Trial Pegase 03*

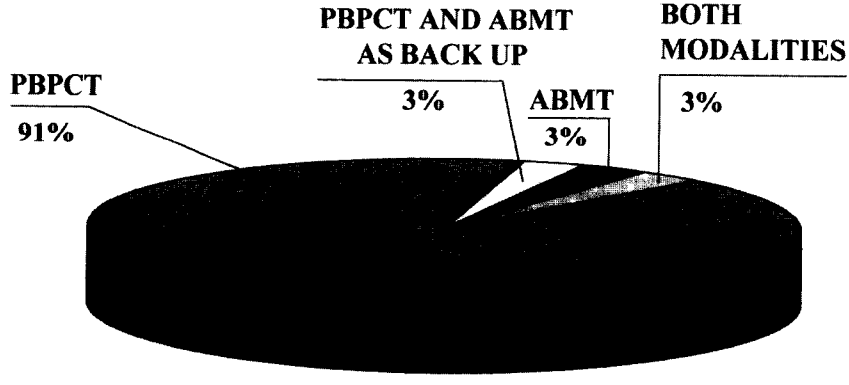

Figure 2. *EBMT Questionnaire Project: Type of rescue employed*

trials' entry varies considerably: except for the three German studies that include cases with >9 nodes, generally a lower number is required. The final results of the studies listed in Table 1 will be ready a minimum of three years from now, due to the long required follow-up for adjuvant breast cancer trials[4].

The use of ABMT as hematopoietic rescue is completely abandoned and today PBPCT is the only modality of hematopoietic rescue. It must be

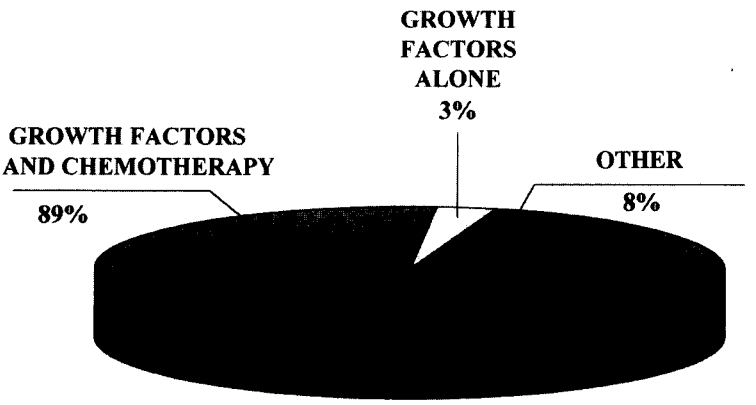

Figure 3. *EBMT Questionnaire Project: Methods of mobilization*

remembered that in Europe the policy of combination of ABMT + PBPCT was rarely used even in early times of progenitor cell development. The situation for metastatic disease is quite different. Only four randomized trials are planned or ongoing (one in Spain, and three on a wider European basis: two on behalf of the French Society for Bone Marrow Transplantation together with the EBMT and one from Ireland and Great Britain). In the French/EBMT trial Pegase 03, patients with metastatic disease receive 4 courses of 5-Fluorouracil, Epidoxorubicin and Cyclophosphamide (FEC) and at the time of restaging in case of PR or CR, they receive intensification with a single dose of Cyclophosphamide 6.4 gr/sqm and Thiotepa 800 mg/sqm and adequate hydration and mesna for uroprotection or no further chemotherapy. Randomization occurs up-front at the time of registration in this study (Figure 1).

In the trial Pegase 04, patients are referred only if they achieve PR or CR after any induction treatment and are subsequently randomized to receive CAM (Cyclophosphamide 120 mg/kg, Melphalan 140 mg/sqm and Mitoxantrone 45 mg/sqm) or to continue with free standard-dose regimens(s).

A new European proposal will be available by October, 1996 (Chairmen Dr. John Crown and Prof. Lothar Kanz).

In this study, patients with metastatic disease will be randomized up-front between Adriamycin and Docetaxel for four courses plus Cyclophosphamide, Methotrexate and 5-Fluorouracil -CMF- for eight courses versus Adriamycin and Docetaxel every two weeks followed by Cyclophosphamide 3 gr/sqm with PBPC mobilizing intent, and then high doses of Carboplatin, Ifosfamide and Etoposide plus PBPCT, and finally the last segment with high-dose Cyclophosphamide and Thiotepa + PBPCT.

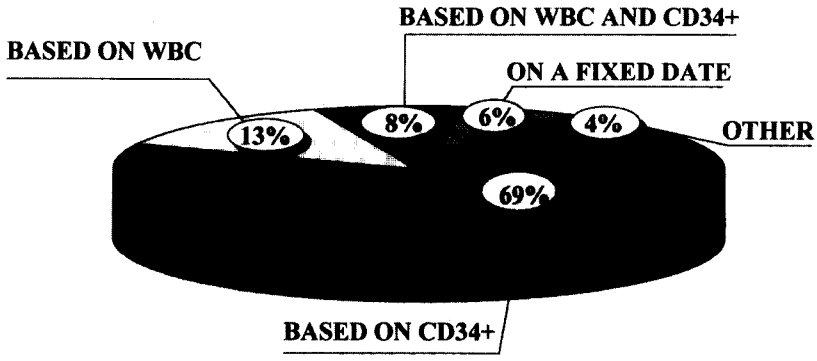

Figure 4. *EBMT Questionnaire Project: Timing of apheresis*

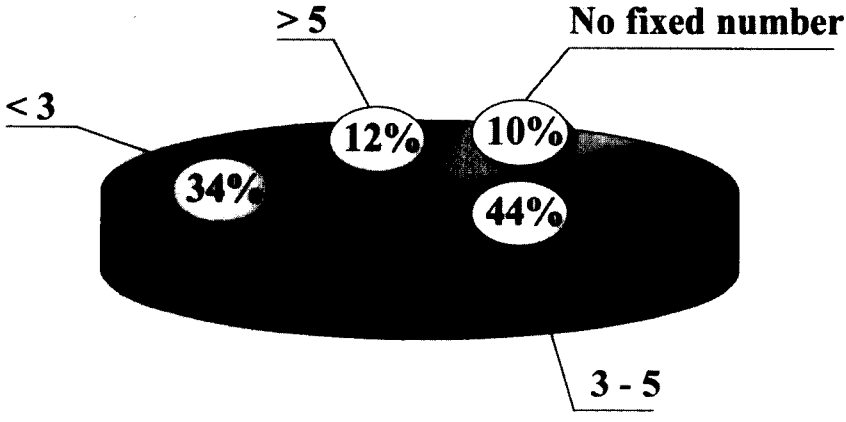

Figure 5. *EBMT Questionnaire Project: Minimum amount of $CD34^+$ cells $\times 10^6$/Kg for autotransplantation for solid tumors and for breast cancer in particular*

THE EBMT REGISTRY

The EBMT Solid Tumors Registry was set up in 1984 to obtain information and data about European activity in the field of solid tumors. Up to March 1996, 4536 patients had been registered (1075 with breast carcinoma). From the Registry files some points deserve consideration:

1) 44% of the patients with inflammatory breast carcinoma are alive and disease-free at 3.5 years; 2) 20% of the patients with metastatic disease grafted in complete remission are disease-free at a mean follow-up of nearly three years; and 3) 62% of the patients are alive and disease-free in the adjuvant setting (mean

follow-up 27 months). These last data await confirmation by the final analysis foreseen for October, 1996, after a full reanalysis and clearance of the files.

THE QUESTIONNAIRE PROJECT

In November 1995 a survey was conducted and sponsored by the EBMT Solid Tumors Working Party. The aim of the study was to know what was going on in Europe in the field of HDCT for solid tumors. One hundred and thirty-one out of 172 centers answered the questionnaire.

For the purpose of this report, the centers dealing with adult patients only will be reported, these centers treat nearly 100% of the cases of breast cancer (70/131).

The first question we addressed was the type of hematopoietic rescue employed. The results indicated that 91% of the institutions prefer to use peripheral blood progenitor cells alone, leaving only 3% to ABMT and 3% to both modalities and to marrow backup.

Eighty-eight percent of the interviewed centers have harvesting facilities in their own institution. Those centers answering that they use PBPC were requested to state their choice of mobilization. Figure 3 shows a clear preference for the combination of hematopoietic growth factors and chemotherapy is reported compared with other modalities. Nearly 30% of the breast cancer patients are treated in clinical protocols and this figure is superior if compared with standard chemotherapy; however, if we consider that HDCT is an experimental treatment, most centers should probably enter their patients in clinical protocols.

Figure 4 shows the different policies of peripheral progenitors collection. In Europe, $CD34^+$ cells determination is considered sufficient for harvesting by the majority of the centers (69%), while others prefer to utilize WBC alone (13%), WBC and $CD34^+$ cells (8%), fixed date (6%) or other unspecified methods. Another point we took into consideration was the minimum amount of $CD34^+$ cells required for autotransplantation for solid tumors and breast cancer in particular. The results are shown in Figure 5. A minority still require a high number of precursors (i.e., $>5\times10^6$/kg including some leading centers like the National Cancer in Milan), while 44% normally consider sufficient a quantity of $CD34^+$ cells between 3 and 5×10^6/kg. Interestingly, one-third of the participants in this survey have a lower threshold of <3 (generally between 2 and 3 $CD34^+$ cells $\times$ 10^6/kg).

A requirement for a lower minimum number of precursors may reduce the number of aphereses leading to a reduction in costs for the entire procedure.

FINAL CONSIDERATIONS

HDCT in Europe is expanding year after year for breast cancer patients despite the fact that no trial has been completed so far in the adjuvant setting and only a relatively small phase III study was published in metastatic disease[5]

(which, in any case was in favor of the high-dose arm).

By the year 2000 the final results of our efforts will become available, showing if "more" is better. But which "more" and which "less"? Different schedules are used for the intensification arms than in the adjuvant and in the advanced settings as reported above. Also, standard arms might have a different impact on survival (i.e., are 5 courses of FEC equal to or worse than 8 courses; or is Adriamycin + Docetaxel combination better the FEC × four courses). Moreover, surprisingly in Europe the role of contamination in the rescue product is not studied as it is overseas.[6] Some efforts in this area should be made in the near future.

Another point to be kept in mind: if the randomized trials ongoing in Europe will confirm the extremely good results of the pilot studies in the adjuvant setting, how shall we manage to offer the best treatment to thousands of women? Every single effort has to be made in Europe to reduce the costs (which are considerably lower than in North America) in order to be ready to offer this treatment to all women who need it.

REFERENCES

1. Gratwohl A, Hermans J, Baldomero H: Hematopoietic precursor cell transplants in Europe: Activity in 1994. Report from the European Group for Blood and Marrow Transplantation. *Bone Marrow Transplant* 17:137–148,1996.
2. Gianni AM, Siena S, Bregni M et al.: Five-year results of high-dose sequential (HDS) adjuvant chemotherapy in breast cancer with ≥10 positive nodes. *Proc Am Soc Clin Oncol* 14 [abstr 61], 1995.
3. Peters WP, Berry D, Vredenburgh JJ et al.: Five year follow-up of high-dose combination alkylating agents with ABMT as consolidation after standard-dose CAF for primary breast cancer involving ≥10 axillary lymph nodes (Duke/CALGB 8782). P*roc Am Soc Clin Oncol* 14 [abstr 933], 1995.
4. Bonadonna G, Zambetti M, Valagussa P et al.: Sequential of alternating doxorubicin and CMF regimen in breast cancer with more than three positive nodes. Ten years results. *JAMA* 273:542–547, 1995.
5. Bezwoda WR, Seymour L, Dansey RD: High-dose chemotherapy with hematopoietic rescue as primary treatment for metastatic breast cancer: A randomized trial. *J Clin Oncol* 13:2483–2489, 1995.
6. Fields KK, Elfenbein GJ, Trudeau AD et al.: Clinical significance of bone marrow metastases as detected using the polymerase chain reaction in patients with breast cancer undergoing high-dose chemotherapy and autologous bone marrow transplantation. *J Clin Oncol* 14:1868–1876, 1996.

HIGH-DOSE CYCLOPHOSPHAMIDE (CTX), MITOXANTRONE (MXT) AND PACLITAXEL (TAXOL®, TXL) FOR THE TREATMENT OF METASTATIC BREAST CANCER WITH AUTOLOGOUS BLOOD CELL (ABC) SUPPORT

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ABSTRACT

A phase I/II study is being performed using CTX 6 g/m^2, MXT 70 mg/m^2, in combination with TXL at a starting dose of 250 mg/m^2 IV over 3 hours in dose escalation for the purpose of determining dose-limiting toxicity (DLT), maximum tolerated dose (MTD), and efficacy of this drug combination in a transplantation setting. Patients with advanced breast cancer and chemosensitive disease are eligible. Up to the 3rd level (TXL 350 mg/m^2), few patients have experienced grade II and III toxicity other than hematotoxicity. One patient, who had pericardial involvement, received adjuvant radio/chemotherapy with epirubicin (EPI) and had pre-existing cardiac problems, died 5 weeks after transplantation. No other toxic or early deaths occurred on study. The autologous blood cell (ABC) reinfusion and subsequent recovery occurred in the outpatient setting. Recovery for ANC ≥0.5/nL was 10–16 (median 12) days. Recovery for platelets ≥20/nL was 18–20 (median 12) days. Fifteen patients developed neutropenic fever that required IV antibiotics and readmission. Transfusion frequency for PRBC was 0–5 (median 2) units and for platelets, 1-5 (median 1) encounters. At the 4th dose level (TXL 400 mg/m^2), 6 patients were treated; three of them experienced DLT; accrual is therefore continuing on an extended infusion schedule delivering the 400 mg/m^2 TXL over 6 rather than 3 hours. One out of six patients had a minor reaction only; the dose escalation is ongoing until DLT is reached. So far, 49 patients were enrolled in this study: 10 were taken off study because of progressive disease during induction chemotherapy (IDC), or withdrawal of consent, and one for cardiotoxicity from IDC; 38 have completed the whole protocol: four are too early to evaluate; 11 complete responses; two stable disease and 19 partial remissions were

documented. Two patients progressed within 4 weeks after high-dose chemotherapy (HDCT). The median duration of progression-free survival (PFS) is 13.5 months; the median duration of overall survival (OS) has not been reached yet. This study will continue until DLT is determined. Pharmacokinetic analysis of TXL is also performed. Phase II portion of this study will continue at MTD level in order to evaluate efficacy and further toxicity.

INTRODUCTION

Breast cancer is the most common malignancy in women, contributing to around 25% of malignant tumors and 20% of cancer death in female patients[1] in Canada. Once breast cancer becomes metastatic, the disease becomes essentially incurable with a median survival of 15 to 19 months at the time of indication for chemotherapy.[2–5] The overall response rates for single drug regimens are between 20–70%. Only a few patients (under 20%) can achieve complete remissions. In spite of chemotherapy, the majority of patients (80%) have signs of disease progression within 2 years.[2–6] Most of these patients have died within 3 years from documentation of metastasis and there is no report in the literature concerning long-term survival in this subgroup of patients. One way of improving chemotherapy efficacy is the dose effect relationship when analyzing numerous studies in breast cancer.[7,8] The evaluation of these data suggest that dose response effect exists within the range of conventional chemotherapy. Further dose intensification using high-dose chemotherapy (HDCT) leads to even more responses and remissions. It has been shown by ourselves and others that HDCT followed by infusion of autologous blood cells (ABC) mobilized by various growth factors can lead to almost 90% response rates with around 50% complete responses.[9–20] It has been shown that similar to lymphoma, chemosensitive tumors tend to have a better overall response rate than chemotherapy resistant tumors. Recently, a new group of chemotherapeutic agents, the taxanes, have been introduced for treatment of malignant diseases.[21–25] Previous in vitro and later clinical phase I/II studies confirmed the activity of one of these agents, paclitaxel (Taxol, TXL), as an active agent in breast cancer. In fact, paclitaxel seems to be one of the most promising and most active agents in the treatment of breast cancer. Our group has previously shown that the combination of several drugs active in breast cancer can lead to additive and, in some instances, to synergistic effects.[26,27] The data clearly show that the combination of TXL and MXT in vitro on malignant breast cancer cell lines can show a synergistic effect; and TXL in combination with CTX, an additive effect. Although the extrapolation of in vitro data on cell lines cannot be applied on clinical trials, it is a reasonable rationale for using this drug combination.[26,27] Therefore, we conducted a phase I study, using high-dose CTX (6 g/m^2) and MXT (70 mg/m^2) with dose escalation of TXL.

Table 1. The total number of sites of metastasis is listed. Since 31 patients had more than one site involved, the total number of sites exceeds the total number of 49 patients enrolled into our study.

LN	11
bone	27
lung	19
liver	17
chest wall	5
breast	4
>2 Sites	16
2 Sites	15
1 Site	17

PATIENTS AND METHODS

Female patients age 18–55 years with metastatic breast cancer were eligible to enter this phase I study. Major end points of the study were defining the dose-limiting toxicity (DLT) and maximum tolerated dose (MTD), identifying side effects and toxicities. Minor end points included response rates after induction chemotherapy (IDC) and after HDCT, ability to mobilize and collect sufficient numbers of ABC and time to hematologic recovery. Moreover, progression-free survival (PFS) and overall survival (OS) were recorded. No previous chemotherapy for metastatic disease was allowed. Adjuvant chemotherapy, if any, had to be at least 6 months prior to metastatic disease. Patients were excluded if they had exposure to taxanes. Performance status according to Karnofsky was 60% or more, and cardiopulmonary and hematopoietic functions were normal. Between January, 1995, and September, 1996, 49 patients were enrolled into the study. Thirty-eight patients are evaluable for toxicity; 34 patients for response. The median age was 40 years (29–55 yrs). Before developing metastatic disease, 14 patients were diagnosed with stage I; 18 patients with stage II; and 6 patients with stage III breast cancer; 11 patients were initially stage IV (metastatic disease). The time to progressive disease ranged between 0 (stage IV) and 96 months, median time 20 months. Twenty-nine patients were premenopausal and 23 hormone receptor negative. Table 1 summarizes the sites of metastasis as well as the number of sites involved in our patient population.

One third of our patients had three or more sites involved; 19 had lung metastasis and 17 liver metastasis. When patients entered the study, met all eligibility criteria and signed the informed consent form, they received the initial IDC consisting of 5-fluorouracil (5-FU) 750 mg/m^2 body surface area (BSA), epirubicin (EPI) 100 mg/m^2 BSA, CTX 750 mg/m^2 BSA, all delivered on day 1 as IV infusion. Starting on day 2, 10 μg/kg B.W. subcutaneously was administered

Table 2. Hematologic support and use of IV antibiotics during the pancytopenic phase after HDCT and BC reinfusion. The episodes of IV antibiotics, red blood cell transfusions, and the number of platelet transfusions in the 5 different cohorts are given as the median and range.

Dose	*Cohort*	*Pts*	*IV antibiotics (Pts)*	*PRBC (units)*	*Platelets*
250	1	7	5	2, 0–4	1, 1–4
300	2	5	1	2, 0–3	2, 1–3
350	3	10	4	1, 0–2	2, 1–5
400	4	6	2	2, 1–5	2, 1–4
400/6h	4a	6	3	2, 1–4	2, 1–2
total		34	15	2, 0–5	2, 1–5

daily for approximately 2 weeks until the total WBC reached 2.5/nL. On this day, standard apheresis procedure was performed using a double lumen catheter connected to a Baxter CS3000+. The blood flow on average was 70 mL/min to a blood volume of 10 L. Our target was to collect at least 5×10^8 mononucleated cells per kg/B.W. and 2×10^6 $CD34^+$ cells/kg B.W.[28] After this first cycle of IDC, the patients obtained a further 2–3 cycles of chemotherapy consisting of 5-FU 600 mg/m^2 BSA, EPI 60 mg/m^2 BSA, CTX 600 mg/m^2 BSA all administered IV. The treatment was delivered every three weeks if ANC was ≥1.5/nL and platelets ≥100/nL. After this IDC all these patients were restaged with a physical examination, imaging studies and blood work. Only patients who did not progress on IDC and did not experience severe toxicity and/or organ damage proceeded to HDCT. HDCT consisted of CTX 6 g/m^2 IV delivered on 3 consecutive days; MXT 70 mg/m^2 IV delivered on the same 3 consecutive days. On day 4, the paclitaxel dose starting at 250 mg/m^2 was delivered on a 3 hour infusion. The usual premedication including antiemetics and dexamethasone was delivered on each day. After 24–48 hours of rest, the ABC were reinfused and rhG-CSF was administered at the

Table 3. Summarizes the severity and frequency of mucositis in all 5 cohorts tested. The numbers represent the highest grade in each cohort according to WHO toxicity evaluation and the number of patients who actually experience the highest grade in the according cohort.

	Cohort	*Highest grade*	*# of pts. in highest grade*
mucositis			
	1	2	3
	2	3	1
	3	3	3
	4	2	4
	4a	3	1

Table 4. In the upper part of Table 4, the response rates after IDC just prior to HDCT and ABC are summarized for each cohort and as a total for the group. In the lower part, the response rates after HDCT and ABC are listed for each cohort and as a total for the group. With a median observation time of 10 months, the median PFS according to the Kaplan-Meier estimate is 13.5 months; the OS has not been reached.

Cohort	*PD*	*SD*	*PR*	*CR*
1	0	0	6	1
2	0	0	3	2
3	0	1	8	1
4	0	1	5	0
4a	0	2	4	0
total	0	4	26	4

Cohort	*PD*	*SD*	*PR*	*CR*
1	1	0	5	1
2	0	0	1	4
3	0	1	6	3
4	1	0	5	0
4a	0	1	2	3
total	2	2	19	11

PD: progressive disease; SD: stable disease; PR: partial remission; CR: complete remission.

dose of 5 μg/kg B.W. daily until hematologic recovery (ANC ≥1.5/nL for 3 consecutive days). The ABC was cryopreserved in 10% DMSO as previously described.[29] The four days of chemotherapy were delivered in the hospital. The patients were usually discharged on the day after HDCT just prior to the reinfusion of the autograft.[30,31]

RESULTS

Recovery for ANC ≥ 0.5 /nL was 10–16 (median 12) days. Recovery for platelets ≥20/nL was 18–20 (median 12) days. Table 2 summarizes patients and doses per cohort with the use of IV antibiotics and transfusions of red blood cells and platelets. When reaching the cohort no. 4, three of six patients experienced approximately 20–40 minutes after completion of infusion, diaphoresis, bradycardia, mild hypertension and diarrhea; two of these three patients lost consciousness for a few minutes. It was therefore decided that the infusion of TXL was increased to 6 hours (cohort 4a). This complication was not observed in the next 6 patients. Other toxicities like nausea and vomiting, gastrointestinal, genitourinary, cardiac and neurologic toxicities were only mild and infrequently observed. Very few patients experienced bone pain, fatigue, peripheral edema,

redness at site of G-CSF injection, insomnia and occasional reaction to transfusion. One patient who had pericardial involvement with metastatic disease, and who had adjuvant radiotherapy to the left chest as well as adjuvant chemotherapy with a total dose of 720 mg/m^2 EPI, died 5 weeks after transplantation with clinical signs of cardiac failure. No other early or toxic deaths occurred on study.

DISCUSSION

We have dose escalated the breast cancer-active drug, paclitaxel, to a dose of 400 mg/m^2 in combination with high-dose CTX and MXT followed by ABC transplantation. In this phase I dose escalation study we did not yet reach the DLT and therefore accrual to this protocol is continuing. This protocol was designed to be delivered on an outpatient basis. Therefore, a longer infusion of any of these drugs was not planned. Although toxicities described in the Results section are tolerable, minor and not life threatening, some important observations have been made: infusion of TXL more than 350 mg/m^2 over 3 hours are not possible. No toxic deaths directly associated to the treatment protocol were observed. Nevertheless, due to the very short infusion time, doses over 350 mg/m^2 in this setting are not possible. This may be due to the TXL itself but alternatively, to its dilution vehicle cremaphor EL. Also, the high content of ethyl alcohol in the solution may contribute to the reactions described above. The prolonging of the infusion time from 3 to 6 hours would not jeopardize an outpatient protocol and yet abolish entirely the side effects observed at the 400 mg/m^2 dose level during the 3-hour infusion. Although this study was designed as a phase I study, the response rates observed so far both after IDC and after HDCT are extremely high and comparable to other HDCT regimens. Since the highest dose level of taxol, which is one of the most active drugs in breast cancer, has not been reached yet, it is anticipated that the response rates after further dose escalation might be even higher. The North American Autologous Blood and Marrow Transplantation Registry has described that differences between different protocols are statistically not different when using multivariate analysis. At this point, the response rates lead to a median PFS of more than 1 year. Compared with other studies, the usual PFS using standard dose chemotherapy lies between 5–8 months only. After median observation time of 10 months and maximum observation time of 20 months, the median survival time has not yet been reached. Since DLT has not yet been reached and therefore the MTD is not identified, this study continues accruing patients on higher dose levels. A pharmacokinetic study is being performed simultaneously. The results at this point are not yet available. Any possible differences regarding survival are of a speculative nature and need to be addressed in well designed prospective randomized phase III studies.

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REFERENCES

1. Canadian Cancer Statistics 1995: Produced by National Cancer Institute of Canada; Statistics Canada; Provincial Cancer Registries; Health Canada. January 1995; ISSN 0835-2976.
2. Clavel M, Catimel G: Breast cancer: Chemotherapy in the treatment of advanced disease. *Eur J Cancer* 29A:598–604, 1993.
3. Sledge GW, Antman KH: Progress in chemotherapy for metastatic breast cancer. *Sem Oncology* 19:317–332, 1992.
4. Ross MB, Buzdar AU, Smith TL, Eckles N, Hortobagyi GN, Blumenschein GR et al.: Improved survival of patients with metastatic breast cancer receiving combination chemotherapy, comparison of consecutive series of patients in 1950s, 1960s, and 1970s. *Cancer* 55:341–346, 1985.
5. Falkson G, Gelman RS, Leone L, Falkson CI: Survival of premenopausal women with metastatic breast cancer. Long-term follow-up of Eastern Cooperative Group and cancer and leukemia group B studies. *Cancer* 66:1621–1629, 1990.
6. Cancer in Ontario 1994-1995: The Ontario Cancer Treatment and Research Foundation. Published by D. Blackstone and K. Kwiatkowska, OCTRF, Toronto.
7. Antman K, Corringham R, Devries E, Elfenbein G, Gianni AM, Gisselbrecht C et al.: Dose intensive therapy in breast cancer. *Bone Marrow Transplant* 10:67–73, 1992.
8. Osborne CK: Dose-intensity as a therapeutic strategy in breast cancer. *Breast Cancer Research and Treatment* 20:11–14, 1991.
9. Kessinger A, Armitage JO: The use of peripheral stem cell support of high-dose chemotherapy. In: DeVita VT, Hellman S, Rosenberg SA (eds) Important Advances in Oncology 1993. Philadelphia: J. B. Lippincott Company, 1993, pp 167-175.
10. Glück S, Ho AD: High dose chemotherapy (HDCT) with autologous blood cell (BC) support in metastatic breast cancer (MBC). *Eur J of Cancer* 32A(2):35:PP-5-3, 1996.
11. Antman K, Ayash L, Elias A, Wheeler C, Hunt M, Eder JP et al.: A phase II study of high-dose cyclophosphamide, thiotepa, and carboplatin with autologous marrow support in women with measurable advanced breast cancer responding to standard-dose therapy. *J Clin Oncology* 10:102–110, 1992.
12. Ayash LJ, Elias A, Wheeler C, Reich E, Schwartz G, Mazanet R et al.: Double dose-inten-

sive chemotherapy with autologous marrow and peripheral-blood progenitor-cell support for metastatic breast cancer: A feasibility study. *J Clin Oncology* 12:37–44, 1994.

13. Ghalie R, Richman CM, Adler SS, Cobleigh MA, Korenblit AD, Manson SD et al.: Treatment of metastatic breast cancer with a split-course high-dose chemotherapy regimen and autologous bone marrow transplantation. *J Clin Oncology* 12:342–346, 1994.
14. Peters WP, Ross M, Vredenburgh JJ, Meisenberg B, Marks LB, Winer E et al.: High-dose chemotherapy and autologous bone marrow support as consolidation after standard-dose adjuvant therapy for high-risk primary breast cancer. *J Clin Oncology* 11:1132–1143, 1993.
15. Somlo GS, Doroshow JH, Forman SJ, Leong LA, Margolin KA, Morgan RJ et al.: High-dose cisplatin, etoposide, and cyclophosphamide with autologous stem cell reinfusion in patients with responsive metastatic or high-risk primary breast cancer. *Cancer* 73:125–134, 1994.
16. Dunphy FR, Spitzer G, Buzdar AU, Hortobagyi GN, Horwitz LJ, Yau JC et al.: Treatment of estrogen receptor-negative or hormonally refractory breast cancer with double high-dose chemotherapy intensification and bone marrow support. *J Clin Oncology* 8:1207–1216, 1990.
17. Shpall EJ, Jones RB, Bearman SI, Franklin WA, Archer PG, Curiel T et al.: Transplantation of enriched CD34-positive autologous marrow into breast cancer patients following high-dose chemotherapy: Influence of CD34-positive peripheral-blood progenitors and growth factors on engraftment. *J Clin Oncology* 12:28–36, 1994.
18. Kennedy MJ, Beveridge RA, Rowley SD, Gordon GB, Abeloff MD, Davidson NE: High-dose chemotherapy with reinfusion of purged autologous bone marrow following dose-intense induction as initial therapy for metastatic breast cancer. *J Nat Cancer Inst* 83:920–926, 1991.
19. Williams SF, Gilewski T, Mick R, Bitran JD: High-dose consolidation therapy with autologous stem-cell rescue in stage IV breast cancer: Follow-up report. *J Clin Oncology* 10:1743–1747, 1992.
20. Mulder NH, Mulder POM, Sleijfer D Th, Willemse PHB, van der Ploeg E, Dolsma WV, de Vries EGE: Induction chemotherapy and intensification with autologous bone marrow reinfusion in patients with locally advanced and disseminated breast cancer. *Eur J Cancer* 29A:668–671, 1993.
21. Holmes A, Walters RS, Theriault RL, Forman AD, Newton LK, Raber MN et al.: Phase II trial of Taxol, an active drug in the treatment of metastatic breast cancer. *J Nat Cancer Inst* 83:1797–1805, 1991.
22. Taxol. *Lancet* 339:1447–1448, 1992.
23. Bissett D, Kaye SB: Taxol and taxotere-current status and future prospects. *Eur J Cancer* 29A(9):1228–1231, 1993.
24. Rowinsky EK, Onetto N, Canetta RM, Arbuck S: Taxol: The first of the taxanes, an important new class of antitumor agents. *Sem in Oncology* 19(6):646–662, 1992.
25. Seidman AD, Norton L, Reichman BS et al.: Preliminary experience with paclitaxel (TAXOL®) plus recombinant human granulocyte colony-stimulating factor in the treatment of breast cancer. *Sem in Oncology* 20(4):40–45, 1993.
26. Glück S, Sharan N, Chadderton T, Dietz G, Bewick M, Köster W et al.: In vitro combi-

nation of paclitaxel (Taxol®, TXL) with 5 different cytotoxic drugs: Effect on three different cell lines. *Proc of AACR* 35:335 (#1992), 1994.

27. Glück S, Köster W, Sharan N, Wilson C, Chadderton T, Dietz G et al.: Combination of paclitaxel (Taxol®, TXL) with different cytotoxic drugs: Effect on malignant cell lines in vitro. *Can J of Infectious Disease* (6):397C:#3041, 1995.
28. Ho AD, Glück S, Germond C, Sinoff C, Dietz G, Maruyama M et al.: Optimal timing for collections of blood progenitor cells following induction chemotherapy and granulocyte-macrophage colony-stimulating factor for autologous transplantation in advanced breast cancer. *Leukemia* 7:1738–1746, 1993.
29. Glück S, Porter K, Chadderton T, Dietz G, Germond C: Depletion of DMSO (dimethylsulfoxide) prior to autografting after high-dose chemotherapy for metastatic breast cancer. *Blood* 82:430a:#1705, 1993.
30. Glück S, Gagnon A: Neutropenic fever in patients after high-dose chemotherapy followed by autologous transplantation and GM-CSF. *Bone Marrow Transplant* 14:989–990, 1994.
31. Glück S, DesRochers S, Cano C, Dorreen M, Germond C, Gill K, Lopez P, Sinoff C: High-dose chemotherapy followed by autologous blood cell transplantation: A safe and effective outpatient approach. Submitted for publication September 1996.

THE FEASIBILITY OF THREE COURSES OF "TINY CTC" (tCTC) IN PATIENTS WITH ADVANCED BREAST CANCER

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ABSTRACT

We have recently reported that three subsequent courses of cyclophosphamide (6 g/m^2), thiotepa (480 mg/m^2) and carboplatin (CTC) (1600 mg/m^2), may lead to severe organ toxicity [*J Clin Oncol 1996, 14:1473–1483*], including hemorrhagic cystitis, veno-occlusive disease (VOD) and a late hemolytic uremic syndrome. A sufficient number of peripheral blood progenitor cells (PBPCs) could be harvested from nearly all patients to be divided over three transplantations. Although there was a suggestion of delayed bone marrow (BM) recovery after the third course, organ toxicity proved to be the (cumulative) dose-limiting toxicity of the alkylating agents in the regimen, rather than myelosuppression.

We have administered 45 courses of "tiny CTC" (tCTC) (containing two thirds of each of the three agents of CTC) to 17 patients with advanced breast cancer: 17 first, 15 second and 13 third courses. tCTC was given every 4–5 weeks and was followed by autologous PBPC-transplantation and granulocyte colony-stimulating factor (G-CSF) (300 μg) until neutrophil recovery. Selected patients were discharged from the hospital on the day after PBPC transplantation. The toxicity was generally mild, but one second course was followed by (nonlethal) VOD of the liver. No other severe end-organ toxicity was encountered. Bone marrow recovery was rapid after all courses. Three courses of tCTC are feasible in advanced breast cancer but the ability of this sequence to induce long-term survival remains to be studied.

INTRODUCTION

High-dose therapy is frequently used in breast cancer, but this is the subject of considerable controversy.[1,2] Based on retrospective analyses of bone marrow (BM) transplant registries, it has been suggested that high-dose therapy may be beneficial in a small subgroup of advanced breast cancer patients: those who have achieved a complete remission (or a "very good partial remission") with standard-dose

chemotherapy. Of these, approximately one-fourth will survive 3 years or more without relapse after transplantation.[3–6] It is not clear whether comparable long-term survival rates could be achieved with conventional therapy in similarly highly selected patients. Even if high-dose therapy would eventually be shown to be more effective than standard-dose therapy in this situation, its efficacy is far from satisfactory: if one assumes that 20% of patients with advanced disease can achieve a complete remission with standard therapy, and 25% of these can be effectively consolidated with high-dose therapy, then only 5% of patients would experience long-term survival.

One strategy to improve on these figures is to further increase the dose of chemotherapy delivered. The advent of peripheral blood progenitor cell transplantation has allowed much faster recovery from BM depression than after BM transplantation, and multiple transplants are now under investigation in a number of centers. Multiple transplantation procedures, however, may lead to cumulative dosages of cytotoxic agents that are much higher than in previous experience. Most of the agents used in high-dose therapy are associated with specific forms of extramedullary toxicity that may include potentially lethal organ toxicities such as interstitial pneumonitis or veno-occlusive disease (VOD) of the liver (for a recent review see reference 7). We have recently reported that three subsequent courses of CTC may lead to severe organ toxicity,[8] including hemorrhagic cystitis, VOD and a late hemolytic uremic syndrome. A sufficient number of peripheral blood progenitor cells (PBPCs) to be divided over three transplantations could be harvested from nearly all patients. Although there was a suggestion of delayed BM recovery after the third course, organ toxicity proved to be the (cumulative) dose-limiting toxicity of the alkylating agents, rather than myelosuppression.

To be able to safely administer three high-dose courses of the same agents with stem cell support, we have lowered the dose of CTC to two-thirds of its original level ("tiny CTC"). Here we report the feasibility of this approach in the first nineteen patients.

PATIENTS, MATERIALS AND METHODS

Patient selection

All patients had biopsy-proven stage IV or recurrent breast cancer, were less than 56 years of age and had excellent performance status (WHO 0 or 1). Previous chemotherapy was not allowed, unless it had been limited to nonanthracycline-based adjuvant therapy that had been discontinued at least one year before relapse. Only patients with estrogen receptor-negative tumors were eligible, unless they had failed at least one line of hormonal therapy. Bone metastases were acceptable, but there could be no signs of BM failure and both plain roentgenograms and radioisotope bone scans were required to be negative

for bilateral pelvic lesions. Written informed consent was obtained from all patients, and the study was approved by the Committee on Medical Ethics of the Netherlands Cancer Institute.

Treatment plan

Therapy was started with two courses of $FE_{120}C$ (fluorouracil 500 mg/m^2, epirubicin 120 mg/m^2 and cyclophosphamide 500 mg/m^2, all given as intravenous push on day 0 and repeated on day 21). This is a relatively intensive anthracyclin-based regimen that we previously reported to be highly efficacious in locally advanced breast cancer.[9] The second of these $FE_{120}C$ courses was used to mobilize stem cells (see below). In patients with at least a minimal response to chemotherapy, the first high-dose therapy course (tCTC) was begun three weeks later, to be followed by the second and third after 4–5 weeks each. After recovery from the last high-dose therapy course, resection or irradiation of residual disease was attempted and, when possible, prior sites of disease were irradiated.

Delays and dose adaptations of the chemotherapy courses were executed as described previously for full-dose CTC.[8] Evaluable patients who did not respond to a $FE_{120}C$ or tCTC course were taken off study.

Peripheral blood progenitor cell (PBPC) mobilization and harvest

For stem cell mobilization, the $FE_{120}C$ chemotherapy regimen was used, which consists of fluorouracil (500 mg/m^2), epirubicin (120 mg/m^2) and cyclophosphamide (500 mg/m^2), all given as IV push on day 1. Filgrastim was started on day 2. Leukocytaphereses began when the WBC count exceeded 3.0×10^9/L and the CD34$^+$ cell count in the peripheral blood was at least 0.5%. To facilitate apheresis, all patients had 13.5 French double-lumen Hickman catheters. A continuous flow blood cell separator was employed (Fenwal CS 3000, Baxter Deutschland GmbH, Germany). Both the number of CD34$^+$ cells and the number of granulocyte-macrophage colony-forming units (GM-CFU) were determined in the cell collections. All methods employed in the stem cell harvests have been described previously.[10]

Based on earlier findings,[10] we considered a graft size of 3.0×10^6 CD34$^+$ cells/kg body weight sufficient for sustained BM recovery, and 1.0×10^6 CD34$^+$ cells/kg body weight sufficient for rapid (but possibly transient) granulocyte recovery after high-dose therapy. Using these criteria, a simple algorithm could be used to determine the distribution of the available CD34$^+$ cells over the three transplants (Table 1).

High-dose chemotherapy regimen: "Tiny CTC"

The high-dose chemotherapy regimen tCTC was administered as published previously,[11] but with three major modifications: 1) the doses of all three agents were lowered to two-thirds of their original levels; 2) the period between the last

Table 1. Distribution of $CD34^+$ cells harvested over three transplants

$CD34^+$ cells harvested	*Action*
$<3.0\times10^6$/kg	Patient must be taken off study.
	Bone marrow harvest before first transplant.
$>3.0\times10^6$/kg	Divide harvested cells into 3 equal portions, 1
$<5.0\times10^6$/kg	for each transplant. Reinfuse bone marrow together with stem
	cells after third course.
	Reserve 3.0×10^6 $CD34^+$ cells for third
$>5.0\times10^6$/kg	transplant. Divide remainder of cells into
$<9.0\times10^6$/kg	equal halves for first and second transplants, respectively.
$>9.0\times10^6$/kg	Reserve 3.0×10^6 $CD34^+$ cells for each of the
$<11.0\times10^6$/kg	transplants and add the remaining cells to the
	reinfusion after the third course.
$>11.0\times10^6$/kg	Reserve 5×10^6/kg for third course, divide
$<15.0\times10^6$/kg	remaining cells into two equal halves for first and
	second transplants.
$>15.0\times10^6$/kg	Divide into three equal portions for each transplant.

infusion and the transplantation was reduced by one day and 3) the carboplatin dose was corrected for renal function employing a modified Calvert formula.[12] We have previously shown that such a modified formula adequately predicts the carboplatin area under curve (AUC) in this patient group.[13,14]

Briefly, carboplatin was administered IV as daily two-hour infusions on days –6, –5, –4 and –3. The total dose of carboplatin was 1170 mg/m^2 in patients with normal renal function, but in those with creatinine clearances of 110 mL/min or less, it was determined by the formula:

DOSE (mg) = 13.3 × (Creatinine Clearance + 25)

Cyclophosphamide (total dose 4000 mg/m^2) was divided over 4 daily one-hour infusions and thiotepa (total dose 320 mg/m^2) was divided over 8 twice-daily one-hour infusions. Both agents were given on days –6, –5, –4 and –3. Mesnum (500 mg total dose) was given 6 times daily for a total of 36 doses, beginning one hour before the first cyclophosphamide infusion. All infusions were administered through double-lumen Hickman catheters inserted in a subclavian vein. The peripheral blood progenitor cells were reinfused on day 0.

Antiemetics were employed both prophylactically and as needed and usually included dexamethasone and granisetron. All patients received prophylactic antibiotics, including ciprofloxacin and itraconazole orally. In addition, all patients received acyclovir, in an oral dose of 400 mg twice daily. To prevent

Table 2. Peripheral blood progenitor cell harvests

	Median	*Range*
mononuclear cells (10^6/kg)	657	315–1661
$CD34^+$ cells (10^6/kg)	15	9.8–36.2
CFU-GM (10^4/kg)	176	101–357
number of leukaphereses	3	2–4

Data from 18 patients, prior to cryopreservation. The leukapheresis product of the 19th patient was subjected to positive selection for $CD34^+$ cells, yielding non-comparable results.

gram-positive infections, roxitromycin was given orally from day 0 and was discontinued when the neutrophil count exceeded 0.5×10^9 cells/L. Irradiated platelet transfusions were administered to maintain platelet counts of at least 10×10^9/L and leukocyte-free irradiated red blood cells were given to maintain hemoglobin levels at or above 5.5 mmol/L. Whenever possible, patients were discharged from the hospital on the day following PBPC reinfusion, but returned daily to the clinic for check-ups.

Granulocyte colony-stimulating factor (G-CSF) (filgrastim, received as a gift from Amgen-Roche, Breda, The Netherlands) was administered as a daily s.c. injection of 300 μg, regardless of body weight, from day 1 until the WBC count exceeded 5.0×10^9/L.

RESULTS

By August, 1996, 19 patients had completed protocol treatment. Of these, two had been taken off study, when evaluation after two courses of $FE_{120}C$ revealed stable disease. Seventeen patients received at least one course of tCTC. Of these, 2 patients did not proceed to a second course of tCTC: one because of lack of response to the first tCTC course and one because of biochemical signs of liver toxicity that took many weeks to clear. Fifteen patients received a second course of tCTC. Two of these discontinued protocol treatment before the third course was given: one patient did not respond to the second course and a second patient developed reversible VOD of the liver. As a result, thirteen patients (72%) are currently evaluable for toxicity and BM reconstitution after three courses of tCTC. Only two patients (11%) discontinued high-dose therapy because of toxicity, in both cases because of reversible liver toxicity.

Progenitor cell harvests and hematopoietic reconstitution

Sufficient PBPCs could be harvested in all patients, and no more than 2–4 leukocytapheresis sessions were required in any of them. The cell yields are given in Table 2.

Table 3. Bone marrow reconstitution and transfusion requirements after first, second and third courses of CTC

	tCTC course number		
	I	*II*	*III*
day of neutrophil recovery[1]	10 (9–13)[2]	10 (9–15)	10 (9–13)
days to platelet transfusion independence[3]	13 (10–27)	16 (10–47)	15 (9–21)
number of platelet transfusions[4]	4 (1–15)	4 (2–37)	3 (2–12)
number of blood transfusions[5]	6 (2–10)	7 (4–42)	8 (5–10)

[1]*First day after transplantation (=day 0) with at least 500 neutrophils/mm^3.*
[2]*Median (range).*
[3]*First day after transplantation on which platelets > 20×10^9/l without platelet transfusions.*
[4]*Five to six donor units per transfusion.*
[5]*Units.*

BM reconstitution was rapid in all patients (Table 3), except in the single patient who developed VOD of the liver after her second course. There were no significant difference in rate of engraftment or in transfusion requirements after the first or subsequent courses in the other patients.

Treatment delays and dose reductions

In the first 10 patients, the second and third tCTC courses were planned to be administered 5 weeks after the previous ones. This was successful in all of the eight second courses that were actually administered, and in 6 of 7 third courses. One third course had to be postponed for one week because of persistent anorexia and weakness. In the last seven patients, we attempted to begin the tCTC courses every 4 weeks, hoping to increase the dose intensity and to shorten the duration of treatment. This was possible in 4 of 7 second courses. Two patients had to be delayed for one week, in both cases because of fevers of undetermined origin that had not cleared in time. One of these patients also had a pulmonary infiltrate. Three of six third courses could be given in time, but the other three had to be delayed for either one week (one patient because of fever of undetermined origin, one because of slowly resolving weakness and nausea) or for 2 weeks (slowly resolving weakness and nausea).

All second courses were given at full dose, but in five third courses, dose reductions were applied. Two patients experienced hypersensitivity reactions to the third course, characterized by fever, rash and facial edema. In one patient, carboplatin was discontinued after 50% of the dose had been given; the second received only the first day of the 4-day chemotherapy course. In three further

Table 4. Toxicity comparison of full-dose CTC and tCTC

	No. of courses	*Toxic deaths*	*Major nonlethal toxicity*
CTC-1	35	0	exhaustion syndrome (1)
CTC-2	28	VOD[1] (1)	hemorrhagic cystitis (4),VOD (1)
CTC-3	10	VOD (1)	HUS[2] (1)
		sepsis (1)	VOD (1)
		HUS (1)	
tCTC-1	17	0	none
tCTC-2	15	0	VOD and renal failure[3] (1)
tCTC-3	13	0	none

[1]*VOD, veno-occlusive disease.*
[2]*HUS, hemolytic uremic syndrome.*
[3]*Same patient, renal failure possibly due to mild HUS.*

patients, the carboplatin dose was reduced according to protocol in the third course, because grade 4 diarrhea had occurred after the second tCTC course.

DISCUSSION

Multiple courses of high-dose alkylating therapy have become feasible with PBPC transplantation, and this type of therapy is no longer limited by BM toxicity but rather by end-organ toxicity. We have shown previously that three subsequent courses of full-dose CTC are frequently associated with severe and sometimes lethal toxicity.[8] This lower-dose regimen, designated "tiny CTC", appears to be tolerable and no fatal toxicities were noted in the 45 courses administered to 17 patients in this report. There was one patient with VOD of the liver that developed after the second course. The patient recovered but developed renal failure, possibly a mild form of the hemolytic uremic syndrome, four months after the last transplantation. The syndrome was characterized by easily manageable hypertension and a glomerular filtration rate decrease to 25 mL/min. It is possible that pharmacokinetic monitoring during the first cycle of tCTC may help to identify patients at risk for this type of organ damage.

Although the dose of tCTC is only 33% lower than that of full-dose CTC, the decrease in subjective and objective toxicity is striking (Table 4). Most patients were able to leave the hospital on the day following PBPC reinfusion and there was markedly less nausea and vomiting. Despite this, weight loss was considerable over the three months of high-dose therapy and averaged 10% of pre-treatment weight. The improved subjective tolerance and treatment in the outpatient setting allowed a further reduction in time between courses, and a 4-week interval was feasible in

over half of the second and third. The main reasons for delay were slow recovery of performance status because of fevers of undetermined significance or because of gastrointestinal toxicity. Further experience with the regimen may eventually lead to more effective prevention of these common problems.

On the basis of these preliminary results, we believe that triple tCTC with PBPC transplantation is feasible in most patients under 60 years of age with advanced breast cancer, who have excellent performance status and organ function. Its efficacy in achieving long-term disease-free survival cannot be judged at this point in time because of insufficient follow-up. We are currently expanding our experience by continuing the phase II study.

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REFERENCES

1. Peters WP: High-dose chemotherapy with autologous bone marrow transplantation for the treatment of breast cancer: Yes. In: DeVita VT Jr, Hellman S, Rosenberg SA (eds) Important Advances In Oncology. Philadelphia: Lippincott Company, 1995, pp 215–230.
2. Smith GA, Henderson IC: High-dose chemotherapy with autologous bone marrow transplantation (ABMT) for the treatment of breast cancer: The jury is still out. In: DeVita VT Jr, Hellmannn S, Rosenberg SA (eds) Important Advances In Oncology. Philadelphia: Lippincott Company, 1995, pp 201–214.
3. Ayash LJ: High dose chemotherapy with autologous stem cell support for the treatment of metastatic breast cancer. *Cancer* 4:532–535, 1994.
4. Ayash LJ, Wheeler C, Fairclough D, Schwartz G, Reich E, Warren D, Schnipper L, Antman K, Frei III E, Elias A: Prognostic factors for prolonged progression-free survival with high-dose chemotherapy with autologous stem-cell support for advanced breast cancer. *J Clin Oncol* 13:2043–2049, 1995.
5. Livingston RB: Dose intensity and high-dose therapy. Two different concepts. *Cancer* 74:1177–1183, 1994.
6. De Vries EGE, Rodenhuis S, Schouten HC, Hupperets PSGJ, Dolsma WV, Lebesque JV, Blijham GH, Bontenbal M, Mulder NH: Phase II study of intensive chemotherapy with autologous bone marrow transplantation in patients in complete remission of disseminated breast cancer. *Breast Cancer Res Treatm* 1996, in press.
7. Van der Wall, Beijnen JH, Rodenhuis S: High-dose chemotherapy regimens for solid tumors. A review. *Cancer Treatm Rev* 21:105–132, 1995.
8. Rodenhuis S, Westermann A, Holtkamp MJ, Nooijen WJ, Baars JW, van der Wall E, Slaper-Cortenbach ICM, Schornagel JH: Feasibility of multiple courses of high-dose cyclophosphamide, thiotepa and carboplatin for breast cancer or germ cell cancer. *J Clin Oncol* 14:1473–1483, 1996.
9. Van der Wall E, Rutgers EJT, Holtkamp MJ, Baars JW, Schornagel, Peterse JL, Beijnen JH, Rodenhuis S. Efficacy of up-front FEC-chemotherapy with an increased dose of

epirubicin in high risk breast cancer. *Brit J Cancer* 73:1080–1085, 1996.

10. Van der Wall E, Richel DJ, Holtkamp MJ, Slaper-Cortenbach ICM, Dalesio O, Nooijen WJ, Schornagel JH, Rodenhuis S: Bone marrow reconstitution after high-dose chemotherapy and autologous peripheral stem cell transplantation: Correlation with graft size. *Ann Oncol* 5:795–802, 1994.
11. Rodenhuis S, Baars J, Schornagel JH, Vlasveld LT, Mandjes I, Pinedo HM, Richel DJ: Feasibility and toxicity study of a high-dose chemotherapy regimen incorporating carboplatin, cyclophosphamide and thiotepa. *Ann Oncol* 3:855–860, 1992.
12. Calvert AH, Newell DR, Gumbrell LA, O'Reilly S, Burnell M, Boxall FE, Siddik ZH, Judson IR, Gore ME, Wiltshaw E: Carboplatin dosage: Prospective evaluation of a simple formula based on renal function. *J Clin Oncol* 7:1748–1756, 1989.
13. Van Warmerdam LJC, Rodenhuis S, Van Tellingen O, Maes RAA, Beijnen JH: Validation of a limited sampling model for carboplatin in a high-dose chemotherapy combination. *Cancer Chemother Pharmacol* 35:179–181, 1994.
14. Van Warmerdam LJC, Rodenhuis S, Van der Wall E, Maes RAA, Beijnen JH: Pharmacokinetics and pharmacodynamics of carboplatin administered in a high dose combination regimen with thiotepa, cyclophosphamide and peripheral stem cell support. *Br J Cancer* 73:979–984, 1996.

TANDEM DOSE INTENSIVE THERAPY FOR METASTATIC BREAST CANCER: PRELIMINARY RESULTS FROM THE NORTH AMERICAN MARROW TRANSPLANT GROUP (NAMTG)

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INTRODUCTION

Advanced breast cancer is incurable with conventional chemotherapy, most likely because of the presence of cancer cells that exhibit resistance to chemotherapy. This inherent resistance may be overcome by escalating doses of chemotherapy. The dose-response relationship is evident for breast cancer cell lines in vitro and multiple dose-intensive chemotherapy trials have supported this concept in vivo.[1,5–7] We have previously reported a novel dose-intensive chemotherapy regimen consisting of etoposide (VP) and cyclophosphamide (CY).[2] This regimen is unique in that the dose-limiting toxicities are nonhematologic and therefore the maximally tolerated doses may be given without hematopoietic stem cell support. This regimen was initially used for patients with histologic evidence of bone marrow involvement. To patients for whom bone marrow transplant insurance coverage was denied, this regimen offered an alternative dose-intensive therapy. Encouraging clinical results with this regimen (66% complete and partial response) in patients with metastatic breast cancer, led us to pursue additional treatment with thiotepa (TT) and carboplatin (Cb) with hematopoietic stem cell support as a second chemotherapy regimen in an effort to improve response rates and duration of response. We report here the preliminary results of our tandem dose-intensive therapies for metastatic breast cancer using dose-intensive VP-CY followed by TT-Cb.

METHODS

Between July, 1991, and May, 1995, 36 patients with advanced breast cancer have enrolled in our tandem dose-intensive therapy trial after signing written

informed consent for these IRB approved protocols. The initial component of the trial consisted of etoposide 3600–4200 mg/m^2 (54–60 hour continuous infusion) and cyclophosphamide 200 mg/kg (50 mg/kg/d × 4d) as previously reported. This regimen was followed for a median (range) of 73 (41–173) days by thiotepa 900 mg/m^2 (300 mg/m^2/d × 3d), carboplatin 1600 mg/m^2 (4-day continuous infusion) and hematopoietic progenitor cell support.

The median (range) age of the patients was 45 (32–58) years. Twenty-one patients had two or more metastatic sites, 13 had one metastatic site and two patients were treated in remission (no evidence of disease). The most frequent sites of metastases were bone (64%), skin and/or lymph node (28%), lung (28%), liver (22%) and bone marrow (22%). One patient had CNS metastases. The median (range) number of prior chemotherapy regimens was 2 (1–5). At the onset of dose-intensive therapy, 19 patients had chemosensitive disease (17 responding relapse, 2 no evidence of disease), 12 patients had chemoresistant disease (11 resistant relapse, 1 primary refractory) and 5 had unknown responsiveness to chemotherapy (4 untested relapse, 1 "stable" disease). Toxicity was graded using the NCI marrow transplant toxicity criteria: grade 0 represented no toxicity, grade 1 represented mild toxicity, grade 2 represented moderate toxicity, grade 3 was severe and potentially life-threatening and grade 4 was fatal regimen-related toxicity (RRT). Recovery of marrow function was measured as the recovery of granulocytes to >500/μL and platelets to >20,000/μL untransfused. A complete response (CR) was defined as the complete disappearance of all measurable disease. Any response less than 50% was considered no response (NR). Patients with bone involvement who had normalization of laboratory parameters, clinical resolution of bone pain, but had stable bone scans with no new bone scan abnormalities were designated with an asterisk qualifier, i.e., CR*, PR*. Patients who died from RRT were excluded from the tumor response evaluation.

RESULTS

Toxicity data

All 36 patients completed the tandem therapy. The RRT data for the etoposide-cyclophosphamide component of the therapy were similar to our previous reports.[2–4] Specifically, 75% of the patients had moderate to severe RRT, the majority of this (80%) represented stomatitis requiring narcotic analgesics. Marrow function recovery was similar to our previous reports. The median (range) day for granulocyte and platelet recovery was day 14 (11–37) and 15 (7–39), respectively. Compared with our historic control patients treated with this regimen without the use of growth factors, hematologic recovery was unaffected by the administration of various combinations of growth factors (G-CSF, GM-CSF, or IL-3/GM-CSF). The median (range) length of stay was 27 (15–46) days. There were no regimen-

related toxic deaths attributable to the initial component of this trial.

The TT-Cb regimen was generally well tolerated. There were 2 RRT deaths (6%), one due to veno-occlusive disease of the liver and the other to sepsis. There was one death due to documented progressive disease. In the 36 patients treated, the most commonly observed toxicity was grade 2 stomatitis in 19 patients (52%). Grade 3 stomatitis was observed in 2 (6%) patients. Other observed, and reversible, toxicities included: grade 2–3 renal, hepatic and neurologic in 5 (14%) patients, grade 2-3 cardiovascular toxicity in 4 (11%) patients, grade 2-3 gastrointestinal toxicity in 3 (8%) patients, and grade 2–3 pulmonary toxicity in 1 (3%) patient. Hematologic recovery data after TT-Cb were available in 33 patients. Hematopoietic support consisted of the following: bone marrow alone (5 patients), peripheral blood progenitor cells (16 patients) or both (12 patients). Growth factor support after hematopoietic progenitor cell reinfusion consisted of G-CSF (21 patients), GM-CSF (7 patients) or both (5 patients). The median (range) day for granulocyte recovery to absolute neutrophil recovery (ANC) >500/μL was day +14 (11–29), +11 (9–20) and +13 (10–14), for patients rescued with bone marrow, peripheral blood progenitor cells or both, respectively. There was no difference in days to granulocyte recovery with respect to different growth factors. The median (range) day to platelet recovery was day +24 (21–25), +21 (8–56) and +23 (11–60), for patients rescued with bone marrow, peripheral blood progenitor cells or both, respectively. The median (range) length of stay was 27 (10–80) days.

Response data

The response rates to the etoposide-cyclophosphamide therapy were as follows: CR+ CR* in 7/36 patients (19%), PR in 18/36 (50%), and NR in 11/36 patients (31%). Response rates to VP-CY varied depending upon the chemosensitivity status at initial therapy. Of the 19 patients deemed chemosensitive upon induction therapy with VP-CY, there were 5 CRs (26%), 10 PRs (53%) and 4 NRs (21%). Of the 12 patients deemed chemoresistant upon induction therapy with VP-CY there were 0 CRs, 6 PRs (50%) and 6 NRs (50%). Of the 5 patients with unknown sensitivity to induction therapy there were 2 CRs (40%), 2 PRs (40%) and 1 NR (20%). The overall response rates to VP-CY were slightly higher in the group deemed chemosensitive prior to therapy than in the group deemed chemoresistant (79% versus 58%).

The response rates to the TT-Cb component of this trial were as follows: CR+ CR*+CCR (patient in CR from initial therapy) in 13/33 patients (39%), PR in 10/33 patients (30%) and NR in 10/33 patients (30%). Of the 19 patients deemed chemosensitive upon induction therapy, 11 improved their response rates with the second dose-intensive regimen, 4 of these patients were converted to CRs. Also, of the initial 12 patients with disease that was deemed chemoresistant, 6 had PRs to the induction regimen and 2 of these were converted to CRs with the TT-Cb

regimen. The 3 patients with unknown sensitivity to induction (2 PR, 1 NR) therapy all improved their response rates with the second dose-intensive therapy.

The median (range) duration of response and follow-up in this study is relatively brief (10 months [2–36+]) and further evaluation is required to determine efficacy of this approach to treating patients with advanced breast cancer.

DISCUSSION AND SUMMARY

Even with the advent of newer chemotherapy agents over the past 50 years, the median survival for metastatic breast cancer remains unchanged.[1] The principles of dose-intensive therapy, i.e., utilizing high-dose combination noncross-resistant chemotherapy with nonoverlapping nonhematologic end organ toxicities, have been applied to the treatment of advanced breast cancer with only moderate success. Multiple trials reported in the medical literature have shown higher response rates with dose-intensive therapy compared to conventional chemotherapy.[1,3–6] Unfortunately, the majority of these patients will relapse and succumb to complications of progressive disease. Certain prognostic factors associated with advanced breast cancer may portend a better outcome with dose-intensive therapy. Of 2336 patients with metastatic breast cancer, treated with dose-intensive therapy between 1989 and 1994, and reported to the Autologous Blood and Marrow Transplant Registry, the 3-year probability of survival was 28 ± 3%. Patients who achieved a complete remission to conventional induction chemotherapy have a significantly better survival.[8] Long-term survival may be achieved in a group of patients that have chemosensitive disease.

We utilized a tandem dose-intensive treatment strategy in an attempt to improve response rates and potentially, the duration of response, in patients with advanced metastatic breast cancer. This strategy allowed us the opportunity to utilize four active chemotherapeutic agents given at high doses, over a relatively short duration. Thirty-six patients enrolled in the trial. The RRT death rate (6%) was acceptable. The toxicity profile was moderately severe with stomatitis requiring parenteral narcotics being the most common toxicity (80%). The majority of patients had improvement of their response with the second dose-intensive therapy, and several patients that were deemed resistant at initial therapy were converted to a CR with sequential therapy. These responses have proven to be durable (>1 year). The response rates have proven to be acceptable (70%), and certainly patients with chemosensitive disease fared better with respect to response rates. Median follow-up of the entire cohort of patients is relatively short and further evaluation of this patient population to determine efficacy of this treatment strategy is ongoing.

REFERENCES

1. Vahdat L, Antman KH: Dose-intensive therapy in breast cancer. In: Armitage JO, Antman KH (eds) High-Dose Cancer Therapy: Pharmacology Hematopoietins, Stem Cells. Baltimore: Williams & Wilkins, 1995, pp 802–823.
2. Brown RA, Herzig RH, Wolff SN et al.: High-dose etoposide and cyclophosphamide without bone marrow transplantation for resistant hematologic malignancy. *Blood* 76:473–479, 1990.
3. Herzig RH, Lynch JP, Christiansen NP et al.: Dose-intensive chemotherapy with etoposide-cyclophosphamide without stem cell support for advanced breast cancer: Preliminary results. VII International Symposium for Autologous Bone Marrow Transplantation, Arlington, TX, 1994.
4. Herzig RH, Lynch JP, Christiansen NP et al.: Dose-intensive chemotherapy with etoposide-cyclophosphamide for advanced breast cancer. *Semin Oncol* 23:28–32, 1996.
5. Peters WP, Shpall EJ, Jones RB et al.: High dose combination alkylating agents with bone marrow support as initial treatment for metastatic breast cancer. *J Clin Oncol* 6:1368–1376, 1986.
6. Williams SF, Mick R, Desser R, et al.: High-dose consolidation chemotherapy with autologous stem cell rescue in stage IV breast cancer. *J Clin Oncol* 7:1824–1830, 1989.
7. Bezwoda WR, Seymour L, Dansey RD: High dose chemotherapy with hematopoietic rescue as primary treatment for metastatic breast cancer: A randomized trial. *J Clin Oncol* 13:2483–2489, 1995.
8. Horowitz M: 1995 IBMTR/ABMTR Summary slides: Current status of blood and marrow transplantation. PP4, 1995, July.

AUTOTRANSPLANTS FOR BREAST CANCER IN NORTH AMERICA

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The ABMTR-NA now has data on over 7000 patients receiving autotransplants for breast cancer since 1989. These transplants were performed by over 170 teams in North and South America with a small number of teams from other continents also contributing patients. Changes in patient characteristics and early mortality from 1989 to 1995 are as follows.

The changes over time documented in the table are all significant at $p<0.0001$. Three-year probabilities of survival (95% confidence interval) for stages II, III and inflammatory disease are 74 (68–80)%, 70 (63–77)% and 52 (40–64)%, respectively. Survival of patients with metastatic disease varies by response to chemotherapy prior to high dose therapy; 3-year probabilities of survival for patients in complete remission, partial remission and with non-responsive disease are 46 (40–52)%, 29 (25–33)% and 16 (12–20)%, respectively.

Results will soon be available from detailed ABMTR analyses of prognostic factors affecting outcomes of autotransplants for patients with metastatic or high-risk primary breast cancer.

Table 1.

	1989–90	*1991–2*	*1993–4*	*1995*
average number of patients transplanted per year	310	920	1400	1700
median age, years (range)	42 (23–66)	44 (22–72)	45 (24–66)	45 (22–71)
disease stage at transplant				
adjuvant/neoadjuvant	12%	30%	35%	49%
metastatic	88%	70%	64%	50%
other	<1%	<1%	1%	1%
graft type				
bone marrow	80%	52%	24%	10%
peripheral blood stem cell	14%	20%	49%	72%
BM + PBSC	6%	28%	27%	18%
100-day mortality	18%	8%	5%	5%

DETECTION OF BREAST CANCER CELLS IN THE APHERESIS PRODUCT: POTENTIAL CLINICAL SIGNIFICANCE

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ABSTRACT

In this retrospective study we assessed the clinical significance of detecting contaminating breast cancer cells in the peripheral blood stem cell apheresis product of patients undergoing high-dose chemotherapy followed by autologous blood cell transplantation. We analyzed 172 apheresis products from 17 patients who underwent these procedures between June, 1991, and February, 1993. All patients had metastatic breast cancer and were enrolled into a phase II clinical study. The mobilization induction chemotherapy consisted of 3 cycles of cyclophosphamide 750 mg/m^2 IV, epirubicin 100 mg/m^2 IV and 5-FU 750 mg/m^2 IV followed by 5 μg/kg BW of granulocyte-macrophage colony-stimulating factor (GM-CSF) daily until apheresis was completed. On average, 10 apheresis products were obtained from each patient. Samples from these apheresis products were immunostained with a cocktail of anti-cytokeratin antibodies using an immunoalkaline phosphatase staining procedure. A minimum of 10^6 cells were directly examined by light microscopy by at least 2 blinded observers. Cells were considered positive when immunostaining was observed in the plasma and on the cell membrane and cellular morphology was consistent with a malignant phenotype. One hundred seventy-two samples were analyzed; 13/57 (23%) samples were positive after first mobilization; 3/60 (5%) after second; and 4/55 (7%) after third mobilization. The contamination by breast cancer cells after first mobilization is significantly higher than after second and third mobilization (p=0.0052). After high-dose chemotherapy and reinfusion of the autograft, the median progression-free survival (PFS) for 9 patients with tumor-free apheresis products was 18.25 months for 8 patients with tumor cell positive apheresis products, PFS was 9.8 months. The median overall survival for patients with tumor cell contaminated apheresis products was 28.1 months; and 44.3 months for patients without tumor cell contamination. These differences did not reach statistical significance. Our study shows that apheresis products after second or third cycles of induction chemotherapy carry a significantly lower likelihood of tumor cell contamination.

INTRODUCTION

Autologous blood cell (ABC) transplantation is being increasingly used as a hematopoietic rescue for patients undergoing myeloablative high-dose chemotherapy (HDCT). It has been previously reported that ABC collections from patients with advanced stage breast cancer contain fewer tumor cells than do simultaneously collected bone marrow.[1] These data further indicate that tumor cells may not be present each day of apheresis and that additional courses of chemotherapy may be able to reduce the number of tumor cells present in bone marrow but also in peripheral blood.[2,3]

A recent report by Brugger et al.[4] suggests that tumor cells may be mobilized into the peripheral blood by treatment with chemotherapy followed by recombinant human granulocyte colony-stimulating factor (G-CSF) in previously untreated breast cancer patients. Similar tumor cell contamination effects have been reported by others in a variety of stages of lymphoma.[5–8] However, in a preliminary study of 28 patients with breast cancer undergoing HDCT with ABC reinfusion, no mobilization of tumor cells into a single large volume AP following mobilization with cyclophosphamide (CTX) and recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) was observed.[9] Thus, different mobilization procedures may produce different tumor cell mobilization results.

The presence of tumor cells in the autograft has been reported to be traceable to sites of disease relapse[10] and/or to appear to be associated with poor prognosis in the post-transplant outcome. Although conclusive proof that contaminated autografts lead to the recurrence of breast cancer has not been published, it seems prudent to develop stem cell mobilization protocols that minimize, or even abolish, the presence of contaminating and viable tumor cells.

We had the unique opportunity to retrospectively investigate 172 apheresis products (AP) derived from 17 patients with metastatic breast cancer. These samples were mobilized, apheresed and thawed, as previously published.[12] All patients were entered into a phase II clinical study between June, 1991, and February, 1993. Complete clinical data are available for our patients, including identification of AP each patient received at the time of reinfusion.

With this sample size and clinical information of all patients, we asked the following questions: Do successive cycles of chemotherapy followed by GM-CSF affect the mobilization of tumor cells? Does the presence of tumor cells vary with each apheresis? Is it possible to design an optimal protocol with a highest possible yield of hematopoietic stem cells and, at the same time, the lowest possible count of contaminating breast cancer cells? Finally, what is the clinical significance of tumor cell infusion for progression-free (PFS) and overall survival (OS)?

PATIENTS AND METHODS

Between June, 1991, and February, 1993, 17 patients were enrolled into a phase II study using three cycles of induction chemotherapy (IDC) followed by HDCT and ABC. Patients who entered the study had metastatic breast cancer and were between 29–55 years, median age 40 years. They did not have previous chemotherapy for metastatic disease but some of them had received adjuvant chemotherapy which was at least 6 months before developing metastatic disease. Performance status, according to Kanofsky, was 60% or more. The cardiopulmonary and hematopoietic functions were normal. The end points of this study were: 1) defining the feasibility and identifying side effects and toxicities of the regimen; 2) the ability to mobilize and collect sufficient numbers of ABC after each of the three IDC; 3) time to hematologic recovery; 4) response rates; and 5) PFS and OS. When patients entered the study, met all eligibility criteria and signed the informed consent form, they received the initial IDC consisting of 5-fluorouracil (5-FU) 750 mg/m^2 body surface area (BSA), epirubicin (EPI) 100 mg/m^2 BSA, CTX 750 mg/m^2 BSA, all delivered on day 1 as IV infusion. Starting on day 2, 5 μg/kg GM-CSF (Sandoz Canada Inc., Montreal, Canada) BW was administered subcutaneously daily for approximately 2 weeks until the total WBC reached 2.5/nL. On this day, standard AP was performed using a double lumen catheter connected to a Baxter CS3000+. The blood flow on average was 70 mL/min to a blood volume of 10 L. Our target was to collect at least 5×10^8 mononucleated cells per kg/BW with at least 2×10^6 CD34$^+$ cells /kg BW. This IDC was repeated twice. After final IDC all patients were restaged with a physical examination, imaging studies and blood work. Only patients who did not progress on IDC and did not experience severe toxicity and/or organ damage proceeded to HDCT.

HDCT consisted of CTX 6 g/m^2 IV, mitoxantrone (MXT) 70 mg/m^2 IV and carboplatin 800 mg/m^2 BSA delivered on 4 consecutive days. After 24–48 hours of rest, the ABC were reinfused and rhGM-CSF was administered at the dose of 5 μg/kg BW daily until hematologic recovery (ANC → 1.5 /nL for 3 consecutive days). The ABC was cryopreserved in 10% DMSO as previously described.[13] The four days of chemotherapy were delivered in the hospital. The patients were usually discharged on the day after HDCT just prior to the reinfusion of the autograft.[14,15] The protocol was approved by the institutional review board and all patients gave written informed consent.

Methods for detection of breast cancer cells in the AP

Cryopreserved specimens were rapidly thawed in a 37°C water bath. Cells were washed twice in serum-free RPMI medium (BioWhitaker, Walkersville, MD).

Table 1. Frequency of contaminated AP

	Mobilization regimen		
Day of apheresis	*#1*	*#2*	*#3*
1	4/16 (25%)	1/15 (6.7%)	0/14 (0%)
2	4/16 (25%)	0/15 (0%)	2/13 (15.4%)
3	2/13 (15.4%)	1/13 (7.7%)	1/13 (7.7%)
4	2/8 (25%)	1/11 (9.1%)	0/10 (0%)
5	2/4 (50%)	0/4 (0%)	1/5 (20%)
all apheresis	13/57 (22.8%)	3/60 (5.0%)	4/55 (7.3%)
patients with positive AP	6/17 (35.3%)	3/17 (17.6%)	4/17 (23.5%)

DNAase (Sigma Chemical, St. Louis, MO) was added at concentration of 2000 Kunitz U/mL to all wash steps to prevent clotting. Cells (5.0×10^6) were re-suspended in PBS (BioWhitaker) and cytocentrifuged onto glass slides and stored at 4°C. Slides were fixed in a paraformaldehyde-Histochoice (AMRESCO, Solon, OH) fixative, and immunostained with a cocktail of anti-cytokeratin antibodies (AE-1, AE-3, Boehringer-Mannheim, Mannheim, Germany; CAM 5.2, Becton Dickinson, San Jose, CA; Keratin 8+ 18+ 19, Monosan, Uden, The Netherlands) using an immunoalkaline phosphatase staining procedure. Nonimmune mouse serum was used as negative control. Positive control specimens consisted of the breast cancer cell line CAMA-1 admixed with AP cells from a normal, mobilized donor processed as above. For each specimen, a minimum of 10^6 cells was directly examined by light microscopy. At least two blinded observers read all slides. Cells were considered positive when immunostaining was observed in the cytoplasm and on the cell membrane and cellular morphology was consistent with a malignant phenotype. Unless all staining criteria were met, samples were coded as inconclusive, or as invalid if too few cells remained on the slides for adequate evaluation.

Statistical analysis was performed using chi-squared test for independence with contingency table for the differences between the first, second and third mobilization regimens. For comparison of progression-free survival (PFS) and overall survival, the logrank test for trend was calculated with two degrees of freedom.

RESULTS

Table 1 shows the number of contaminated AP over the total number of AP as different days of apheresis after the 3 consecutive mobilization regimens. Thirteen of the 57 (22.8%) APs obtained after the first mobilization regimen showed contaminating breast cancer cells. The distribution between days 1–5 does not show a particular pattern. In contrast, only 3 of 60 (5%) apheresis products after the

Table 2. Breast cancer cells present (+) or absent (–) in the apheresis product/autograft

	Patients	*PFS*	*Overall survival*
–/–	9	19.9 (4.8–61.7+)	59.1 (6.8–61.7+)
+/–	4	13.1 (5.7–44.7+)	28.3 (6.1–44.7+)
+/+	4	8.6 (6.9–38.9)	27.6 (9.5–53.5+)
total	17	13.1 (4.8–61.7+)	29.2 (6.1–61.7+)

second and 4 of 55 (7.3%) after the third mobilization regimen were contaminated by breast cancer cells. The differences are statistically different using chi-squared test for independence (contingency table p= 0.0052). In the lower part, the number of patients with AP contaminated with breast cancer cells are shown. The number of patients with at least one positive AP after the first mobilization regimen was 6 of 17 (35.3%), after the second and third mobilization regimens 3 and 4, respectively (17.6% and 23.5% respectively).

The progression-free and overall survivals in patients with breast cancer cells present or absent in their apheresis product or autografts: "–/–" depicts all AP were negative for breast cancer cells and the autograft did not contain any breast cancer cells; "+/–" depicts some AP contained breast cancer cells but the autograft did not contain any breast cancer cells; "+/+" depicts some AP contained with breast cancer cells and were actually reinfused to the patient. The PFS and OS are shown in months with the range in brackets. There is a trend towards increased PFS and OS for patients who did not have any breast cancer cells in the AP analyzed as shown in Table 2. However, this difference did not achieve statistical significance (p=0.746 for PFS and p=0.63 for OS, respectively).

DISCUSSION

In our retrospective study we assessed the number of AP that contained contaminating breast cancer cells; we observed a decrease of the contaminating breast cancer cells that were detectable after the first IDC from on average 29.8% to 5% and 7.3% after second and third IDC, respectively. The differences achieved statistical significance with sufficient power (p=0.0052, > 80% power). These results likely represent the in vivo tumor purging effects of consecutive IDC therapy. In a previous study of breast cancer patients with metastatic disease,[3] we reported a similar tumor purging phenomenon after consecutive cycles of cyclophosphamide, doxorubicin and 5-fluorouracil. Thus, the inclusion of IDC treatment prior to the autologous transplantation setting appears to have an in vivo tumor-purging benefit.

Since its introduction in 1991 as the first Canadian center using peripheral blood AP for hematologic rescue after HDCT, without a bone marrow backup, we collected

a relatively high number of APs in order to ensure engraftment and potentially having a sufficient backup for a second autograft. Therefore, not all APs were reinfused into patients for hematologic rescue after HDCT. This gave us a unique opportunity to retrospectively subdivide our patients into three groups: nine patients whose APs were negative for breast cancer cells by ICC analysis, and consequently did not contain any contaminating breast cancer cells. In eight of our 17 patients, we were able to detect contaminating breast cancer cells in the AP but by random (the tests were not available between 1991 and 1993) only 4 patients actually obtained APs for autografting that contained breast cancer cells. When we analyzed these three subgroups of patients (Table 2) we are able to identify a trend toward increase PFS and OS for patients who did not have any breast cancer cells in the AP detected. However, this difference did not achieve statistical significance due to low patient numbers (p=0.746 for PFS, p=0.63 for OS, respectively). Interestingly, those 4 patients whose autografts were negative for breast cancer cells but whose AP's contained breast cancer cells had a very similar OS to those patients who actually contained breast cancer cells in their autograft. This observation seems to be quite important. The detection of tumor cells in the AP may merely represent a function of residual disease in the patient, and therefore, may be a prognostic factor rather than the infusion of tumor cells contributing to relapse. Unfortunately, due to the low numbers of patients investigated, our study does not have the power to detect a statistically significant difference.

In contrast to Brugger's study,[4] the tumor cell detection was performed in a mobilized AP only. No baseline values were collected; neither were samples from peripheral blood available at the time of the apheresis. Therefore, this study cannot address the question whether mobilization of stem cells leads also to mobilization of cancer cells into the peripheral blood. Nevertheless, one study by J. Passos-Coelho et al. shows no mobilization of tumor cells into a single large volume AP following mobilization using CTX with rhGM-CSF, or G-CSF alone.[9] The risk of tumor cell contamination in peripheral blood stem cell (PBSC) collections has been outlined as early as 1992 by Moss and Ross in neuroblastoma, breast cancer and lymphomas.[16] Moreover, it was shown that circulating breast cancer and neuroblastoma cells are present in peripheral blood and have clonogenic properties in vitro as well.[1,17] Apart from the studies using immunocytochemistry[1,3,9,16,18,19] some other techniques have been used for detection of tumor cells in the bone marrow, in the peripheral blood or in the AP.[10,20–24] They are based on detecting the breast cancer cells through flow cytometry (monoclonal antibodies against cytokeratins) but also using PCR for K19. Fields et al. show that the presence of K19 (as detected by PCR) in the bone marrow of patients undergoing HDCT and ABMT is associated with a poor prognosis. Vredenburgh et al. compared immunohistochemistry, two color immunofluorescence and flow cytometry with cell sorting for the detection of breast cancer in the bone marrow.[24] Since none of these methods

shows substantial sensitivity and specificity differences in detecting contaminating breast cancer cells in the AP and ICC renders results with high reliability and visible control for the phenotype, in addition to our large experience with this method, we chose this method for our retrospective analysis. Also, substantial experience with this method in experiments using in vitro expansion of $CD34^+$ selected population[19] as well as ex vivo purging using 4-hydroperoxycyclophosphamide[24] has been published.

Our study clearly shows that the presence of tumor cells varies with each apheresis, but successive cycles of chemotherapy followed by rhGM-CSF show an "in vivo purging effect" by reducing the occasions of contaminating breast cancer cells in the APs. In this context, methods that deplete tumor cells, by positive selection of hematopoietic cells and/or negative depletion of tumor cells, warrant investigation. Compared with our previously published paper,[12] the optimal window with the highest possibility of collection of stem cells coincides with the highest number of contaminating breast cancer cells. Therefore, an optimal protocol has not yet been identified which would have the highest yield of stem cells and, at the same time, the lowest level of breast cancer cells in the AP.

The question of clinical significance of infusion of tumor cells in our patient population cannot be sufficiently answered at this time. Although there is a trend towards increased PFS and OS, more studies are necessary with higher numbers of patients in order to achieve sufficient power to detect such a difference with a statistical significance. We are currently in the process of analyzing additional patient specimens in an attempt to answer this question.

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REFERENCES

1. Ross AA, Cooper BW, Lazarus HM et al.: Detection and viability of tumour cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques. *Blood* 82:2605–2610, 1993.

2. Vredenburgh JJ, Peters WP, Rosner G et al.: Detection of tumour cells in the bone marrow of stage IV breast cancer patients receiving high-dose chemotherapy: The role of induction chemotherapy. *Bone Marrow Transplant* 16:6924–6925, 1995.
3. Passos-Coelho J, Ross AA, Davis JM et al.: Bone marrow micrometastases in chemotherapy-responsive advanced breast cancer: Effect of ex vivo purging with 4-hydroperoxycyclophosphamide. *Cancer Res* 54:2366, 1994.
4. Brugger W, Bross KJ, Glatt M et al.: Mobilization of tumour cells and haematopoietic progenitor cells into peripheral blood of patients with solid tumours. *Blood* 83:636–640, 1994.
5. Hardingham JE, Kotasek D, Sage RE et al.: Molecular detection of residual lymphoma cells in peripheral blood stem cell harvests and following autologous transplantation. *Bone Marrow Transplant*, 11:15–20, 1993.
6. Berinstein NL, Reis MD, Ngan BY et al.: Detection of occult lymphoma in the peripheral blood and bone marrow of patients with untreated early-stage and advanced-stage follicular lymphoma. *J Clin Oncol* 11:1344–1352, 1993.
7. Negrin RS, Pesando J: Detection of tumour cells in purged bone marrow and peripheral-blood mononuclear cells by polymerase chain reaction amplification of *bcl-2* translocations. *J Clin Oncol* 12:1021–1027, 1994.
8. Billadeau D, Blackstadt M, Greipp P et al.: Analysis of B-lymphoid malignancies using allele-specific polymerase chain reaction: A technique for sequential quantitation of residual disease. *Blood* 11:3021–3029, 1991.
9. Passos-Coelho JL, Ross AA, Kahn DJ et al.: Similar breast cancer cell contamination of single-day peripheral-blood progenitor-cell collections obtained after priming with haematopoietic growth factor alone or after cyclophosphamide followed by growth factor. *J Clin Onc* 14:2569–2575, 1996.
10. Rill DR, Santana VM, Roberts WM et al.: Direct demonstration that autologous bone marrow transplantation for solid tumours can return a multiplicity of tumorigenic cells. *Blood* 84:380–383, 1994.
11. Gribben JG, Neuberg D, Freedman AS et al.: Detection by polymerase chain reaction of residual cells with the bcl-2 translocation is associated with increased risk of relapse after autologous bone marrow transplantation for B-cell lymphoma. *Blood* 81:3449–3457, 1993.
12. Ho AD, Glück S, Germond C et al.: Optimal timing for collections of blood progenitor cells following induction chemotherapy and granulocyte-macrophage colony-stimulating factor for autologous transplantation in advanced breast cancer. *Leukemia* 11:1738–1746, 1993.
13. Glück S, Porter K, Chadderton T et al.: Depletion of DMSO (dimethylsulfoxide) prior to autografting after high-dose chemotherapy for metastatic breast cancer. *Blood* 82(suppl 1):430a, 1993.
14. Glück S, DesRochers C, Kaitila B et al.: Safe and effective approach to an out-patient high-dose chemotherapy and peripheral blood progenitor/stem cell transplantation in patients with metastatic breast cancer. *J Clin Apheresis* 36:27 [abstr], 1995.
15. Glück S, Des Rochers C: High-dose chemotherapy followed by blood cell transplantation: A safe and effective outpatient approach. Submitted.

16. Moss TJ, Ross AA: The risk of tumour cell contamination in peripheral blood stem cell collections. *J Hematother* 1:225–232, 1992.
17. Moss TJ, Cairo MS, Bostrom B et al.: Using bone marrow (BM) immunocytochemical (ICC) analysis to determine optimal timing of peripheral blood stem cell (PBSC) harvest for patients with breast cancer and neuroblastoma. *Blood* 84:354a [abstr], 1994.
18. Moss TJ, Cairo M, Santana VM et al.: Clonogenicity of circulating neuroblastoma cells: Implications regarding peripheral blood stem cell transplantation. *Blood* 83:3085–3089, 1994.
19. Ross AA, Loudovaris M, Hazelton B et al.: Immunocytochemical analysis of tumour cells in pre- and post-culture peripheral blood progenitor cell collections from breast cancer patients. *Exp Hematol* 23:1478–1483, 1995.
20. Simpson SJ, Vachula M, Kennedy MJ et al.: Detection of tumor cells in the bone marrow, peripheral blood, and apheresis products of breast cancer patients using flow cytometry. *Exp Hematol* 23:1062–1068, 1995.
21. Datta YH, Adamas PT, Drobyski WR et al.: Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. *J Clin Oncol* 12:475–482, 1994.
22. Fields KK, Elfenbein GJ, Trudeau WL et al.: Clinical significance of bone marrow metastases as detected using the polymerase chain reaction in patients with breast cancer undergoing high-dose chemotherapy and autologous bone marrow transplantation. *J Clin Oncol* 14:1868–1876, 1996.
23. Gross HJ, Verwer B, Houck D et al.: Model study detecting breast cancer cells in peripheral blood mononuclear cells at frequencies as low as 10^7. *Proc Natl Acad Sci* 92:537–541, 1995.
24. Vredenburgh JJ, Silva O, Tyer C et al.: A comparison of immunohistochemistry, two-colour immunofluorescence, and flow cytometry with cell sorting for the detection of micrometastatic breast cancer in the bone marrow. *J Hematother* 5:57–62, 1996.

MINIMAL RESIDUAL DISEASE IN THE BONE MARROW OF PATIENTS WITH BREAST CANCER UNDERGOING BONE MARROW TRANSPLANT: PROGNOSTIC SIGNIFICANCE

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ABSTRACT

Using a reverse transcriptase polymerase chain reaction (RT-PCR) to detect the presence of cytokeratin 19 (K19) in bone marrow (BM), we have studied the prognostic significance of minimal residual disease in patients with breast cancer undergoing high-dose chemotherapy (HDCT) and autologous bone marrow transplantation (autoBMT). This assay can reliably detect 1 breast cancer cell in 10^7 normal BM cells on serial dilution with a low incidence of false positives (approximately 3% in normal healthy donors). Cryopreserved, diagnostic BM aspirates obtained immediately before autologous BM harvesting were evaluated retrospectively. All patients were considered to have histologically negative BM prior to harvesting. Patients studied had stage II (8 or more positive nodes) and stage III disease and were evaluated following anthracycline-based adjuvant therapy but prior to high-dose therapy (n=29). Patients with stage IV breast cancer had disease responsive to anthracyclines or other salvage therapy if anthracycline refractory and were evaluated prior to high-dose therapy (n=43). Patients received one of two high-dose regimens: ifosfamide, carboplatin and etoposide (all stages, n=67); or mitoxantrone and thiotepa (MITT) (stage III and IV only, n=5). Of 29 patients with stage II and III breast cancer, 9 (31%) were K19 negative and 20 (69%) were K19 positive; of 43 patients with stage IV breast cancer, 10 (23%) were K19 negative and 33 (77%) were K19 positive. For patients with stage II and III breast cancer, event-free survival (EFS) at 3 years following autoBMT was 89% for patients who were K19 negative compared to 49% for patients who were K19 positive (p=0.1019), and the risk of relapse at 3 years was 11% for patients who were K19 negative compared to 38% for K19 positive patients (p=0.215). For patients with stage IV breast cancer, EFS at 3 years was 56% for K19 negative patients and 11% for K19 positive patients (p=0.0008), and the risk of relapse at 3 years was 44% for K19 negative patients compared with 88% for K19 positive patients (p=0.0009). We conclude that the presence of minimal residual disease in

BM confirmed by PCR to detect the presence of K19 prior to high-dose therapy and autoBMT is associated with an increased risk of relapse in patients with stage IV breast cancer but not in patients with stage II and III breast cancer. These data represent further follow-up of some previously described patients and confirm our earlier observations.

INTRODUCTION

The presence of contaminating breast cancer cells detected in bone marrow (BM) and peripheral blood stem cell pheresis products has been reported as a risk factor for relapse following standard and high-dose chemotherapy (HDCT).[1–3] Current methods of detection include standard histologic evaluation with or without the addition of immunohistochemistry using monoclonal antibodies directed at a variety of antigens. The use of more sensitive immunocytochemical techniques has led to improved detection of breast cancer cells in BM and peripheral blood with estimates of sensitivity of up to 1 breast cancer cell in 10^6 normal BM cells under optimal conditions; however, achieving this degree of sensitivity is relatively labor intensive.[4,5] Cell culture techniques have been reported as a method to detect minimal breast cancer in the BM and peripheral blood.[3] Flow cytometry has also been reported to be a sensitive and specific, as well as a less time consuming, method to detect breast cancer cells in the BM but has been less widely studied.[6]

Recently we and other investigators have reported the sensitivity and specificity of a reverse transcriptase polymerase chain reaction (RT-PCR) technique to detect the presence of cytokeratin 19 (K19), a protein expressed by epithelial-derived tissues and tumors without expression by normal hematopoietic precursors.[1,7] To determine the clinical significance of minimal residual disease detected in the BM of patients with breast cancer undergoing HDCT and autologous bone marrow transplantation (autoBMT), we performed a retrospective analysis of cryopreserved, diagnostic BM aspirates of patients with stage II, III and IV breast cancer obtained immediately prior to HDCT and autoBMT and compared the results of K19 with event-free survival (EFS) and risk of relapse following transplant. This communication represents further follow-up from our initial report.

PATIENTS AND METHODS

Patients

From October, 1989, through December, 1994, BM aspirate specimens from 72 patients with breast cancer were cryopreserved at the time of diagnostic BM evaluation performed prior to autologous BM harvesting. All patients gave written informed consent according to a protocol that was approved and reviewed annually

by the Institutional Review Board of the University of South Florida. Patients up to 65 years of age with pathologically documented breast cancer were eligible to receive HDCT consisting of ifosfamide, carboplatin and etoposide (ICE) or mitoxantrone and thiotepa (MITT) followed by autoBMT.[9,10] Patients met standard physiologic parameters for high-dose therapy and were assessed for stage of disease using standard radiographic examinations. Routine histologic evaluation of the BM was performed on all patients, which included, in the majority of cases, immunohistochemical evaluation of the biopsy specimen for the presence of cytokeratin using a panel of anticytokeratin antibodies. Patients with evidence of BM metastases or inadequate BM cellularity were not eligible for BM harvesting and transplantation.

Patients included in this study had a diagnosis of stage II breast cancer with eight or more nodes positive for metastatic breast cancer, stage III breast cancer excluding patients with inflammatory breast cancer, or metastatic (stage IV) breast cancer. Patients with stage II and III breast cancer were eligible for high-dose therapy following primary surgery and adjuvant chemotherapy consisting of 4 to 6 cycles of an anthracycline-based regimen. Patients also received chest wall irradiation prior to or following the completion of HDCT. Estrogen and/or progesterone receptor positive patients received tamoxifen for a minimum of 2 years following completion of chemotherapy. Patients with metastatic breast cancer were eligible for high-dose therapy if they had chemosensitive disease defined by responsiveness to an anthracycline-based regimen or, failing anthracyclines, to two cycles of attenuated doses of ifosfamide, carboplatin and etoposide (miniICE)[11] or if they had a solitary metastatic lesion which was resected or responsive to radiation therapy.

High-dose treatment regimens

ICE and MITT were developed as phase I/II dose escalation trials within our institution.[9,10] ICE was administered daily over 6 days in evenly divided doses with the following ranges: ifosfamide 6 to 24 g/m^2 (maximum tolerated dose, 20.1 g/m^2), carboplatin 1200 to 2100 mg/m^2 (maximum tolerated dose, 1800 mg/m^2) and etoposide 1800-3000 mg/m^2 (maximum tolerated dose, 3000 mg/m^2). Mesna was used as a uroprotectant at 100% of the daily ifosfamide dose. MITT consisted of mitoxantrone 45–105 mg/m^2 (maximum tolerated dose, 90 mg/m^2) and thiotepa 900–1350 mg/m^2 (maximum tolerated dose, 1200 mg/m^2) given in equally divided doses over 3 days. All patients in this study underwent autoBMT only.

Performance of RT-PCR for Cytokeratin 19

Complete details of the methods employed and nucleotide sequences for primers and probes used in the K19 and -actin controls have been described elsewhere.[1,7]

Table 1. Three-year EFS and risk of relapse based on presence or absence of K19

Stage	*K19 status*	*Number (%)*	*EFS at 3 years*	*p*	*Relapse risk at 3 years*	*p*
II/III	negative	9 (31%)	89%	–	11%	–
	positive	20 (69%)	59%	0.1019	38%	0.215
IV	negative	10 (23%)	70%	–	30%	–
	positive	33 (77%)	11%	0.0008	88%	0.00096

Briefly, total cellular RNA was prepared using RNA STAT-60 (Tel-Test "B", Inc., Friendswood, TX). One g of RNA was subjected to reverse transcription in the presence of the K19 outer downstream and upstream primers for 35 cycles of PCR followed by 35 cycles of PCR in the presence of inner upstream and downstream primers. β-actin served as the control for RNA amplification. Following completion of PCR, K19 and β-actin probes were end-labeled with ^{32}P-ATP, annealed with their respective probes and visualized using electropheresis.

RESULTS

Sensitivity and specificity of the K19 assay

The sensitivity and specificity of this assay has been previously described. RT-PCR for K19 could routinely detect 10 fg of T47D RNA in 1 g of normal donor RNA, which is equivalent to 1 breast cancer cell in 10^7 normal BM cells from T47D breast cancer cell lines.[1,7] False positives have been seen in 2.9% of normal healthy donor marrows.

Patient characteristics

In the current study, twenty-nine patients had stage II and III breast cancer and 43 patients had metastatic breast cancer responsive to anthracyclines (n=19) or refractory to anthracyclines and responsive to miniICE (n=24). Sixty-seven patients were treated with ICE and 5 patients were treated with MITT.

Treatment outcomes

Table 1 describes the 3-year EFS and the risk of relapse based on the presence or absence of K19 for patients with stage II and III breast cancer compared with patients with chemosensitive metastatic breast cancer. An event is defined as a transplant-related death or relapse of disease. Probability (p) values are calculated as two-tailed values of p from the logrank method. The median follow-up of the

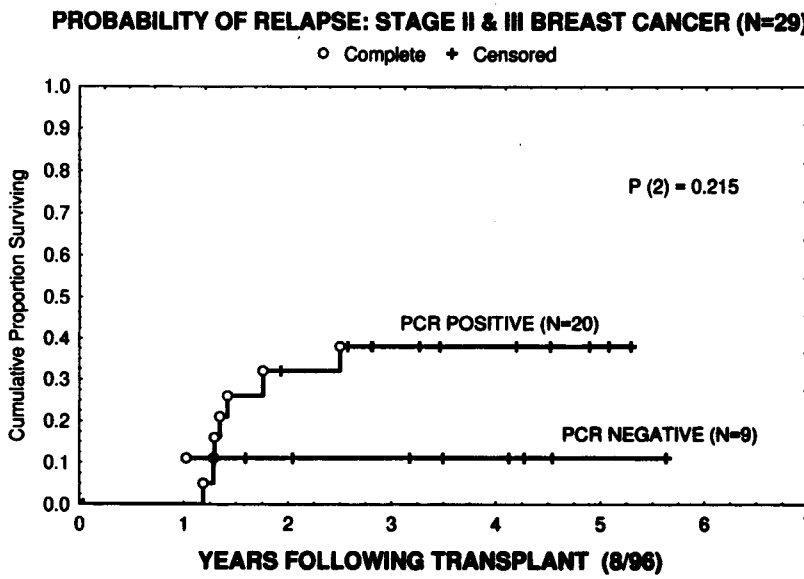

Figure 1. *The time to relapse following autoBMT for patients with stage II and III breast cancer based on the presence or absence of K19.*

event-free survivors with stage II and III breast cancer was 34 months with a range of 4 to 68 months. The median follow-up of the event-free survivors with stage IV breast cancer was 10.5 months with a range of 0.2 to 68.5 months.

The time to relapse following autoBMT based on the presence or absence of K19 in the BM for patients with stage II and III breast cancer is illustrated in Figure 1. Figure 2 illustrates the time to relapse following transplant for patients with metastatic breast cancer. In these figures, "complete" refers to patients who have relapsed, and "censored" refers to patients who have not relapsed or died of a toxic death. Of note, there are 13 patients with stage II and III breast cancer and 8 patients with stage IV breast cancer greater than 3 years from the time of transplant with no evidence of relapse. Also of note, 62% of patients with stage II and III breast cancer reinfused with K19 positive BM have not relapsed following transplant.

DISCUSSION

This study illustrates that the presence of K19 in the BM detected by RT-PCR is associated with an increased risk of relapse in patients with chemosen-

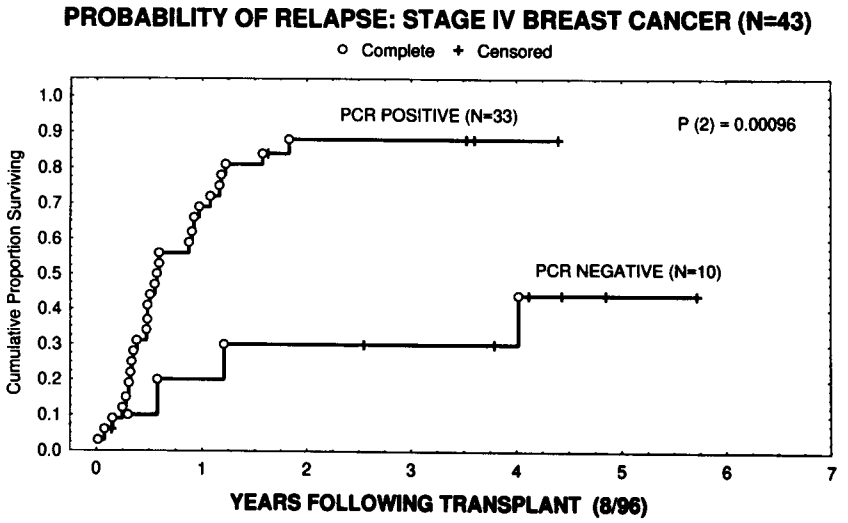

Figure 2. *The time to relapse following autoBMT for patients with metastatic breast cancer with stage IV disease based on the presence or absence of K19.*

sitive metastatic breast cancer undergoing HDCT and autoBMT. The risk of relapse following autoBMT was not significantly increased, however, in patients with stage II and III breast cancer with K19 in the BM compared to patients with no detectable K19. The present, limited study confirms our previous observations concerning the importance of K19 and minimal residual disease in the BM of patients with breast cancer undergoing autoBMT and, of note, these differences remain significant within two different HDCT regimens and with further clinical follow-up. These data suggest that K19 in the BM detected by RT-PCR is a marker of overall tumor burden in patients with breast cancer. In this study, BM was evaluated following the completion of standard adjuvant chemotherapy, in the case of patients with stage II and III breast cancer, or induction chemotherapy, in the case of patients with metastatic breast cancer, and the presence of K19 is also likely to represent persistent and, therefore, chemotherapy resistant disease. However, since the majority of patients with stage II and III breast cancer who were infused with K19 BM remain in continuous complete remission following transplantation, the role of contaminating tumor cells in the autologous BM graft remains unclear.

At least one investigator has demonstrated that contaminating tumor cells play a role in relapse following transplant for the treatment of breast cancer.[12] In the

present study, for patients with early stage breast cancer (i.e., patients with less overall tumor burden at the time of transplant), the reinfusion of a contaminated stem cell product was not associated with a significant increase in relapse in contrast to patients with advanced breast cancer (i.e., patients with greater overall tumor burden at the time of transplant). These findings suggest that purging of the autograft may not improve clinical outcomes following transplant, especially in patients with stage II and stage III disease where one would logically expect the optimal circumstances to test the value of this maneuver. Rather, these data suggest that improved anticancer therapy directed specifically toward chemoresistance and residual disease following high-dose therapy is needed to improve clinical outcomes following transplant for the treatment of breast cancer.

REFERENCES

1. Fields KK, Elfenbein GJ, Trudeau WL, Perkins JB, Moscinski LC: The clinical significance of bone marrow metastases as detected using polymerase chain reaction methods in patients with breast cancer undergoing high dose chemotherapy and autologous bone marrow transplantation. *J Clin Oncol* 14:1868–1876, 1996.
2. Harbeck N, Untch M, Pache L, Eiermann W: Tumour cell detection in the bone marrow of breast cancer patients at primary therapy: Results of a 3-year median follow-up. *Br J Cancer* 69:566–571, 1994.
3. Kessinger A, Reed E, Vaughan W, Sharp J: Clinical outcome of patients (pts) with breast cancer and gynecological epithelial tumors undergoing high-dose therapy and peripheral stem cell transplantation with or without minimally contaminated apheresis harvests. *Proc Am Soc Clin Oncol* 12:A97, 1993.
4. Osborne MP, Wong GY, Asina S et al.: Sensitivity of immunocytochemical detection of breast cancer cells in human bone marrow. *Cancer Research* 51:2706–2709, 1991.
5. Ross AA, Cooper BW, Lazarus HM et al: Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques. *Blood* 82:2605–2610, 1993.
6. Hood DL, Dicke KA, Donnell P et al.: Detection of metastatic disease in bone marrow using flow cytometry and the effect on overall survival of stage IV and breast cancer patients. *In*: Dicke KA, Keating A (eds.): *Autologous Blood and Marrow Transplantation, Proceedings of the 8th International Symposium.* Arlington, TX , 1996. In press.
7. Moscinski LC, Trudeau WL, Fields KK, Elfenbein GJ: High sensitivity detection of minimal residual breast carcinoma using the polymerase chain reaction and primers for cytokeratin 19. *Diagn Mol Pathol* 5:173–180, 1996.
8. Datta YH, Adams PT, Drobyski WR et al.: Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. *J Clin Oncol* 12:475–482, 1994.
9. Fields KK, Elfenbein GJ, Lazarus HM et al.: Maximum tolerated doses of ifosfamide, carboplatin, and etoposide given over six days followed by autologous stem cell rescue: Toxicity profile. *J Clin Oncol* 13:323–332, 1995.
10. Fields KK, Elfenbein GJ, Perkins JB et al.: Two novel high-dose treatment regimens for

breast cancer—ifosfamide, carboplatin, plus etoposide and mitoxantrone plus thiotepa: Outcomes and toxicities. *Sem Oncol* (suppl 6): 59–66, 1993.

11. Fields KK, Zorsky PE, Hiemenz JW et al.: Ifosfamide, carboplatin, and etoposide: A new regimen with a broad spectrum of activity. *J Clin Oncol* 12:544–552, 1994.
12. Brenner MK, Rill DR, Moen RC et al.: Gene marking and autologous bone marrow transplantation. *Ann of the New York Acad Sciences* 716:204–214, 1994.

DETECTION OF METASTATIC DISEASE IN BONE MARROW USING FLOW CYTOMETRY AND THE EFFECT ON OVERALL SURVIVAL OF STAGE IV BREAST CANCER PATIENTS

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ABSTRACT

Our technique for immunofluorescent labeling of cytokeratin with analysis by flow cytometry is a rapid, sensitive and cost-effective approach for detecting micrometastases in the bone marrow of breast cancer patients. In an initial study of 95 marrow aspirates, we compared our assay with cytokeratin staining with pathology. Both methods identified the same 9 positive patients; however, we detected 17 additional positive cases and had a maximum sensitivity ten-fold higher than pathology. In an effort to determine if our assay has biologic relevance, we performed a retrospective analysis of 40 Stage IV breast cancer patients who had no pathologic history of marrow involvement and were either progression-free or without evidence of disease at the time we performed our assay on their aspirate. Flow cytometry identified cytokeratin positive cells in 20% (8/40) of these patients with a median tumor frequency of 1 in 5.9×10^4 cells. Routine pathology reported metastatic disease in 5% (2/40) of the patients, all of whom were positive with our method and demonstrated the highest tumor frequencies of 1 in 5×10^3 cells and 1 in 7.5×10^3 cells. Samples found positive by flow cytometry alone had considerably lower tumor frequencies ranging from 1 in 2.5×10^4 cells to 1 in 3×10^5 cells, our highest sensitivity at that time. Within the limits of a univariate, retrospective analysis, there seems to be a favorable trend in the overall survival (OS) for patients who were cytokeratin-negative. With a maximum follow-up of 17 months from aspiration, patients determined to be cytokeratin-negative have not reached their median OS, whereas positive patients had a median OS of 8 months. Analysis is now routinely performed on 1×10^6 cells, producing a 50% (17/34) positivity rate with a median tumor frequency of 1 in 5×10^5 cells for aspirates from Stage IV breast cancer patients. Visual examination with fluorescent and brightfield microscopy confirms the presence of positive cells and minimizes false positive results. To further characterize cytokeratin-positive cells we are developing a protocol for dual staining of p-glycoprotein to identify metastatic cells with multidrug resistance-1 and determine their impact on clinical outcome.

INTRODUCTION

The ability to detect micrometastases in the marrow and blood of breast and lung cancer patients would be invaluable for staging and identifying patients who should receive adjuvant therapy, for monitoring response to treatment, and for determining optimal times to collect autologous stem cells thereby reducing the risk of relapse due to reinfusion of tumor cells.[1–3] Radiological techniques detect bone involvement only when destruction of the matrix has taken place and therefore are not useful in identifying occult metastases. Morphologic examination of marrow smears with light microscopy is labor intensive and not very sensitive in detecting minimal disease. Immunohistochemical staining can increase sensitivity but still requires the preparation and examination of numerous slides, making this approach inefficient for routine practice.[4–8] Immunofluorescent staining of epithelial cells allows easy visual identification but at the expense of cellular morphology and confirmation of malignant status.[1,4] Culturing marrow samples can expand occult tumor cells to detectable numbers in histologically negative specimens but requires 7 or more days for a result.[6–8] Reverse-transcriptase polymerase chain reaction (RT-PCR) has been used to detect breast cancer cells through amplification of keratin 18 or 19 messenger-RNA with reported sensitivities as high as 1 tumor cell in 10^7 cells.[9,10] This approach has also been applied in the detection of specific genetic mutations and carcinoembryonic antigen in colon cancer cells; however, these transcripts may not be ubiquitous.[11,12] Furthermore, the instability of messenger-RNA and meticulous technical demands make widespread use of RT-PCR unlikely at this time.[9–13] A recent report confirmed the potential of flow cytometry for detecting cytokeratin positive cells in breast cancer patients; however, the preparation and staining of the specimens was labor intensive and 8% of cytologically positive samples were negative using their method.[14]

In our current study, we report a rapid method for labeling unprocessed specimens with immunofluorescent antibodies against cytokeratin with flow cytometric analyses of 1.0×10^6 cells followed by visual confirmation with both fluorescent and light microscopy. Commercially available reagents for lysing, permeabilization and antibody-labeling make this technique cost effective and reproducible. The use of whole samples minimizes the loss of pertinent cells and reduces technical time since laborious separation procedures are unnecessary.

MATERIALS AND METHODS

Bone marrow aspirates

Bone marrow aspirates were performed on 169 consecutive Stage IV breast cancer patients. Two microliters of marrow were aspirated from a single bone

puncture. One microliter was placed in a tube containing EDTA and sent for cytokeratin-labeling and flow cytometry. The other mL was used to prepare ten slides and a clot, which was sent to pathology for morphologic examination. Samples from the first 95 patients were also sent to pathology for immunocytochemical detection of cytokeratin positive cells using an alkaline phosphatase anti-alkaline phosphatase technique.[15,16]

Labeling of cells

Whole marrow (approximately 10×10^6 white blood cells) was incubated for 20 minutes with 20 μL of CD45 antibody (Becton Dickinson, Mountain View, CA) at room temperature in the dark. After two washes in phosphate buffered saline (PBS), 4 mLs of 1× Facslyse (Becton Dickinson) was added for a 30-minute incubation followed by 2 additional washes with PBS. Cells were split between two tubes and incubated with 15 μL of either Cam 5.2 anti-cytokeratin FITC antibody or an IgG2 FITC isotype control (Becton Dickinson) for 45-60 minutes in the dark at room temperature. Cam 5.2 reacts with human cytokeratin (CK-18) ranging in molecular weight from 39–50 K daltons. Its reactivity with most adenocarcinomas, including breast and lung, and lack of reactivity with epidermis and hematopoietic cells is documented.[15] After washing twice, cells were resuspended in 1 mL of PBS + 0.2% paraformaldehyde. A positive control using the MCF7 breast cancer cell line was processed concurrently with each run of patient specimens. During initial development, ten marrow aspirates from acute myelogenous leukemia patients, four aspirates from ovarian patients and ten normal blood samples were analyzed using our methodology; all yielded negative results.

Flow cytometric analysis

Cells were analyzed on a Facsort flow cytometer using Lysis II and Paint-a-Gate software (Becton Dickinson). As many as 1×10^6 patient cells were acquired and gated from a plot of CD45 expression versus side scatter. Quad stat position was individually based on the isotypic control for each sample. Background fluorescence typically appeared within the first two decades. Cytokeratin positive cells emerged primarily in the 4th decade; however, fluorescence intensity did diminish into the 3rd and 2nd decades with increasing numbers of positive events. The MCF7 cell line was gated from a plot of forward versus side scatter and was always >80% positive with mean fluorescence intensity in the 3rd decade, clearly distinct from the background. Remaining specimens after acquisition were spun down, decanted and resuspended in 100 μL of PBS for visual confirmation under fluorescent and bright field microscopy (Nikon, Melville, NY). Results are expressed as % positive cells, mean fluorescent channel and minimum tumor frequency, which is the ratio of negative events to each positive event.

Table 1. Flow cytometry versus pathology in the detection of cytokeratin positive cells

Flow cytometry	*Pathology*
analyzed 1.2×10^5 cells	5–10 stained slides
27% Positive (26/95)	10% positive (9/95)
9 positive samples	positive pathology
17 positive samples	negative pathology
maximum sensitivity:	maximum sensitivity:
1 tumor cell/1.2×10^5 cells	1 tumor cell/1.5×10^4 cells

RESULTS

Flow cytometry versus pathology

The first 95 bone marrow aspirates analyzed using our method were sent for cytology plus immunohistochemical staining of cytokeratin to assess the efficacy of our staining procedure and level of detection relative to accepted routine pathologic procedures. Flow cytometry detected cytokeratin positive aspirates 3× more often than pathology (26 versus 9 positive specimens). Both methods identified the same 9 positive samples thereby validating our labeling method and further confirming the identity of "fluorescent events" as malignant epithelial cells. These 9 samples had the highest estimated tumor frequencies ranging from 1/3000 cells to 1/15,000 cells. As the frequency decreased to a minimum of 1/120,000 cells only flow cytometry recognized the presence of cytokeratin positive cells, indicating our analysis was 8× more sensitive than pathology.

In comparison to routine pathology (Table 2), which does not ordinarily include immunohistochemical staining, our technique was 40× more sensitive and identified positive specimens 4× more often. The maximum sensitivity of routine pathology decreased from 1 tumor cell/15,000 cells when morphology and cytokeratin staining were performed to 1/7,500 cells when only morphologic examination was used.

Further increases in detection and sensitivity were achieved by increasing the number of events analyzed by flow cytometry to 1×10^6 (Table 3). Positive cells were identified in half of the aspirates, which was almost 6× more often than morphology reported. The level of sensitivity using immunofluorescence and flow cytometry on 1×10^6 cells appears to be as much as 200× greater than standard morphologic evaluation.

DISCUSSION

Our method for detecting minimal disease in the marrow of breast cancer patients has several advantages over other approaches. By working with unprocessed samples,

Table 2. Flow cytometry on cytokeratin-stained aspirates versus routine pathology

Flow cytometry	*Pathology*
analyzed 3×10^5 cells	smears and clot sections
20% Positive (8/40)	5% positive (2/40)
2 positive samples	positive pathology
6 positive samples	negative pathology
maximum sensitivity:	maximum sensitivity:
1 tumor cell/3×10^5 cells	1 tumor cell/7.5×10^3 cells

preparation is simplified, technical time and cost are reduced and the risk of losing relevant cells is minimized. The use of commercially available, quality assured reagents and monoclonal antibodies adds to the reproducibility and efficiency of this procedure.

Our technique for permeabilization and cytoplasmic labeling does not destroy cell morphology and allows consistent staining patterns in the positive and negative controls. Due to the intense fluorescence of cytokeratin positive cells, 10^5–10^6 marrow cells can be concentrated on a slide and examined under fluorescence and brightfield microscopy. In addition to eliminating false negatives and positives, a qualitative result can be determined thereby allowing the use of this approach in routine practice without a stringent need of flow cytometry. Precise quantitation of tumor cells using flow cytometry is difficult since colonies of epithelial cells are not completely processed to single cell suspensions. Microscopic examination of positive specimens revealed mixtures of individual cells and clusters containing 2–15 cells, which were reported as single positive events. Our calculation of a tumor frequency based on the total number of positive events relative to the total number acquired is actually an estimation of minimal tumor presence.

Any procedure that is based on a 2 mL unilateral aspirate may not adequately disclose the presence of micrometastases because solid tumor cells are not likely to be distributed uniformly throughout the marrow cavity. This sampling problem may be reduced by analyzing larger numbers of cells. As we increased from 1.2×10^5 to 1×10^6 cells, the number of positive aspirates also increased from 27 to

Table 3. Flow cytometry on 10^6 cytokeratin-stained marrow cells

Flow cytometry	*Pathology*
analyzed 10^6 cells	smears and clot sections
50% positive (17/34)	9% positive (3/34)
3 positive samples	positive pathology
14 positive samples	negative pathology
maximum sensitivity:	maximum sensitivity:
1 tumor cell/1.0×10^6 cells	1 tumor cell/5.0×10^3 cells

50%. At this level of detection we compared single site aspirates with samples pooled from four sites. In ten Stage IV breast cancer patients the results were exactly the same; there was no advantage of multi-site sampling compared with single site specimens (data not shown).

Direct comparison to standard immunocytochemical and morphologic methods validated our results in patients with relatively high levels or undetectable levels of marrow involvement. It also confirmed the malignant character of cells immunofluorescently labeled for cytokeratin. When the estimated tumor frequency fell below $1/1.5\times10^4$ cells, positive samples could still be identified by flow cytometry but not by pathology. Our approach is more sensitive with a detection level of $1/1\times10^6$ cells and represents a reliable method by which to identify micrometastatic disease and ultimately determine its biologic relevance. This may be especially important in Stage I and II patients since 4–48%, using immunocytochemistry, have demonstrated marrow involvement at initial diagnosis; 33% of positive patients recurred within 2 years versus only 3% of the negative patients.[17,18] We performed a retrospective analysis of 40 Stage IV patients who had no pathologic history of marrow involvement and were progression-free or without evidence of disease at the time of their marrow aspirate. With a maximum follow-up of 17 months we found a favorable trend in the overall survival of negative patients compared with positive patients who had a median survival of only 8 months from the date of their aspirate. A recent study[9] using RT-PCR for detecting cytokeratin positive cells in marrow reported a detection level of 1 breast cancer cell in 10^7 cells. After high dose therapy and autologous stem cell transplant, 38 of 41 of the positive patients relapsed at a median of 6 months. None of the patients with a negative marrow have relapsed with a median follow-up of 38 months. Although ten-fold more sensitive than flow cytometry, RT-PCR does not allow further analysis of specimens.

Initial studies at our facility indicate that previously labeled samples using our technique can be counterstained with other immunofluorescent antibodies to further characterize metastatic disease. Using two-color flow cytometry we have identified cytokeratin-positive cells that also express p-glycoprotein, indicative of multi-drug resistance. Efforts are underway to use three-color analysis to simultaneously determine cell cycle status. The ability to assess these parameters could be invaluable in choosing effective chemotherapy regimens, monitoring patient responses and identifying the evolution of resistant clones.

REFERENCES

1. Osborne MP, Rosen PP: Detection and management of bone marrow micrometastases in breast cancer. *Oncology* 8:25–31, 1994.
2. Rill DR, Santana VM, Roberts WM et al.: Direct demonstration that autologous bone mar-

row transplantation for solid tumors can return a multiplicity of tumorigenic cells. *Blood* 84:380–383, 1994.

3. Leonard RCF, Duncan LW, Hay FG: Immunocytological detection of residual marrow disease at clinical remission predicts metastatic relapse in small cell lung cancer. *Cancer Res* 50:6545–6548, 1990.
4. Molino A, Colombatti M, Bonetti F et al.: A comparative analysis of three different techniques for the detection of breast cancer cells in bone marrow. *Cancer* 67:1033–1036, 1991.
5. Pantel K, Izbicki JR, Angstwurm M et al.: Immunocytological detection of bone marrow micrometastases in operable non-small cell lung cancer. *Cancer Res* 53:1027–1031, 1993.
6. Joshi SS, Kessinger A, Mann SL et al.: Detection of malignant cells in histologically normal bone marrow using culture techniques. *Bone Marrow Transp* 1:303–310, 1987.
7. Passos-Coelho JL, Ross AA et al.: Absence of breast cancer cells in a single-day peripheral blood progenitor cell collection after priming with cyclophosphamide and granulocyte-macrophage colony-stimulating factor. *Blood* 85:1138–1143, 1995.
8. Ross AA, Cooper BW, Lazarus HM et al.: Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques. *Blood* 82:2605–2610, 1993.
9. Fields KK, Elfenbein GJ, Trudeau WL et al.: Clinical significance of bone marrow metastases as detected by the polymerase chain reaction in patients with breast cancer undergoing high-dose chemotherapy and autologous bone marrow transplantation. *J Clin Oncol* 14:1868–1876, 1996.
10. Datta YH, Adams PT, Drobyski WR et al.: Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. *J Clin Oncol* 12:475–482, 1994.
11. Gerhard M, Juhl H, Kalthoff H et al.: Specific detection of carcinoembryonic, antigen-expressing tumor cells in bone marrow aspirates by polymerase chain reaction. *J Clin Oncol* 12:725–729, 1994.
12. Hardingham JE, Kotasek D, Farmer B et al.: Immunobead-PCR: A technique for the detection of circulating tumor cells using immunomagnetic beads and the polymerase chain reaction. *Cancer Res* 53:3455–3458, 1993.
13. Cote RJ, Chatterjee S, Glaspy JA: Breast cancer contamination in bone marrow or peripheral blood progenitor cells. *Hemato Therapy* 4:1–14, 1995.
14. Simpson SJ, Vachula M, Kennedy MJ et al.: Detection of tumor cells in the bone marrow, peripheral blood, and apheresis products of breast cancer patients using flow cytometry. *Exp Hematol* 23:1062–1068, 1995.
15. Makin CA, Bobrow LG, Bodmer WF: Monoclonal antibody to cytokeratin for use in routine histopathology. *J Clin Pathol* 37:975, 1984.
16. Osborne MP, Wong GY, Asina S et al.: Sensitivity of immunocytochemical detection of breast cancer cells in human bone marrow. *Cancer Res* 51:2706–2709, 1991.
17. Cote RJ, Rosen PP, Lesser ML et al.: Prediction of early relapse in patients with operable breast cancer by detection of occult bone marrow micrometastases. *J Clin Oncol* 9(10):1749–1756, 1991.
18. Vredenburgh JJ, Silva O, Tyer C et al.: A comparison of immunohistochemistry, two-color immunofluorescence, and flow cytometry with cell sorting for the detection of micrometastatic breast cancer in the bone marrow. *J Hemato* 5:57–62, 1996.

CHAPTER 7
Solid Tumors

HIGH-DOSE CHEMOTHERAPY IN GERM CELL TUMORS

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ABSTRACT

The role of high-dose chemotherapy (HDCT) in germ cell tumors is being refined. Evidence from many phase II trials demonstrates that HDCT is curative in 15–20% of patients with testicular primaries who fail multiple cisplatin-based regimens. There are also emerging data regarding prognostic factors in this setting. Such factors as primary site, degree of chemotherapy sensitivity and marker elevation have been found to be important in predicting outcome with HDCT.

The most important current investigation of HDCT in germ cell tumors is the ongoing comparison of HDCT with standard dose chemotherapy in patients who have failed initial cisplatin-based chemotherapy (Europe) and the comparison of standard BEP versus two cycles of BEP followed by two high-dose cycles of carboplatin, etoposide and cyclophosphamide in patients presenting with poor-risk germ cell tumors (USA). The results of these trials should define the role of HDCT in less heavily treated patients or those with very poor risk features.

INTRODUCTION

Since the mid 1980s, high-dose chemotherapy (HDCT) has been explored in germ cell tumors. Initially studies were conducted in patients with highly refractory disease to define tolerable doses and to discern whether HDCT could cure patients with disease resistant to standard dose chemotherapy. Single institution and cooperative group studies demonstrated that such attempts were warranted.[1–3] Despite initial high therapy-related mortality rates (15–20%), a significant fraction of patients were cured (10–20%). Efforts then began to improve the preparative regimen and improve patient selection for these procedures. Outlined below are the major studies of this era.

Indiana University studies

Investigations into the use of high-dose carboplatin (CBCDA) and etoposide (VP-16) with autologous bone marrow support began at Indiana University in 1986. Initial investigations were in patients who were heavily pretreated and for whom no other curative therapeutic options existed. Subsequent studies have explored modification of the initial regimen in refractory patients and the efficacy of this regimen in patients in first relapse after conventional therapy. Important

insights into the need for patient selection, the particular problem of primary nonseminomatous mediastinal germ cell tumors and the value of intervention early in the course of the disease for these toxic, expensive, yet potentially curative modes of therapy have been gained.

The initial phase I/II dose escalation study examined the use of two courses of high-dose CBDCA and VP-16 in patients with germ cell tumors refractory to cisplatin (defined as progression on or within 4 weeks of the last cisplatin dose) or recurrent after a minimum of two prior regimens containing cisplatin.[1] Thirty-three patients were entered on this trial. The initial 13 patients were treated with escalating doses of CBDCA to establish the maximum tolerated dose in combination with VP-16 at a fixed dose of 1200 mg/M^2. The subsequent 20 patients were treated with VP-16 1200 mg/M^2 and the phase II dose of CBDCA of 1500 mg/M^2 given in three divided doses on days –7, –5, –3. Toxicities seen in the protocol were the expected severe myelosuppression, moderate enterocolitis and stomatitis. Grade 3 hepatic toxicity (more than five-fold increase in liver enzymes) usually in association with massive infections was observed in 8/33 (24%) patients. Of interest, significant ototoxicity, neurotoxicity or nephrotoxicity were not seen despite extensive prior exposure to cisplatin in this patient population. Overall, 7/33 (21%) of patients died as a consequence of treatment, two on the phase II portion of the study. Deaths were primarily due to infection, although one patient died of veno-occlusive disease of the liver. Of note, this was a very heavily pretreated patient population with over half of the patients having received three or more prior chemotherapy regimens and 67% of patients were cisplatin-refractory. There were eight patients who obtained a complete remission (CR) and six a partial remission (PR) for an overall response rate of 44% (95% confidence intervals, 27% to 63%). Of the eight patients obtaining CR, three are long-term disease-free survivors, and a fourth patient died at 22 months free of germ cell cancer from a therapy-related acute nonlymphocytic leukemia. More recently, an overview of the experience at Indiana University with the first 40 patients with multiply-relapsed and refractory germ cell cancer treated with double autologous transplant demonstrated a 15% long-term disease-free survival.[4]

Following the phase I/II study, a larger multi-institutional phase II trial was carried out through the Eastern Cooperative Oncology Group (ECOG) utilizing the same dose and schedule of agents as in the phase II portion of the initial study.[5] Again patients had to have failed at least two prior cisplatin-based regimens, at least one of which contained ifosfamide, or be cisplatin-refractory. Forty patients were entered on this multi-institution cooperative effort between July, 1988, and September, 1989. Nine patients (24%) achieved a CR including two patients disease-free with post bone marrow transplant (BMT) surgical resection, and eight achieved a partial remission for an overall response rate of 45%. Three of the CR's occurred on BMT #1 and four patients converted to CR on BMT #2. Five of nine

are alive and free of disease with a minimum follow-up of 18 months. Of note, all PRs recurred with a median duration of remission of 2.5 months.

In view of the known activity of ifosfamide (IFX) in recurrent and refractory germ cell tumors and its favorable side effect profile for dose-escalation in the setting of BMT, investigators at Indiana University added high-dose ifosfamide to the CBDCA/VP-16 treatment template. Seven patients were entered on a phase II trial of CBDCA/VP-16 in the previously described doses and schedule with the addition of ifosfamide beginning at 10 gm/ M^2 daily $\times$ 5 with mesna.[6] The patients were treated with one or two courses of high-dose therapy. Due to excessive renal toxicity at the first dose level, escalation of the ifosfamide dose was impossible. Of the seven patients treated, four had a marked decline in their renal function with three of the four requiring hemofiltration or hemodialysis. Six of the seven patients had decline of serum biomarkers indicating a response to treatment, but all responses have been brief; perhaps due to the truncated treatment course necessitated by the toxicity encountered.

Recent trials include further attempts to escalate doses of CBDCA and VP-16 in patients with refractory germ cell tumors. Escalation has been possible since patients currently undergoing this therapy are much less heavily pretreated than those in the initial phase I trial. Thirty-two patients were on a careful dose escalation of each of these agents. The maximum tolerated dose level was carboplatin 700 mg/m^2 and VP-16 750 mg/m^2 given daily on days –6, –5 and –4. Dose-limiting toxicity for this regimen was mucositis. There were five treatment-related deaths: four due to sepsis and multi-organ failure and one central nervous system hemorrhage. A number of conclusions can be drawn from the series of studies performed at Indiana University. First, a small percentage of patients with multiply relapsed or cisplatin-resistant germ cell cancer can be cured with HDCT. Second, the initial attempt to increase the therapeutic ratio of the regimen with the addition of ifosfamide was unsuccessful in this patient population. Furthermore, analysis of prognostic factors from these and other studies suggest that patients with primary mediastinal nonseminomatous germ cell cancer have a particularly poor prognosis and such patients should be entered into clinical trials of more intense therapy or combinations with newer agents.

Institut Gustave-Roussy studies

Similar studies from other institutions provide further substantiation of the curative potential of HDCT in refractory germ cell cancer. Serial studies at the Institut Gustave-Roussy have demonstrated activity in recurrent germ cell cancer. These investigators developed a regimen using cisplatin 40 mg/M^2 day 1–5, etoposide 350 mg/M^2 day 1–5 and cyclophosphamide 1600 mg/M^2 day 2–5 (PEC). Sixteen patients with recurrent germ cell cancer were enrolled.[7] All had received prior therapy with cisplatin-based treatments. Five of the 15 evaluable for

response were long-term disease survivors. The succeeding study enrolled untreated patients felt to be at high risk of treatment failure with conventional therapy.[8] Brief conventional induction therapy was followed by a single round of HDCT with PEC. Of 32 poor risk patients entered, 15 remain free of disease at a median follow-up of 18 months.

Other European studies

Rosti and colleagues have expanded the CBDCA/etoposide skeleton with the addition of ifosfamide.[9] In this study, 21 patients were entered after failing primary, and often, secondary chemotherapy. In addition to the CBDCA and etoposide as given at Indiana University, ifosfamide was added at a dose of 12 gm/M^2 over 3 days. No significant renal toxicity was encountered in this study. Thirteen of the 21 patients received one course, seven received two courses and one patient received three courses. There was one therapy-related death due to veno-occlusive disease. There were eight complete remissions ranging from one to 33 months. Five of the complete remissions are ongoing.

A preliminary report from Linkesch and colleagues in Austria combines features of the PEC protocol and the protocols from Indiana University along with recombinant granulocyte-macrophage colony-stimulating factor (rhGM-CSF).[10] In this study, HDCT with carboplatin (2000 mg/M^2), etoposide (1500 mg/M^2) and cyclophosphamide (60 mg/kg $\times$ 2) was given to patients with recurrent and refractory germ cell cancer. All patients were deemed incurable with standard therapy and 62% had advanced disease by the Indiana University classification system. Twelve patients received the HDCT with autologous bone marrow rescue and an additional 30 patients received the same treatment with hematopoietic growth factors along with autologous BMT. Response was assessed in 38 patients and reported by state of disease at time of transplant. In patients with recurrent disease, 11/17 patients (64%) obtained a remission including 5 partial and 6 complete responses. In patients with refractory disease, 10/15 patients (67%) obtained a response including seven complete remissions. Of the six patients with progressive disease on chemotherapy, only one patient obtained a brief partial response. Of the patients obtaining complete remission 10/13 remain in complete response of whom seven have been greater than one year disease-free.

INITIAL SALVAGE THERAPY

Since the overall cure rate for recurrent testis cancer with ifosfamide-cisplatin based therapy is 20–25%, the proper next investigation seems to be incorporation of HDCT as a component of initial salvage therapy. A recent pilot study at Indiana University enrolled 25 patients with cisplatin-sensitive disease who were treated with conventional salvage therapy (usually vinblastine, ifosfamide and cisplatin

[VeIP]) for two courses followed by a single course of high-dose carboplatin and etoposide. Several preliminary results of this trial merit emphasis. First, there was only one transplant-related death in this series. Only six of the 25 patients enrolled did not enter the transplantation portion of the protocol. Overall, 8 patients obtained a CR, 14 PR, 2 stable disease and one progressive disease. There was one therapy-related death. With a median follow-up of 18 months, 14/25 (56%) are progression-free. Three additional patients who progressed after protocol treatment are disease-free after subsequent surgeries or additional chemotherapy. It is unclear that these results are superior to conventional salvage approaches as these patients are highly selected, but the excellent tolerance of therapy and the high response rate were encouraging.

Siegert and colleagues in Germany report the results of high-dose carboplatin, etoposide and ifosfamide in the treatment of recurrent testicular cancer.[11] Patients had received a median of six cycles of cisplatin-based chemotherapy. Patients were given two induction courses of conventional dose cisplatin, etoposide and ifosfamide before receiving escalated therapy. Fifty-five patients received treatment with conventional therapy followed by carboplatin 1500–2000 mg/M^2, etoposide 1200–2400 mg/M^2 and ifosfamide 0–10 gm/M^2. Two patients died of treatment-related causes. Responses included 12 patients (21%) with complete remission and 16 patients (28%) with marker negative partial remission. Twenty-one of these patients (38%) have maintained their response from 3+ to 26+ months. While the precise degree of chemotherapy-resistance in this patient population is not given, it is encouraging that a high percentage of recurrent patients remain progression-free.

The value of HDCT is rigidly tested in a randomized pan-European trial comparing VIP as standard salvage therapy to an experimental arm of VIP plus HDCT. The trial is ongoing and accrual hopefully will be completed in 1998.

HIGH-DOSE CHEMOTHERAPY AS INITIAL TREATMENT IN POOR RISK PATIENTS

Memorial Sloan Kettering has begun to use HDCT as a portion of initial treatment in selected patients.[12] Patients are given conventional chemotherapy (VAB-6) and those patients in whom there is a sub optimal decline in serum HCG or AFP after 2–3 cycles of treatment are given high-dose carboplatin and etoposide with autologous marrow support. To date, the majority of patients entered on the protocol have required transplantation and there is early evidence of improved outcome relative to a comparable group of patients from earlier trials. To date, 16 patients have been treated with high-dose carboplatin and etoposide after sub optimal response to VAB-6. Nine patients (56%) have obtained a complete remission and eight remain free of disease ranging from 8+ to 27+ months. These

reports compare favorably with a similar prognostic group treated with VAB-6 alone in whom only 14% have durable response to treatment.

A more recent extension of this trial at Memorial Sloan Kettering enrolled a similar population of poor-risk patients with sluggish decline in serum markers. Patients with poor-risk features were begun on VIP and if markers failed to decline by predicted half-life, conventional dose therapy was discontinued and the patient proceeded to two high-dose cycles of carboplatin (1800 mg/M^2), etoposide (1800 mg/M^2) and cyclophosphamide (150 mg/kg). Thirty untreated patients were enrolled and sixteen received VIP alone. Fourteen had conventional therapy truncated and moved to HDCT due to poor marker decline. Overall, 18/30 (60%) are continuously progression-free; a result that compares favorably with a historical group of poor-risk patients from the same institution. Again, whether this represents a therapeutic advance is being assessed in a randomized clinical trial in poor risk patients.[13]

Investigators at Institut Gustave-Roussy (IGR) have recently completed a phase III trial testing the addition of HDCT to conventional dose induction therapy for patients with untreated poor risk germ cell cancer.[14] Patients with poor risk features as assigned by the IGR prognostic system were randomly allocated to receive PVeBV as described by Ozols and colleagues or a modified PVeBV $\times$ 2 cycles followed by high-dose intensification with PEC.[15] Preliminary results suggest no benefit for patients receiving high-dose intensification. Of 49 patients randomized to receive PVeBV $\times$ 4, there were two early deaths and one refusal. Complete response was obtained in 30/49 (61%) and 82% of patients were 2-year survivors. Of 53 patients randomized to two cycles of modified PVeBV plus consolidation, there were eight early deaths and two refusals. Complete response was obtained in 21/53 (41%) and 61% of patients were 2-year survivors. A statistically significant improvement in complete remissions ($p=0.01$) and a trend toward improved survival ($p=0.1$) were seen in the standard arm relative to the "dose intense" arm.

The trial reported from IGR incorporated principles that were sound at the time of the initiation of the trial. However, subsequent evidence of the ineffectiveness of double-dose cisplatin, the availability and demonstrated activity of high-dose carboplatin and evidence of benefit of HDCT in refractory patients suggested that it was important to repeat the trial using more modern concepts. Thus, a large inter-group trial has been started in the United States enrolling patients with poor risk disease by the new International Prognostic System and randomizing them to receive either standard therapy (BEP $\times$ 4) or high-dose therapy (BEP $\times$ 2 followed by carboplatin, etoposide and cyclophosphamide $\times$ 2). This trial is ongoing and will either support or refute the role of HDCT in patients presenting with poor risk disease.

PROGNOSTIC FACTORS

Throughout the initial studies of HDCT in germ cell tumors, investigators analyzed outcome in various subgroups in an attempt to better define those who had a significant chance of benefitting from chemotherapy. In initial studies, primary site and degree of chemotherapy resistance seemed to be the most important predictors of outcome. These attempts at defining prognosis have recently been formalized in a large cooperative study by Beyer and colleagues.[16] Clinical data on 310 patients from Berlin, IGR, Austria and Indiana University who underwent HDCT for germ cell tumor were analyzed. For the group as a whole the failure-free survival was 32, 30 and 29% at one, two and three years after treatment. Clinical parameters that obtained significance in the multivariate model were mediastinal primary site, progressive disease prior to treatment, HCG >1000 U/L, refractory disease (response to cisplatin, but progression within 4 weeks) or absolutely refractory disease (no response to cisplatin). The investigators developed three categories of risk using these factors. Good risk patients had 51% FFS at three years compared with 27% and 5% for those with intermediate or poor risk. Thus it seems that patients with absolutely cisplatin-resistant disease, patients with mediastinal primaries or progressive disease prior to transplant have very poor overall outcome even with transplant and should be considered for less toxic palliative approaches.

SUMMARY

Whether HDCT will play a major role in the treatment of untreated poor-risk patients or those failing initial salvage treatments or remain a minor option for rare patients failing multiple chemotherapies is currently being defined by two large multi-center studies. It can be said that new analyses of prognostic variables have led to better understanding of those who are not at all likely to benefit from these treatments. The results of these ongoing trials will be crucial to planning future investigations of HDCT in germ cell tumors.

REFERENCES

1. Nichols CR, Tricot G, Williams SD et al.: Dose-intensive chemotherapy in refractory germ cell cancer—A phase I/II trial of high dose carboplatin and etoposide with autologous bone marrow transplantation. *J Clin Oncol* 7:932-939, 1989.
2. Nichols C, Anderson J, Fisher H et al.: High dose carboplatin and VP-16 with autologous bone marrow transplantation in patients with recurrent and refractory germ cell cancer: An Eastern Cooperative Oncology Group Study. *J Clin Oncol* (in press), 1991.
3. Motzer R, Bosl G: High-dose chemotherapy for resistant germ cell tumors: Recent

advances and future directions. *J Natl Cancer Instit* 84:1703–1709, 1992.
4. Broun E, Nichols C, Kneebone P et al.: Long term outcome of patients with relapsed and refractory germ cell tumors treated with high dose chemotherapy and autologous bone marrow rescue. *Ann Int Med* 117:124–128, 1992.
5. Nichols C, Andersen J, Lazarus H et al.: High-dose carboplatin and etoposide with autologous bone marrow transplantation in refractory germ cell cancer: An Eastern Cooperative Oncology Group Protocol. *J Clin Oncol* 10:558–563, 1992.
6. Broun E, Nichols C, Tricot G et al.: High dose carboplatin/VP-16 plus ifosfamide with autologous bone marrow support in the treatment of refractory germ cell tumors. *Bone Marrow Transplant* 7:53–56, 1991.
7. Pico J, Droz J, Gouyette A et al.: 25 High dose chemotherapy regimens (HDCT) followed by autologous bone marrow transplantation for treatment of relapsed or refractory germ cell tumors. *Proc Am Soc Clin Oncol* 5:111, 1986.
8. Droz J, Pico J, Ghosn M et al.: High complete remission (CR) and survival rates in poor prognosis (PP) non seminomatous germ cell tumors (NSGCT) with high dose chemotherapy (HDCT) and autologous bone marrow transplantation (ABMT). *Proc Am Soc Clin Oncol* 8:130, 1989.
9. Rosti G, Salvioni R, Pizzocaro G et al.: High dose chemotherapy (HDC) with carboplatin (CBP) and VP-16 in germ cell tumors: The Italian experience, *Internat Sym Auto Bone Marrow Transplan*t Omaha, Nebraska, 1990, pp 186.
10. Linkesch W, Krainer M, Wagner A: Phase I/II trial of ultrahigh carboplatin, etoposide, cyclophosphamide with ABMT in refractory or relapsed non-seminomatous germ cell tumors (NSGCT). *Proc Am Soc Clin Oncol* 11:196, 1992.
11. Siegert W, Beyer J, Weisbach V et al.: High dose carboplatin (C), etoposide (E) and ifosfamide (I) with autologous stem cell rescue (ASCR) for relapsed and refractory non-seminomatous germ cell tumors (NSGCT). *Proc Am Soc Clin Oncol* 10:163, 1991.
12. Motzer R, Gulati S, Crown J et al.: High-dose chemotherapy and autologous bone marrow rescue for patients with refractory germ cell tumors: Early intervention is better tolerated. *Cancer* 69:550–556, 1992.
13. Motzer R, Mazumdar M, Lyn P et al.: High dose carboplatin, etoposide and cyclophosphamide in first-line therapy for poor-risk germ cell tumors. *Proc Am Soc Clin Oncol* 15:251, 1994.
14. Droz J, Pico J, Biron P et al.: No evidence of a benefit of early intensified chemotherapy (HDCT) with autologous bone marrow transplantation (ABMT) in first line treatment of poor risk non seminomatous germ cell tumors. *Proc Am Soc Clin Oncol* 11:197, 1992.
15. Ozols RF, Ihde DC, Linehan M et al.: A randomized trial of standard chemotherapy v a high-dose chemotherapy regimen in the treatment of poor prognosis nonseminomatous germ-cell tumors. *J Clin Oncol* 6:1031–1040, 1988.
16. Beyer J, Kramar A, Mandanas R et al.: High dose chemotherapy (HDCT) as salvage treatment in germ cell tumors (GCT): A multivariate analysis of prognostic variables. *Proc Am Soc Clin Oncol* 15:239, 1996.

VERY HIGH-DOSE CHEMOTHERAPY WITH AUTOLOGOUS PERIPHERAL STEM CELL SUPPORT IN PREVIOUSLY UNTREATED ADVANCED OVARIAN CANCER: RESULTS OF A PHASE I-II STUDY

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ABSTRACT

Two consecutive trials were conducted to evaluate a very high-dose chemotherapy program with autologous bone marrow (autoBM) or peripheral stem cell (autoPSC) support in previously untreated advanced ovarian cancer patients with macroscopic (>0.5 cm) residual tumor. Fifty-six patients (median age 46; stage IIIC 84%) underwent induction chemotherapy (IndCT) (2–4 cycles of a cisplatin and cyclophosphamide combination ± granulocyte colony-stimulating factor [G-CSF]) with autoBM and/or autoPSC harvesting (H) followed by intensification chemotherapy (one course of a platinum, etoposide combination ± melphalan, G-CSF and erythropoietin [EPO]) in the absence of clinical progression. Second-look laparotomy was performed in clinical complete responders. Forty-five (80%) and 39 (69%) patients were evaluable, respectively, for toxicity and pathological response (pR), while 3 patients are still under treatment; 7 progressed during treatment, and 3 are awaiting second-look surgery. One toxic death occurred due to systemic mycosis in a patient undergoing autoBM transplantation (T). The number of leukaphereses required for adequate autoPSCH decreased after G-CSF incorporation. Duration of BM aplasia progressively decreased for patients receiving autoBMT, autoPSCT and autoPSCT with G-CSF and EPO, respectively. Eighty-nine percent response (CR 51%, PR 38% of which 23% had microscopic disease) was revealed at second-look. Median follow-up of 30 months (6–85) has been reached from diagnosis. Sixteen of 20 pathologically complete responders (6 with a >5 year follow-up from second-look) are disease-free without further therapy. Treatment proved to be feasible with acceptable toxicity when autoPSCT + G-CSF and EPO were used. The disease-free interval appears longer than expected in patients with chemosensitive tumors. These data, if confirmed after longer follow-up, warrant further investigation in a randomized

setting to determine the therapeutic benefit of this new approach for patients with chemosensitive tumors.

INTRODUCTION

The majority of patients with ovarian cancer present with advanced stage disease at diagnosis. Although clinical responses can be achieved with current platinum-based chemotherapy regimens in over half of patients, the five-year survival is rarely higher than 20%, and only a minority enjoy prolonged disease-free survival. Overall, less than 30% achieve complete remission and of these 40–60% will relapse and have a poor chance of cure. These data suggest that a combination of surgery and standard platinum-based chemotherapy is currently able to cure no more than 10–20% of advanced stage cases, and that there is an urgent need for innovative therapeutic strategies.

In recent years, the concept of the dose-response relationship has formed an integral part of the principles of treatment of hematologic and solid tumors. With regard to ovarian cancer, high-dose cisplatin (DDP) has proved to be effective in some patients failing standard-dose regimens,[1–3] and response rates seem to be increased by dose escalation of platinum in phase I-II studies.[4–7] Moreover, preliminary data on the use of combinations further suggest that multi-agent chemotherapy regimens may yield superior results.[8,9] Combined or sequential DDP and carboplatin (CBDCA) therapy may permit the administration of higher doses of platinum than a single analogue, as these agents have nonoverlapping toxicities.[10,11] Moreover, its negligible nonhematologic toxicity makes CBDCA a potentially useful drug in the setting of very high-dose chemotherapy, when recovery from myelosuppression can be accomplished with the use of adequate hematologic support.[12–17] Of the alkylating agents, etoposide (VP-16) and melphalan (L-PAM) may be selected for combination with platinum compounds, the former showing synergistic activity both in vitro and in vivo with DDP,[18–20] the latter a high anti-tumor response.[21] Furthermore, their dose-limiting myelosuppression makes both these drugs ideal agents to be administered when means for restoring bone marrow are used. In this respect, autologous peripheral stem cell transplantation (autoPSCT) has been proved to be effective in rapidly restoring hematopoietic function after high-dose treatment.[12–17] On the basis of preclinical data available for ovarian cancer, a survival benefit from dose intensification programs is far from expected in patients with large tumor burdens. Conversely, patients with relatively small volume disease and drug-sensitive tumors may potentially benefit from very high-dose chemotherapy trials.[22,23]

In the light of the above, two consecutive phase I-II trials were conducted to test the feasibility and efficacy of very high-dose chemotherapy with autoPSC or bone

Table 1. Patient characteristics

No. of patients entered	56 (100%)	
Study A	20 (36%)	
Study B	36 (64%)	
median age:	46 years (range 29–60)	
FIGO stages:	III B	7 (12%)
	III C	47 (84%)
	IV	2 (4%)
histotype:	serous	45 (80%)
	endometrioid	6 (11%)
	undifferentiated	5 (9%)
grade of differentiation:	moderate	11 (20%)
	poor	45 (80%)
primary surgery:	cytoreductive	42 (75%)
	simple tumor removal	4 (7%)
	exploratory	10 (18%)
residual disease after primary surgery:	0.5–2 cm	42 (75%)
	>2 cm	14 (25%)
residual disease after interval surgery:	0.5–2 cm	14 (100%)

marrow (autoBM) support after initial surgery in selected patients with advanced ovarian cancer. The pooled data from these two trials are presented.

PATIENTS AND METHODS

Fifty-six previously untreated patients with advanced epithelial ovarian cancer were enrolled in two consecutive trials: study A (June 1989–June 1991), and study B (June 1992–July 1996). The following eligibility criteria were adopted: histologically confirmed moderately to poorly differentiated FIGO Stage III-IV ovarian carcinoma; less than 2 cm residual disease after primary surgery; age less than 55 years (study A) and less than 60 (study B); World Health Organization (WHO) performance status of 0–1;[24] written informed patient consent; adequate bone marrow (WBC count >4000 μL; platelet count >100,000 μL), hepatic (bilirubin <2 mg/dL; alkaline phosphatase and serum transaminases <2.5 times normal), renal (serum creatinine <2 mg/dL; creatinine clearance >50 cc/min) and cardiac functions.

Patient characteristics are detailed in Table 1. One stage IV patient had metastasis to the abdominal wall and another presented with pleural effusion. Initial surgery was classified according to Wharton et al.[25] In ten and four patients, primary surgery was limited to explorative laparotomy and simple tumor removal, respectively. Thus, 14 patients (25%) were enrolled in spite of >2 cm residual

disease in an attempt to achieve minimal residual disease by combined induction chemotherapy and surgery. Eligible patients were entered into the study no later than two weeks after primary surgery and first underwent induction chemotherapy consisting of: (study A) 40 mg/m^2 DDP, days 1–4, and 1500 mg/m^2 cyclophosphamide (CTX), day 4, q 4 weeks, for two cycles; treatment was given in an inpatient semi-intensive care setting; autoPSC harvesting was performed by repeated leukaphereses 2–3 weeks from the start of each induction chemotherapy course when recovery from transient myelosuppression became evident (platelets >50,000/μL, WBC >1000/μL); autoBMH was performed no later than 6 weeks after the second course of induction chemotherapy according to the procedure previously reported;[26] (study B) 100 mg/m^2 DDP, day 1, 600 mg/m^2 (1500 mg/m^2 only in the first cycle) CTX, day 1, plus 5 μg/kg s.c. granulocyte colony-stimulating factor (G-CSF), day 2 to the completion of autoPSCH only in the first cycle, q 3 weeks, for four cycles; treatment was given on an outpatient basis while autoPSCH was performed by repeated leukaphereses no later than two weeks after the start of the first induction chemotherapy course only. Given the satisfactory results of autoPSCT,[27,28] autoBMH was no longer included in the treatment plan. Leukaphereses were performed in both studies according to the procedure previously reported.[27]

Of the patients initially suboptimally debulked, those who clinically responded to the first two cycles of induction chemotherapy underwent intervention surgery and, if a <2 cm postoperative residual disease was achieved, were eligible for the intensification chemotherapy. All patients with minimal residual disease after initial surgery—in the absence of progressive disease and with decreasing CA125 levels after induction chemotherapy—were eligible to receive one cycle of intensification chemotherapy (within two weeks after autoBMH in study A, and within four to six weeks after the last course of induction chemotherapy in study B). Intensification chemotherapy consisted of: (Study A) 100 mg/m^2 DDP, day 1, 650 mg/m^2 VP-16, day 2, and 1800 mg/m^2 JM8 by 24-hour infusion, day 3; autoPSC and/or autoBM were infused on day 5; (Study B) 600 mg/m^2 CBDCA, days 1–2, 450 mg/m^2 VP16, days 1–2, 50 mg/m^2 L-PAM, days 3–4; 5 μg/kg s.c. G-CSF, days 6–18, and 150 U/kg s.c. erythropoietin (EPO), every other day, days 6–18; autoPSC were infused on day 5. The anti-emetic treatment consisted of an alizapride-based regimen in the former study, and of a serotonin antagonist-based regimen in the latter. One week before receiving intensification chemotherapy all patients were placed on prophylactic trimethoprim-sulfamethoxazole, ketoconazole and acyclovir until day 30. The patients were given a low bacterial content diet and received total parenteral nutrition during periods of low oral intake. During the period of neutropenia, patients were started immediately on broad-spectrum antibiotics when temperature exceeded 38°C for more than 24 hours; amphotericin-B was added when fever persisted for more than 3 days

despite antibiotic treatment or if blood culture was positive for mycosis. Irradiated RBC and platelets were transfused to maintain platelet (PLT) count $>25\times10^9$/L and hemoglobin (Hb) count >8.5 g/dL. The WHO toxicity and response evaluation criteria were adopted.[24] In the absence of measurable residual disease, clinical response was assessed on the basis of CA125 serum levels. Four to 6 months after intensification chemotherapy, clinically complete responders underwent second-look laparotomy. Overall and PF survivals were calculated from primary surgery to date of death, and of clinical or pathological progression, respectively. All medians and life tables were computed using the product-limit estimate by Kaplan and Meier.[29] Overall, median follow-up times of 30 months (range 6–85) and 26 months (range 8–73) from diagnosis and second-look have been reached, respectively. Analysis was as of July, 1996.

RESULTS

All patients enrolled had completed induction chemotherapy at the time of the present evaluation.

Hematologic toxicity did not seem to be cumulative during induction chemotherapy in either study. Severe neutropenia and thrombocytopenia were documented in 97.5% and 35%, and 4.5% and 1.5% in Study A and B, respectively. Each patient underwent a median number of 8 (7–11) and 2 (2–5) leukaphereses in studies A and B, respectively. In Study A, four patients underwent autoBMH only (one refused the aphereses procedure, two had fever and one did not come in for regular blood tests after induction chemotherapy courses). In accordance with previously reported results,[27,28] autoBMH yielded on average, substantially lower numbers of mononuclear cells (MNC) and colony-forming units-granulocytes macrophages (CFU-GM) than obtained by repeated leukaphereses (data not shown).

All 14 patients with >2 cm residual disease after initial surgery achieved a minimal residual disease state after interventional surgery. Forty-eight (86%) of the 56 patients considered for intensification chemotherapy were in complete remission after induction chemotherapy; 45 (80%) patients have so far received intensification chemotherapy, and the remaining three are still awaiting admission. As expected, hematologic toxicity induced by intensification chemotherapy was severe in all cases.

However, the median duration of leukopenia, granulocytopenia and thrombocytopenia progressively decreased for patients receiving autoBMT, autoPSCT and autoPSCT with G-CSF and EPO, respectively (Table 2). Petechiae and/or epistaxis developed in nine (20%) patients, four of whom had undergone autoBMT. Neutropenic fever was observed in 100% and 46% of study A and B patients, respectively. Severe infections with documented pulmonary infiltrates (two

Table 2. Hematologic toxicity following intensification CT

Study	*A*		*B*
Hematologic support	*autoBMT*	*autoPSCT*	*autoPSCT + GF*
WBC:			
$<1.0\times10^9$/L	17 (15–21)	8 (8–10)	6 (4–9)
granulocytopenia:			
$<0.5\times10^9$/l	16 (15–19)	10 (9–12)	6 (3–9)
$<0.2\times10^9$/l	9 (8–10)	7 (5–8)	5 (3–8)
thrombocytopenia:			
$<50\times10^9$/L	15 (13–19)	6 (2–16)	7 (4–14)
$<20\times10^9$/L	7 (5–15)	2 (0–4)	2 (1–9)

Results are expressed as the median number of days (range) obtained in the different series; autoBMT: autologous bone marrow transplantation; autoPSCT: autologous peripheral stem cell transplantation;GF: growth factors.

patients in study A, one of whom received autoBMT) or sepsis (two patients in study B) developed in 8% of cases. One treatment-related death due to a blood-culture positive candida sepsis occurred in a 42-year-old woman undergoing autoBMT in study A. The median number of transfused platelet and RBC concentrates in studies A and B were 3 (1–4), and 2 (1–5), 1 (0–1) and 0 (0–1), respectively. The additional time and care required for autoBMH and, more importantly, the satisfactory results of the autoPSCT suggested that autoBMH be eliminated from the protocol in the last six patients of study A and in all subsequent patients.

Severe gastrointestinal toxicity was observed in 100% of patients, and required total parenteral nutrition in six (30%) of the patients enrolled in the first study. However, routine total parenteral nutrition was subsequently adopted in the second study. Hepatic toxicity consisted of transient elevation of liver transaminases, and was severe only in two patients of study A. Mild to moderate nephrotoxicity consisting of rapidly reversible serum creatinine elevation was detected only in study A (90%). After intensification chemotherapy, all patients in the first study and six (24%) in the second experienced mild to moderate ototoxicity (such as clinical hearing loss); mild peripheral neuropathy (such as distal sensory deficit)

Table 3. Pathological response

Initial FIGO stage	*No.*	*CR*	*PR (micro)*	*PR (macro)*	*NC*
IIIB	5	5	1	–	–
IIIC	31	14	7	6	4
IV	2	1	1	–	–
total	39 (100%)	20 (51%)	9 (23%)	6 (15%)	4 (11%)

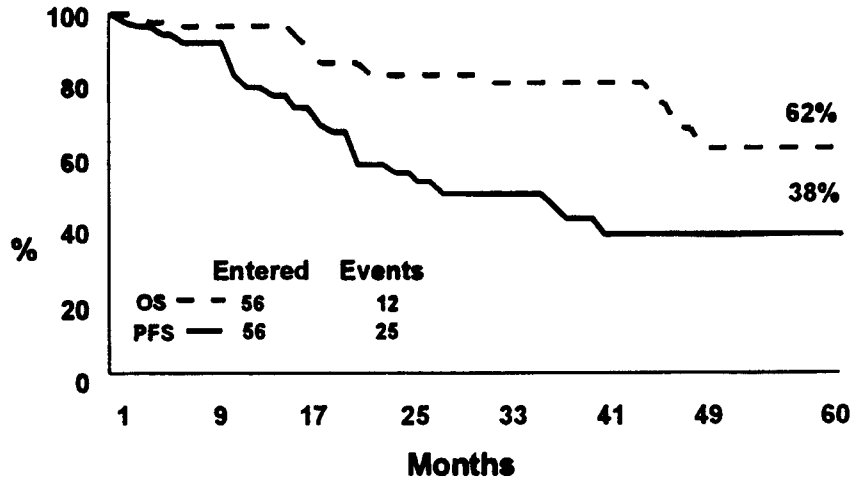

Figure 1. *Overall and progression–free survival.*

was present in 16 (80%) patients in study A and in 4 (16%) patients of study B. Severe neurologic toxicity, defined as gait disturbances, developed in two (10%) cases in study A (without, however, wheelchair-dependence). The median hospital stay from the start of intensification chemotherapy for patients receiving autoBMT, autoPSCT and growth factors was 38 days (32–42), 28 days (27–30) and 19 days (14–29), respectively.

Of the 45 patients who received intensification chemotherapy, 39 (69%) are presently evaluable for pathological response, one died of toxicity, two refused to undergo second-look surgery while three are still awaiting second-look surgery. Pathological complete response (pCR) was found in 20 (51%) evaluable patients, accounting for an overall rate of 36% (Table 3).

Of interest is that among the 15 (38%) patients with pathological partial response (pPR), nine (23%) had only microscopic persistent disease. Forty-two (75%) of the 56 patients who have completed treatment are alive at the time of this analysis. In addition to the toxic death, 12 patients died of progressive tumor and one patient, in complete remission, died of intercurrent disease, 7–38 months and 13 months from second-look, respectively. Sixteen (80%) of the 20 patients with pCR are currently disease-free. Median survival has not yet been reached, while the median progression-free (PF) survival is 26 months. The 5-year survival analysis for study A showed overall survival and PF survival rates of 60% (95% CI: 35.7–52.5) and 51% (95% CI: 26.3–46.2), respectively. The five-year overall and PF survivals are 62% and 38%, respectively (Figure 1).

DISCUSSION

To date no other study has reported results of very high-dose platinum-based chemotherapy with autoPSC support in previously untreated advanced ovarian cancer patients. Therefore, our previous results,[28] together with the update presented here are the only data published in this respect. Given the unfavorable characteristics of our patients, we adopted the therapeutic strategy of salvage combining aggressive surgery and chemotherapy. Maximum surgical effort was pursued in all cases and a 0.5–2 cm residual disease was achieved in 75% of patients by primary surgery and by interventional surgery in the remaining 25%. Thirty-six percent of our patients achieved a pCR, and microscopic disease was the only residual tumor in a further 23%. The pCR rate did not seem to be dramatically higher than that obtained by standard-dose platinum-based combination regimens in stage III–IV patients with minimal residual disease.[30,31] Nevertheless, the following considerations are to be made: 1) series of patients with <2 cm residual disease usually include those with no residual disease, while our patients all presented with macroscopic residual disease; and 2) initial substage according to the new FIGO classification[32] is generally not reported, nor is the kind of surgery required to achieve minimal residual disease; also, despite the 0.5–2 cm residual disease in 75% of our patients, 88% initially presented with stage IIIC-IV. Moreover, only three of the pathologically complete responders have relapsed in the absence of any further treatment and with a median follow-up from second-look of 19 months (12–27). A 40–60% recurrence rate is expected in patients with pCR following standard-dose platinum-based chemotherapy, and most receive further therapy after second-look. Therefore, although the overall follow-up data are still immature, it would seem that very high-dose chemotherapy is able to induce durable responses in sensitive tumors. The 5-year overall and PF survival rates of 62% and 38%, respectively, observed in this study further support the hypothesis of an increased therapeutic effect when compared to the 30% 5-year overall survival reported in optimally debulked stage IIIC patients.[33] Accordingly, results from a recent meta-analysis of randomized trials comparing standard-dose single-agent platinum versus platinum-based combinations and DDP versus CBDCA-based regimens in advanced stage disease showed 4-year overall survival rates never superior to 30%.[34,35] Even though our data are obviously not comparable with the above and the phase I–II setting and size of this study preclude conclusive analyses, it is conceivable that some improvement in disease-free survival (DFS) may be achieved by combining aggressive surgery with a very intensive platinum-based cytoreduction.

In accordance with previous publications,[27,28] patients who underwent autoBMT had delayed engraftment with a significantly longer duration of granulocytopenia and thrombocytopenia than those undergoing autoPSCT. The one case of toxic death caused by systemic candidiasis occurred despite antimycotic

prophylaxis and treatment in a patient receiving autoBMT only, and was probably related to delayed engraftment. Hence, the use of autoBMT seems to be no longer justified, and autoPSCT should be the hematologic support of choice when very high-dose chemotherapy regimens are employed. The use of cytokines, incorporated in study B, decreased myelosuppression in both the induction and intensification phases and shortened the duration of myelosuppression after intensification chemotherapy, further lowering the costs of therapy in terms of supportive care requirements (i.e., transfusion and antibiotic usage), hospitalization and improving patient quality of life. Extrahematologic toxicity consisted mainly of gastrointestinal side effects experienced by all patients despite routine antiemetic treatment. Neurotoxicity was severe in only 4% of cases. Moreover, subsequent to the dose reduction per cycle (induction chemotherapy) and withdrawal of DDP (intensification chemotherapy) in study B, grade 1 ototoxicity and peripheral neuropathy were the sole neurological side effects. The mild, reversible nephrotoxicity observed in almost all patients in study A was most likely due to high-dose DDP in induction chemotherapy and the use of DDP before high-dose CBDCA in intensification chemotherapy. None of the patients in study B, in fact, showed any renal dysfunction.

In conclusion, this study demonstrates that the combination of aggressive surgery and very high-dose chemotherapy with autoPSCT is feasible treatment for previously untreated patients with advanced ovarian cancer. In particular, the toxicity is manageable in those patients undergoing autoPSCT, especially if growth factors are used. Should a longer follow-up period confirm the apparently prolonged disease-free interval, this new approach may prove to have therapeutic benefit for the treatment of chemosensitive tumors. If so, randomized trials comparing very-high dose with standard dose regimens in selected patients will be the order of the day.

REFERENCES

1. Wiltshaw E, Subramarian S, Alexopoulos C, Baker GH: Cancer of the ovary: A summary of experience with cis-dichlorodiammineplatinum(II) at the Royal Marsden Hospital. *Cancer Treat Rev* 63:1545–1548, 1979.
2. Ozols RF, Behrens BC, Ostchega Y, Young RC: High-dose cisplatin and high-dose carboplatin in refractory ovarian cancer. *Cancer Treat Rev* 12(suppl A):59–65, 1985.
3. Ozols RF, Ostchega Y, Curt G, Young RC: High-dose carboplatin in refractory ovarian cancer patients. *J Clin Oncol* 5:197–201, 1987.
4. McGuire WP, Hoskins WJ, Brady MF et al.: A phase III trial of dose intense (DI) versus standard dose (DS) cisplatin (CDDP) and cytoxan (CTX) in advanced ovarian cancer (AOC). *Proc Am Soc Clin Oncol* 11:226, 1992 [abstr 718].
5. Bertelsen K, Bastholt L: High-dose platinum chemotherapy in advanced ovarian cancer:

A phase II study. *Gynecol Oncol* 44:79–82, 1992.
6. Bella M, Cocconi G, Lottici R et al.: Conventional versus high dose intensity regimen of cisplatin in advanced ovarian carcinoma. A prospective randomized study. *Proc Amer Soc Clin Oncol* 11:223, 1992 [abstr 706].
7. Kaye SB: Importance of cisplatin dosage in advanced ovarian cancer: Survival benefit in a randomized trial. *Proc Amer Soc Clin Oncol* 11:226, 1992 [abstr 717].
8. Wiltshaw E, Evans B, Rustin G et al.: A prospective randomized trial comparing high-dose cisplatin with low-dose cisplatin and chlorambucil, in advanced ovarian cancer. *J Clin Oncol* 4:722–729, 1986.
9. Kaye SB, Lewis CR, Paul J et al.: Randomised study of two doses of cisplatin with cyclophosphamide in epithelial ovarian cancer. *Lancet* 340:329–333, 1992.
10. Piccart MJ, Nogaret JM, Marcelis L et al.: Cisplatin combined with carboplatin: A new way of intensification of platinum dose in the treatment of advanced ovarian cancer. *J Nat Cancer Inst* 82:3-7, 1990.
11. Lund B, Hansen M, Hansen OP et al.: High-dose platinum consisting of combined carboplatin and cisplatin in previously untreated ovarian cancer patients with residual disease. *J Clin Oncol* 7:1469–1473, 1989.
12. Lind MJ, Millward MJ, Chapman F et al.: The use of rHGCSF to increase the delivered dose intensity of carboplatin in women with advanced epithelial ovarian cancer. *Proc Amer Soc Clin Oncol* 11:230, 1992 [Abstr 735].
13. Collins RH, Pineiro L, Fay JW: High dose chemotherapy and autologous bone marrow transplantation for advanced ovarian cancer. *Proc Amer Soc Clin Oncol* 11:233, 1992, [abstr 745].
14. Extra JM, Dieras V, Giacchetti S et al.: High dose chemotherapy (HCT) with autologous bone marrow reinfusion (AMBR) as consolidation therapy for patients (pts) with advanced ovarian adenocarcinoma (AO). *Proc Amer Soc Clin Oncol* 11:234, 1992 [abstr 749].
15. Shea TC, Storniolo AM, Mason JR et al.: High-dose intravenous (IV) and intraperitoneal (IP) combination chemotherapy with autologous stem cell rescue for patients with advanced ovarian cancer. *Proc Amer Soc Clin Oncol* 11:236, 1992 [abstr 756].
16. Tepler I, Cannistra S, Anderson K et al.: Repetitive dose-intensive chemotherapy (X4) made possible by initial collection and repetitive rescue with peripheral blood progenitor cells (PBPC) in previously untreated outpatients with ovarian cancer. *Proc Amer Soc Clin Oncol* 11:239, 1992 [abstr 768].
17. Tepler I, Cannistra SA, Frei E III et al.: Use of peripheral-blood progenitor cells abrogates the myelotoxicity of repetitive outpatient high-dose carboplatin and cyclophosphamide chemotherapy. *J Clin Oncol* 11:1593–1591, 1993.
18. Lund B, Hansen OP, Hansen HH et al.: Combination therapy with carboplatin/cisplatin/ifosfamide/etoposide in ovarian cancer. *Semin Oncol* 19:26–29, 1992.
19. Nichols CR, Andersen J, Lazarus HM et al.: High-dose carboplatin and etoposide with autologous bone marrow transplantation in refractory germ cell cancer: An Eastern Cooperative Oncology Group protocol. *J Clin Oncol* 10:558–563, 1992.
20. Barnett MJ, Swenerton KD, Hoskins PJ et al.: Intensive therapy with carboplatin, etoposide and melphalan (CEM) and autologous stem cell transplantation (SCT) for epithelial ovarian carcinoma (EOC). *Proc Amer Soc Clin Oncol* 9:168, 1990 [abstr 654].

21. Viens P, Maraninchi D, Legros M et al.: High dose melphalan and autologous marrow rescue in advanced epithelial ovarian carcinomas: A retrospective analysis of 35 patients treated in France. *Bone Marrow Transplant* 5:227–233, 1990.
22. Ozols RF: Role of chemotherapy in the future treatment of ovarian cancer. *Acta Obstet Gynecol Scand* 71(suppl)155:55–60, 1992.
23. Benedetti-Panici P, Greggi S, Scambia G et al.: Efficacy and toxicity of very high-dose cisplatin in advanced ovarian carcinoma: 4-year survival analysis and neurological follow-up. *Int J Gynecol Cancer* 3:44–53, 1993.
24. World Health Organization. WHO Handbook for reporting results of cancer treatment. WHO offset publication, Geneva, 48 (1979).
25. Wharton JT, Herson J: Surgery for common epithelial tumors of ovary. *Cancer* 48:582–89, 1991.
26. Pierelli L, Menichella G, De Martini M et al.: Autologous bone marrow processing for autotransplantation using an automated cell processor on a semiautomated procedure. *Bone Marrow Transplant* 7:355–361, 1991.
27. Menichella G, Pierelli L, Foddai M et al.: Autologous blood stem cell harvesting and transplantation in patients with advanced ovarian cancer. *Br J Haematol* 79:444-450, 1991.
28. Benedetti-Panici P, Greggi S, Scambia G et al.: Dose intensification chemotherapy with autologous peripheral stem cell support in advanced ovarian cancer. *Annals Med* (in press).
29. Kaplan EL, Meier P: Non parametric estimation from incomplete observation. *J Am Stat Assoc* 53:457–81, 1958.
30. Gruppo Interegionale Cooperativo Oncologico Ginecologia. Randomised comparison of cisplatin with cyclophosphamide/cisplatin and with cyclophospamide/doxorubicin/cisplatin in advanced ovarian cancer. *Lancet* 220:353–359, 1987.
31. Swenerton K, Jeffrey J, Stuart G et al.: Cisplatin-cyclophosphamide versus carboplatin-cyclophosphamide in advanced ovarian cancer: A randomized phase III study of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 10:718–726, 1992.
32. Changes in definitions of clinical staging for carcinoma of the cervix and ovary: International Federation of Gynecology and Obstetrics. *Am J Obstet Gynecol* 263–264, 1987, January.
33. Petterson F (ed): Annual Report on the results of treatment in gynecological cancer. *Int J Gynecol Obstet* 36:1–315, 1991.
34. Williams CJ, Stewart L, Parmar M et al.: Meta-analysis of the role of platinum compounds in advanced ovarian cancer. *Semin Oncol* 19(suppl 2):120–128, 1992.
35. Advanced Ovarian Cancer Trialist Group. Chemotherapy in advanced ovarian cancer: An overview of randomized clinical trials. *Br Med J* 303:884–893, 1991.

HIGH-DOSE CHEMOTHERAPY IN OVARIAN CANCER: THE NORTH AMERICAN EXPERIENCE

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ABSTRACT

The chemotherapy of advanced epithelial ovarian cancer has improved over the last 15 years with the advent of two specific agents, cisplatin and, more recently, paclitaxel. The combination of these compounds or the use of paclitaxel with carboplatin, rather than cisplatin, has now become the standard approach to the treatment of patients with surgically incurable disease. Despite these advances, the long-term disease-free survival (DFS) of patients with stage III or IV disease remains less than 30%. Because of these frequently disappointing results, clinicians have sought additional approaches such as high-dose therapy with stem cell rescue as a means of improving the treatment outcomes for these women. As with diseases such as breast cancer, lymphoma and leukemia, the bulk of disease present at the time of high-dose therapy, the chemotherapy sensitivity of the disease prior to transplant, patient performance status and extent of prior therapy are all important predictors of outcome following high-dose therapy treatment. In one large series, sensitivity to cisplatin or carboplatin at the time of high-dose therapy was the strongest predictor of extended progression-free and overall survival after transplantation and was associated with a 29% two-year progression-free survival (PFS) for patients with low volume and platinum sensitive disease. These and other results will be presented along with a review of upcoming nationwide studies that are planned to rigorously address the role of high-dose therapy with stem cell support in this patient population.

INTRODUCTION

The chemotherapy of advanced epithelial ovarian cancer has improved over the last 15 years with the advent of two specific agents, cisplatin, and more recently, paclitaxel. The combination of these compounds or the use of paclitaxel with carboplatin, rather than cisplatin, has now become the standard approach to the treatment of patients with surgically incurable disease. Despite these advances, the long-term disease-free survival (DFS) of patients with stage III or IV disease remains less than 30%.[1–3] Because of these frequently disappointing results, clinicians have sought additional approaches such as high-dose therapy (HDT)

with stem cell rescue as a means of improving the treatment outcomes for these women. In this review, we will discuss the results of recent studies with standard doses of chemotherapy without growth factor or stem cell support and present data from several trials in which more intensive chemotherapy approaches requiring such support have been utilized.

STANDARD CHEMOTHERAPY FOR ADVANCED DISEASE

In the 1980s and 1990s, standard therapy has consisted of the combination of cisplatin and cyclophosphamide with or without adriamycin.[1,3,4] During the last several years, however, a new standard was established by the report of Gynecologic Oncology Group (GOG) trial 1112. In this study, patients with suboptimally debulked Stage III (>1 cm of tumor mass following initial surgery) or stage IV disease were randomized to receive six cycles of either cyclophosphamide, 750 mg/m^2, plus cisplatin, 75 mg/m^2, or paclitaxel, 135 mg/m^2 by 24-hour infusion and cisplatin, 75 mg/m^2. Both regimens were administered every three weeks and 87% of patients underwent a second-look operation to evaluate residual disease. The combined complete and partial remission (PR) rates for the cyclophosphamide and paclitaxel groups were 60% and 73% respectively ($p<0.05$). Forty percent of the paclitaxel treated group had either pathologically negative or microscopic only disease as opposed to 23% of the cyclophosphamide treated group ($p<0.005$). Lastly, the progression-free and overall survival for the paclitaxel treated group were 17.9 and 36.8 months as opposed to 12.9 and 24.4 months respectively for the cyclophosphamide treated group. Approximately 25% of patients in the paclitaxel group as opposed to approximately 15% of the cyclophosphamide treated patients remain free of recurrence 48 months following initiation of therapy. These results represent the new standard of care in this field and are the benchmark against which new treatments should be compared.

There is general agreement from several large clinical trials that cisplatin and carboplatin are equally active agents.[5,6] Carboplatin is more myelosuppressive and less neuro-, gastro- and nephrotoxic than is cisplatin. In addition, the lower incidence of nephrotoxicity and emesis make carboplatin substantially easier and less expensive to administer in the outpatient setting. While studies comparing cisplatin and paclitaxel with carboplatin and paclitaxel, as well as different schedules (one, three, 24 or 96 hour infusions) and doses (120, 135, 175 or 225 mg/m^2) of paclitaxel are currently underway, many clinicians are using paclitaxel along with carboplatin AUC doses of 6 or 7.5 by the Calvert equation.[7,8]

There are few other agents which are currently being employed as initial treatment for these patients although a recent meta-analysis of adriamycin suggests that there may be a role for that compound in the early therapy of these women.[3] Other agents including docetaxel (taxotere) topotecan and ifosfamide

Table 1. High-dose therapy for advanced disease

Author	*No.*	*Conditioning regimen*	*CR*	*PR*	*Response duration*
Stiff	29	C, MT, CB	16	8	7 months
Shpall	12	C, T, CD	6		6 months
Shea	11	CB	1	5	
Shea	14 (12 eval.)	cycle #1: M	7	3	
		cycle #2: CB, V, MT, T			8 months

C=Cyclophosphamide; MT=Mitoxantrone; CB=Carboplatin; T=Thiotepa, V=VP-16; CD=Cisplatin.

have substantial activity in patients with ovarian cancer, but neither has become part of standard initial treatment for these patients.[9–11] These agents have, however, become increasingly used as salvage therapy for women after progression following therapy with either carboplatin or cisplatin and paclitaxel. Although disease that is refractory to paclitaxel is unlikely to be sensitive to docetaxel, there is clearly some activity in this setting, suggesting a different microtubule target for these two compounds. Other agents such as oral hexamethylmelamine, oral melphalan, 5-fluorouracil and cyclophosphamide have all been used in the salvage setting and provide response rates in the 10–15% range. Despite these attempts at salvage therapy, the median survival duration beyond the time of first recurrence is 12 to 18 months.

HIGH-DOSE THERAPY WITH STEM CELL SUPPORT

The role of dose intensity in ovarian cancer remains controversial. A classic analysis by Hyriniuk and Levin indicated a strong correlation between dose intensity response and survival in these patients.[12] Other trials have not, however, demonstrated a significant advantage for dose increases in the standard therapy range for agents such as cisplatin.[13] The advent of autologous bone marrow and blood stem cell support has permitted the delivery of substantially higher doses of chemotherapy drugs to patients with less toxicity than would otherwise be the case. This approach has been widely applied in the treatment of patients with lymphomas, leukemias, multiple myeloma, testicular and breast cancers and has resulted in high response rates, prolonged disease-free and overall survival, and, in some cases, cure of otherwise incurable disease. High-dose therapy (HDT) has also been used in the treatment of patients with ovarian cancer and has led to very high response rates and some long-term survival in advanced and resistant patients. As outlined in Table I, the majority of U.S. patients treated with this approach have had disease that has recurred at least once following initial treatment and is often both bulky and resistant to standard therapy. Patients with platinum-sensitive

disease as defined by a response to platinum based chemotherapy (cisplatin or carboplatin) immediately prior to transplant, or a prior platinum response that had lasted for more than 6 months without intervening evidence of progression on platinum, and those with disease of less than one cm in largest diameter, achieved response rates of nearly 100%. In the Stiff study, the Phase II dose of chemotherapy included mitoxantrone, 75 mg/m^2, cyclophosphamide, 120 mg/kg, and carboplatin, 1500 mg/m^2. The progression-free survival (PFS) of these patients approached 10 months and the overall survival had not been reached at the time of publication.[14] Approximately 25% of these "good risk" patients remained progression-free two years following their transplant. Bulkier and chemotherapy-resistant tumors also had a response rate exceeding 80%. The median duration of response was on the order of only 5 months, but one patient was reported to be still in remission at 49+ months following transplant. A preliminary report on three patients from this group and 24 additional patients with disease of <1 cm. and platinum-sensitive tumors before transplant (27 patients total), indicated a post-transplant progression-free survival of 19 months and a 67% overall two-year survival for this "best risk" group of patients (P. Stiff, personal communication).

Other regimens that employ different combinations of chemotherapy include a single administration of single agent carboplatin, a tandem approach developed by investigators at UNC, Chapel Hill, a platinum and thiotepa based regimen, and a high-dose paclitaxel program developed at the University of Colorado in Denver.[15–18] The original high dose phase I trial of carboplatin was undertaken at the Dana-Farber Cancer Institute in Boston and identified an MTD of 2 g/m^2. Eleven patients with recurrent ovarian cancer were treated on this phase I trial. One woman achieved a complete remission (CR) and 5 achieved a PR without any response or survival duration provided. The current UNC approach involves a three-step process of stem cell mobilization and collection followed by single agent melphalan at a dose of 100 or 140 mg/m^2 supported by 1/2 the stem cells collected from the mobilization cycle of treatment. Upon count recovery, patients receive a four-drug regimen with fixed doses of VP-16 at 800 mg/m^2 and carboplatin at 1600 mg/m^2. Patients also receive thiotepa and mitoxantrone with current doses of 600 mg/m^2 and 60 mg/m^2, respectively along with blood stem cell and, usually, bone marrow support.[16] Fourteen patients have received this regimen with no treatment-related deaths. Approximately 50% of the patients receiving 100 mg/m^2 of melphalan completed that portion of their treatment as outpatients. Only three patients have received 140 mg/m^2 of melphalan so it is too early to comment on the frequency of hospitalization in this cohort. Two patients remain progression-free more than 15 months after transplant, eight patients have relapsed at a median of 9 months after transplant and four patients remain progression-free within six months of transplant. In this heavily pre-treated population, it appears that this regimen will be comparable, but not necessarily superior, with others that have been used for this patient group.

Table 2. High-dose therapy following second-look surgery in minimal disease patients

Author	*No.*	*Conditioning regimen* [a]	*F/U*	*PFS* [b]
Viens	19	M	23 months	47%
Legros	31	M, C	27 months	50%
Extra	37	C, XRT (33), M (3 pts) CB (14 pts)	32 months	50%

[a]*C=Cyclophosphamide; MT=Mitoxantrone; CB=Carboplatin; T=Thiotepa; V=VP-16; CD=Cisplatin.*
[b]*Progression-free survival.*

Dr. Shpall published a report from Duke University on 11 patients treated with a combination of cyclophosphamide (5.725 mg/m^2), cisplatin (165 mg/m^2) and 600 mg/m^2 of thiotepa.[17] This report described a heavily pretreated population of patients who were able to achieve a high response rate of modest duration. The report appeared before the general availability of stem cells or growth factors and there were two treatment-related deaths. This regimen is currently being tested in a randomized phase II Southwest Oncology Group (SWOG) trial along with the Loyola regimen of mitoxantrone, carboplatin and cyclophosphamide. Drs. Cagnoni, Shpall and their colleagues have continued this work at Colorado and have developed a regimen consisting of fixed doses of 5.625 g/m^2 cyclophosphamide, 165 mg/m^2 of cisplatin and escalating doses of paclitaxel between 135 and 825 mg/m^2 over 24 hours.[18] The phase II dose of paclitaxel is 725 mg/m^2. The dose limiting toxicities at doses of 825 and 775 mg/m^2 included capillary leak, renal insufficiency, polyneuropathy and mucositis. The lack of more severe and debilitating neuropathy and mucositis at lower doses has been surprising given the toxicities of paclitaxel at standard doses. This may partially be explained by the fact that most of the patients have had breast cancer (33 of 39) and have not had prior platinum-based therapy.

While the majority of patients receiving high-dose therapy have received a single course of intensive treatment, several investigators have developed programs that include the delivery of multiple cycles of therapy. Shea and his colleagues have reported on the delivery of multiple cycles of single agent carboplatin at a dose of 1200 mg/m^2 together with blood stem cell and GM-CSF support.[19] This phase I study demonstrated the importance of stem cell support in this approach. A subsequent phase I study utilized a combination of carboplatin and paclitaxel along with G-CSF and blood stem cell support.[20] In this trial the maximum tolerated dose of drugs included 250 mg/m^2 of paclitaxel by 24-hour infusion and a carboplatin AUC dose of 18 by the Calvert equation. These agents were delivered every 21 days for four cycles with minimum cumulative toxicity and a hospitalization rate of 20%. Similar approaches by Crown and Fennelly at Memorial Sloan Kettering have demonstrated a high overall response rate in previously untreated patients with a 60% pathologic disease-free or microscopic disease only state at second-

look surgery.[21] Their regimen currently utilizes a combination of cyclophosphamide and paclitaxel induction therapy followed by stem cell collection. Stem cells are then reinfused following each of three cycles of high-dose carboplatin and paclitaxel and a final cycle of single agent high-dose melphalan. This approach is outlined in Table II and forms the basis for an upcoming pilot study of the Gynecologic Oncology Group (GOG 9501).

Although very high response rates can be achieved in these patients, the majority of them will relapse and progress within one year of therapy. For this reason, some investigators have treated patients earlier in the course of their disease. Several of these studies have been performed in Europe with single agent melphalan or combinations of melphalan, cyclophosphamide and carboplatin for patients with minimal residual disease at the time of second-look surgery following platinum and cyclophosphamide based induction regimens. The number of patients is not large and follow-up is relatively short, but these data suggest that as many as 40–50% of such patients may remain progression-free for two to three years or more following such treatment.[22–24] A trial reported by Benedetti-Panici utilized HDT as part of the initial therapy prior to second-look surgery. In this study, a pathologic CR rate of 42% was achieved in 35 patients who underwent several cycles of standard cyclophosphamide and cisplatin induction therapy followed by two cycles of high-dose therapy consisting of cisplatin, VP-16 and carboplatin followed by melphalan, VP-16 and carboplatin.[25]

Proposed or recently initiated studies

The SWOG has opened a randomized phase II trial comparing toxicities of the mitoxantrone, carboplatin and cyclophosphamide regimen of Loyola with the cisplatin, thiotepa, cyclophosphamide regimen from Colorado. This trial is open to patients with recurrent (more than six months since initial therapy) or residual disease of less than 3 cm following initial therapy with a platinum based chemotherapy program. After initially slow accrual, the activity of that trial has increased markedly in the last six months and approximately 30 patients have now been enrolled (P. Stiff, personal communication). A second cooperative group study through the Gynecologic Oncology Group has recently opened for initial therapy of optimally debulked patients with stage III disease. This trial (GOG 9501) utilizes the sequential regimen developed by Fennelly and Crown at Memorial Sloan Kettering Cancer Center in New York and described in Table 3. The major endpoints of the study include second-look surgery and a pathologic disease-free rate of at least 50%. Additional objectives include the feasibility of delivering multiple cycles of this intensive therapy in a multi-center setting as well as estimating the progression and DFS rate for enrolled patients. This trial is opened as a limited access study at Memorial Sloan Kettering, UNC, Chapel Hill, Fox Chase Cancer Center and Washington University in St. Louis. The last study

in this group is a trial that has been under development for some time with representatives from SWOG, GOG, the Eastern Cooperative Oncology Group (ECOG) and the National Cancer Institute. The study is not yet finalized, but is anticipated to include a randomization between high-dose transplant therapy using the mitoxantrone, cyclophosphamide, carboplatin regimen versus four additional cycles of continued standard doses of platinum and paclitaxel-based therapy. Eligible women would include those with responsive but still identifiable (<1 cm, but not pathologically negative) second look surgeries after four to six cycles of initial platinum-based chemotherapy. Patients with disease that was greater than one centimeter or those with stable or progressive disease after their initial chemotherapy would not be eligible. The role of high-dose therapy will be tested in this population of patients with minimal and chemotherapy-responsive disease. If accrual is adequate, this study should provide crucial information about the value of high-dose therapy in this optimal patient population. This study should be ready for activation by late 1996 or early 1997.

SUMMARY

The role of high-dose therapy remains to be defined in women with ovarian cancer. It is clear that high response rates can be achieved, even in patients with advanced and refractory or bulky disease, but the majority of such patients still die of recurrent malignancy. Phase II and phase III cooperative group trials addressing these issues are underway or are planned and will be able to evaluate the role of multiple cycles of intensive therapy as initial treatment as well as the value of intensive therapy in chemotherapy-sensitive, minimal disease patients (Table 3). Preliminary results suggest that this is the optimal population in whom to test this approach and the planned NCI-sponsored trial should provide important information in this regard. At the same time, it is clear that novel strategies, including the continued evaluation of intraperitoneal therapy for patients with minimal abdominal disease, biologic therapy with intraperitoneal interleukin-2, anti-angiogenes compounds and additional agents such as the camptothecins, need to be developed and tested along with traditional chemotherapy and high-dose approaches. The rational and innovative combination of these approaches will be needed to effectively treat patients with this commonly fatal disease.

REFERENCES

1. Hoskins PJ, O'Reilly SE, Swenerton KD, Spinelli JJ et al.: Ten-year outcomes of patients with advanced epithelial ovarian carcinoma treated with cisplatin-based multimodality therapy. *J of Clin Oncol* 10:1561–1568, 1992.
2. McGuire WP, Hoskins WJ, Brady MF, Kugera PR et al.: Cyclophosphamide and cisplatin

compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. *New Engl J Med* 334:1–6. 1996.

3. Einzig AI, Wiernik PH, Sasloff J, Runowicz D, Goldbert GL: Phase II study of long-term follow-up of patients treated with taxol for advanced ovarian adenocarcinoma. *J Clin Oncol* 10:1748–1953, 1992.
4. A'Hern RP, Gore ME: Impact of doxorubicin on survival in advanced ovarian cancer. *J of Clin Oncol* 13:726–732, 1995.
5. Taylor AE, Wiltshaw E, Gore ME, Fryatt I, Fisher C: Long-term follow-up of the first randomized study of cisplatin versus carboplatin for advanced epithelial ovarian cancer. *J Clin Oncol* 12:2066–2070, 1994.
6. Alberts DS, Green S, Hannigan EV, O'Toole R et al.: Improved therapeutic index of carboplatin plus cyclophosphamide versus: Final report by the Southwest Oncology Group of a phase III randomized trial in stages III and IV ovarian cancer. *J Clin Oncol* 10:706–717, 1992.
7. Bookman MA, McGuire WP III, Kilpatrick D, Keenan E et al.: Carboplatin and paclitaxel in ovarian carcinoma: A phase I study of the gynecologic oncology group. *J Clin Oncol* 14:1895–1902, 1996.
8. Eisenhauer EA, ten Bokkel Huinink WW, Swenerton KD, Gianni L et al.: European-Canadian randomized trial of paclitaxel in relapsed ovarian cancer: High-dose versus low-dose and long versus short infusion. *J of Clin Oncol* 12:12, 2543-2666, 1994.
9. Kavanagh JJ, Kukelka AP, Freedman RS, Edwards CL et al.: A phase II trial of taxotere (RP56976) in ovarian cancer patients refractory to cisplatin/carboplatin therapy. *Proc Am Soc Clin Oncol* [abstr 823] 1993.
10. Kudelka A, Edwards C, Freedman F, Wallin B et al.: An open phase II study of topotecan administered intravenously as 5 daily infusions every 21 days to women with advanced epithelial ovarian carcinoma. *Proc Am Soc Clin Oncol* [abstr 821] 1995.
11. Lorusso V, Catino A, Leone B, Rabinovich M et al.: Carboplatin plus ifosfamide as salvage treatment of epithelial ovarian cancer: A pilot study. *J Clin Oncol* 11:1952–1956, 1993.
12. Levin L, Hryniuk WM: Dose intensity analysis of chemotherapy regimens in ovarian carcinoma. *J Clin Oncol* 5:756–767, 1987.
13. McGuire WP, Hoskins WJ, Brady MF, Homesley HD et al.: Assessment of dose-intensive therapy in suboptimally debulked ovarian cancer: A Gynecologic Oncology Group study. *J Clin Oncol* 13:1589–1599, 1995.
14. Stiff P, Bayer R, Camarda M, Tan S et al.: A phase II trial of high-dose mitoxantrone, carboplatin, and cyclophosphamide with autologous bone marrow rescue for recurrent epithelial ovarian carcinoma: Analysis of risk factors for clinical outcome. *Gynecol Oncol* 57:278–285, 1995.
15. Shea TC, Flaherty M, Elias A, Eder J et al.: A phase I clinical and pharmacokinetic study of carboplatin and autologous bone marrow support. *J Clin Oncol* 7:651–661, 1989.
16. Shea T, Wiley J, Serody J, Mason J, Powell E, VanLe L, Fowler W: High-dose chemotherapy with melphalan, VP-16, carboplatin, thiotepa, and mitoxantrone in patients with advanced epithelial ovarian cancer. *Proc Am Soc Clin Oncol* 14[abstr 807]:284, 1995.
17. Shpall E, Clarke-Peterson D, Soper J et al.: High-dose alkylating agent chemotherapy with autologous bone marrow support in patients with stage III/IV epithelial ovarian cancer.

Gynecol Oncol 38:386–391, 1990.

18. Cagnoni PJ, Stemmer SM, Matthes S, Dufton C et al.: Phase I trial of high dose paclitaxel, cyclophosphamide and cisplatin with autologous hematopoietic progenitor cell rescue. *Proc Am Soc Clin Oncol* 14[abstr 1548]:477, 1995.
19. Shea TC, Mason JR, Storniolo AM, Newton et al.: Sequential cycles of high-dose carboplatin administered with recombinant human granulocyte-macrophage colony-stimulating factor and repeated infusions of autologous peripheral-blood progenitor cells: A novel and effective method for delivering multiple courses of dose-intensive therapy. *J Clin Oncol*, 10:464–473, 1992.
20 Shea T, Graham M, Bernard S, Steagall A et al.: A clinical and pharmacokinetic study of high-dose carboplatin, paclitaxel, granulocyte colony-stimulating factor, and peripheral blood stem cells in patients with unresectable or metastatic cancer. *Sem in Oncol*, 22(suppl 12):80–85, 1995.
21. Fennelly D, Schneider J, Spriggs D, Bengala C et al.: Dose escalation of paclitaxel with high-dose cyclophosphamide, with analysis of progenitor cell mobilization and hematologic support of advanced ovarian cancer patients receiving rapidly sequenced high-dose carboplatin/cyclophosphamide courses. *J Clin Oncol* 13:1160–1166, 1995.
22. Viens P, Maraninchi D, Legros M et al.: High dose melphalan and autologous marrow rescue in advanced epithelial ovarian carcinomas: A retrospective analysis of 35 patients treated in France. *Bone Marrow Transplant* 5:227-233, 1990.
23. Extra JM, Giacchetti S, Bourstyn et al.: High dose chemotherapy with autologous bone marrow reinfusion as consolidation therapy for patients with advanced ovarian adenocarcinoma,. *Proc Am Soc Clin Oncol* 11[abstr 749]:234, 1992.
24. Legros M, Fluery J, Cure H et al.: High dose chemotherapy and autologous bone marrow transplant in 31 advanced ovarian cancers: Long term results. *Proc Am Soc Clin Oncol* 11[abstr]:222, 1992.
25. Benedetti-Painica P, Greggi S, Scambia G et al.: Very high dose chemotherapy with autologous peripheral stem cell as hematologic support in previously untreated advanced ovarian cancer. *Proc Soc Gyn Oncol* p 30, 1995 [abstr 73].

MULTIPLE CYCLES OF HIGH-DOSE CHEMOTHERAPY WITH PERIPHERAL BLOOD STEM CELLS (PBSC) IN SMALL CELL LUNG CANCER (SCLC): EBMT STUDY

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ABSTRACT

Since February, 1994, 50 small cell lung cancer (SCLC) patients with limited or extensive disease were entered into a phase II European study. The treatment plan consisted of a mobilization phase with Epirubicin (75 mg/mq/d, d1 and d2) and granulocyte colony-stimulating factor (G-CSF) 5 μg/kg/d for 14 days, followed by an intensification phase with 3 sequential cycles of high-dose ICE (IFO 10 g/mq, CBDCA 1200 mg/mq and VP16 1200 mg/mq) followed by PBSC reinfusion 48 hours from the end of chemotherapy and G-CSF (5 μg/kg/d) until full hematological recovery. Cycles were repeated every 3 weeks.

Aims of the study were to obtain a 3-fold intensification of chemotherapy compared with standard regimen, to evaluate the power of Epirubicin + G-CSF in mobilization and peripheral blood stem cell (PBSC) harvest, to study the feasibility of multiple sequential cycles of high-dose regimen and to evaluate the survival of treated patients.

Thirty-eight patients have completed the treatment to date and are evaluable for toxicity. Patient features include: median age 54 years (range 36–64), PS 1 (range 0–2), 32 males (84%), 20 (40%) with extensive disease.

A median of 3 leukaphereses were performed (range 0–5); median number of harvested MNC, CFU-GM and $CD34^+$ cells was 6.0×10^8/kg (range 2.2–15.7), 90×10^4/kg (range 3–210.7) and 17×10^6/kg (range 1–42), respectively. Two toxic deaths were registered during the mobilization phase: 1 for cardiac failure and 1 for septic shock. A total of 96 cycles of high-dose ICE were infused. Twenty-eight patients (74%) completed the treatment program (all the 3 planned cycles). No cumulative hematological toxicity was observed comparing the first with the third cycle. Median time to hematological recovery was, respectively, 9 days (range 7–12) for white blood count $>1\times10^9$/L and 10 days (range 7–27) for platelets

$>20\times10^9$/L. There were 3 toxic deaths: 1 for renal failure, 2 for septic shock. Accrual of patients is ongoing. So far, 35 patients are evaluable after high-dose chemotherapy (HDCT). The response rate is 83% (14 CR, 7 near-CR and 8 PR).

Conclusions: Epirubicin with G-CSF gives sufficient mobilization of PBSC to allow for multiple cycles of the high-dose ICE regimen. The sequential model is feasible as all treated patients had full hematological recovery and tolerable toxicity. The five deaths (two in the mobilization phase and three in the intensification phase) may be due to a "learning curve effect." Preliminary results are encouraging with a 60% in CR. The accrual is ongoing and a survival analysis will be conducted when 60 patients have been treated.

INTRODUCTION

Despite the availability of many active cytotoxic drugs, therapeutic strategies based on standard chemotherapy combinations have not improved the prognosis of patients with small cell lung cancer, either extensive- or limited-disease stage. Standard regimens give a high initial response rate (70 to 80% with 20 to 30% of complete remissions) but median survival is only 30 to 50 weeks and long-term survival is unusual.[1]

In the 1980s the mathematical model proposed by Goldie and Coldman describing the development of chemotherapy-resistant clones in malignancies led to many trials of alternating chemotherapy combinations.[2,3]

In our review of the 1067 patients treated according to dose-intensity strategies based on alternating and/or weekly schemes of chemotherapy with or without hematopoietic growth factor support, the median percentage of complete remissions has been calculated to be 23% (range 12–40%) with a median duration of responses of 29 weeks (range 17–43)[4–8] and a maximum treatment-related mortality of 11%.[7]

In view of the chemo-sensitivity of small cell lung cancer, this disease is usually considered suitable for the new phase I–II as well as phase III studies that incorporate the high-dose chemotherapy (HDCT) and the use of peripheral blood stem cells with hematopoietic growth factors.

According to the update presented at the last European Blood and Marrow Transplantation Meeting (Dr. G. Rosti, Wien, March 1996), of the 2551 cases of adult solid tumors treated in Europe with HDCT regimens and autologous hematopoietic support, 90 were small cell lung cancer (3.5%), 42.2% were breast cancers, 19.9% were germ cell tumors, 12.7% gliomas and 8.9% ovarian cancers.

Several high-dose clinical trials are based on the Norton and Simon suggestion. Here, intensification of therapy administered after the maximum response to conventional treatment, so-called "late intensification," might be associated with an improvement in tumor regression and a decreased incidence of tumor recurrence.[9]

In our review of 158 patients with extensive or limited disease treated with high-

dose regimens in the "late setting",[10–16] the median complete response rate has been calculated around 25% (range 0–66%). The median duration of response and the median survival rate, where evaluable,[10–15] are reported, respectively, at 18 months (range 12–24) and 10% (range 0–40%). Of note, treatment-related mortality decreased from 15–30%[10,11] in the first studies, to 0[15]–4%[14] in the more recent trials; this is perhaps due to improvements in supportive care and to a more careful selection of patients.

Several experimental models suggest that resistant tumor cells may be the main cause of treatment failure[17] and emphasize importance of initial dose-intensity in overcoming the development of resistant tumor cells and in improving prognosis mostly in limited-disease stages. In our review of 74 patients treated with high-dose therapy in the "early intensification" setting, the median complete remission rate was 50% (range 56–69%) with a survival rate around 30% at 2-year follow-up.[18–22]

The present trial from the EBMT Working Party Group is a phase II study of sequential high-dose combination chemotherapy, based on the results of a prior feasibility study published in 1993.[23]

It is based on the hypothesis that selected tumor clones may be eradicated by multiple and sequential courses of HDCT in contrast to a single course.

Dose-intensity has been calculated according to the Hryniuk criteria. The average relative dose intensity was projected to be three times higher than 6 cycles of standard Thatcher's regimen consisting of ifosfamide 5 g/m^2, carboplatin 300 mg/m^2 and etoposide 360 mg/m^2 each cycle.[24]

The aims of the study are to evaluate the effect of epirubicin and G-CSF for mobilization and peripheral blood stem cell harvest; to develop an intensive combination chemotherapy to be administered over multiple sequential cycles aiming for a 3-fold increase; and to study the response rate, disease-free survival and overall survival.

METHOD

Patients responding to the following criteria were entered into the study: proven small cell lung cancer; limited or extensive disease; age up to 65 years old; performance status 0–1 by ECOG criteria; no prior treatment; white blood count more than 3.5×10^9/L, platelets more than 100×10^9/L and hemoglobin more than 10.0 g/L; liver enzymes not more than 2.5× normal value, unless due to disease; creatinine clearance minimum of 60 mL/minute; no congestive heart failure and left ventricular ejection fraction more than 50% and informed consent.

The treatment plan consists of an induction-mobilization phase with epirubicin 75 mg/m^2 daily, on day 1 and 2, followed by G-CSF, 5 μg/kg/day, subcutaneously, starting 24 hours after the second dose of epirubicin, and continued until the end of stem cell collection. Apheresis starts when white blood cell count reaches more than

1.0×10^9/L and platelets more than 25×10^9/L. Total collection is divided into three aliquots for rescue after each cycle of HDCT.

HDCT is the ICE regimen, consisting of total dose of ifosfamide, 10 g/m^2, carboplatin, 1200 mg/m^2 and etoposide 1200 mg/m^2, administered in divided doses over four days. Infusion times are seventeen hours for ifosfamide and three hours each for carboplatin and etoposide.

Peripheral blood stem cells are reinfused 48 hours after the last dose of etoposide. G-CSF, 5 μg/kg/day, subcutaneously, is started on the same day. Three cycles are administered to the patients at 28-day intervals. Response is evaluated 4 weeks after the end of chemotherapy using standard radiography, CT scan and bronchoscopy with biopsy when necessary. Response is evaluated according to the Dana-Farber Cancer Institute criteria.[14] Chest radiotherapy and prophylactic cranial irradiation are performed according to Center policy.

RESULTS

Since February, 1994, 50 patients have entered into the study. Patient characteristics are reported in Table 1. Toxicity was assessable for all the 50 patients in the mobilization phase and over the 96 cycles of high-dose ICE. The results are reported in Tables 2 and 3, respectively. Hematological recovery after high-dose ICE is described in Table 4.

DISCUSSION

Of the 50 patients entered into the study, 38 were evaluable: one patient was excluded because of a change in diagnosis to carcinoid after histological re-evaluation and 11 were too early to evaluate. Twenty patients presented extensive disease at diagnosis: the most common sites of metastases were liver (65%), followed by bone (40%), bone marrow (35%), pleura (25%), distant lymph nodes (25%), lung (20%), adrenal gland (20%) and brain (5%).

Toxicity was evaluated according to the standard World Health Organization (WHO) system. All 50 patients treated with epirubicin plus G-CSF in the mobilization phase were assessable for toxicity. The most frequent nonhematologic toxicity was grade 1 and 2 gastrointestinal, with diarrhea. Mucosal toxicity was minimal, occurring in only 10%. Nausea and vomiting occurred in 36% of patients but was grade 3 in less than 10%. Severe infections occurred in 10% of patients: two patients developed a grade 4 infection with sepsis and one died. One patient with a basal arrhythmia developed a fatal cardiac failure.

Hematologic recovery was rapid and the median time from the last dose of epirubicin to first collection was 10 days (range 8 to 13), in agreement with the published data.[25]

Table 1. Patient characteristics

total patients		50	
patients evaluable		38	
too early		11	
ineligible		1	(carcinoid)
median age (year)		54	(range 36-64)
median performance status		1	(range 0-2)
sex:	female	6	(16%)
	male	32	(84%)
disease extension:	limited	18	(48%)
	extensive	20	(52%)

Table 2. Toxicity during mobilization phase evaluated in 50 patients

	WHO grade			
	I	*II*	*III*	*IV*
mucosal	2	3	0	0
gastrointestinal	2	1	1	0
nausea/vomiting	11	3	4	0
infection	0	1	3	2
neurological	0	0	0	0
renal	2	0	0	0
cardiac	0	0	0	1

Table 3. High-dose ICE toxicity evaluated in 96 cycles

	WHO grade			
	I	*II*	*III*	*IV*
mucosal	16	17	6	1
gastrointestinal	16	16	9	2
nausea/vomiting	26	25	21	2
infection	3	8	9	2
renal	6	6	2	0
neurological	2	0	2	1
ototoxicity	4	5	1	1

Table 4. Hematological recovery after high-dose ICE: Cumulative toxicity

Median hematological parameters (range)	*Cycle 1*	*Cycle 3*
days to WBC $>1.0\times10^9$/L	9 (7–12)	9 (8–12)
days to platelets $>20\times10^9$/L	9 (6–18)	10 (7–27)
days with WBC $<0.5\times10^9$/L	5 (1–11)	5 (1–8)
days with platelets $<10\times10^9$/L	3 (1–14)	5 (1–22)*
units platelets transfused	74	62
units packed RBCs transfused	70	46

$p=0.03$ by Kruskal-Wallis test

Leukaphereses were devoid of any significant complication. These procedures yielded a median of 5.9×10^8 nucleated cells/kg (range 2.2 to 15.7), with a median value of 16.9×10^6 total CD34 positive cells/kg (range 1 to 42) and 90.1×10^4 CFU-GM/kg (range 3 to 210.7). The mean number of aphereses was three (range 0 to 5).

Toxicity of high-dose ICE was evaluated in 96 infused cycles. Nausea and vomiting was present in 77% of courses despite antiemetic premedication. However, emesis was grade 1 to 3 in the majority of cases and in only two cycles prolonged vomiting poorly responsive to planned treatment (grade 4) was observed. Mucosal and gastrointestinal toxicity was mostly of low to moderate grade. Severe infections occurred in 11% of cycles; two early deaths were registered for septic shock. Renal dysfunction, mostly grade 1 and 2, occurred after 18% of high-dose regimens. Two patients developed severe grade 3 renal failure and one died.

Time to hematologic recovery was calculated from the day of peripheral blood stem cell reinfusion. The hematologic toxicity of the first cycle compared with the third cycle demonstrated no cumulative toxicity; no difference was observed in white blood count recovery or in transfusion requirements. There was a significant difference in platelet recovery but more platelet transfusions were not required.

Of the 38 evaluable patients, 28 (74%) completed the scheduled treatment plan. The reasons for lack of completion included: three patients did not receive high-dose ICE (one refused and two due to early death in the mobilization phase); two patients received only one cycle of high-dose ICE (one because of early death during the aplasia period and one for refusal); five patients stopped at the second high-dose course (one because of death, two due to severe infection, one for concomitant thrombosis and one for severe gastrointestinal toxicity).

Thirty-five patients had restaging at one month from the end of the last high-dose ICE and were assessable for tumor response. The response rate, including partial remissions, was 86%, with 40% complete remissions and 23% near complete remissions.

Seventeen patients received chest radiotherapy after systemic treatment. At the end of the radiation therapy, complete remissions increased to 48%: three partial remissions were converted to complete remissions.

In conclusion, epirubicin and G-CSF enable sufficient peripheral blood stem cells to be mobilized to administer multiple cycles of high-dose ICE. The high-dose ICE regimen is feasible with an acceptable toxicity, even after repeated courses. The number of toxic deaths registered may be due to a "learning curve effect": in many centers these patients are the first to be treated according to a "high-dose" schedule.

Accrual of patients to the protocol is still ongoing and long-term survival analysis will be completed when 60 patients have been treated. Preliminary results are encouraging and show a response rate of 86% with 71% in complete or near complete remission.

REFERENCES

1. Seifter EJ, Ihde DC: Therapy of small cell lung cancer: A perspective on two decades of clinical research. *Semin Oncol* 15:278–299, 1988.
2. Goldie JH, Coldman AJ: The genetic origin of drug resistance in neoplasms: Implication for systemic therapy. *Cancer Res* 44:3643–3653, 1984
3. Goldie JH, Coldman AJ, Gudauskas GA: Rationale for the use of alternating non-cross resistant chemotherapy. *Cancer Treat Rep* 66:439–449, 1982.
4. Johnson DH, Einhorn LH, Birch R et al.: A randomized trial of high-dose versus conventional dose Cyclophosphamide, Doxorubicin and Vincristine for extensive stage small cell lung cancer: A phase III trial of southwestern Cancer Study Group. *J Clin Oncol* 5:1731–1738, 1987.
5. Murray N, Shah A, Osoba D et al.: Intensive weekly chemotherapy for the treatment of extensive-stage small cell lung cancer. *J Clin Oncol* 9:1632–1638, 1991.
6. Sculier JP, Paesmans M, Bureau G et al.: Multiple-drug weekly chemotherapy versus standard combination regimen in small cell lung cancer: A phase III randomized study conducted by the European Lung Cancer Working Party. *J Clin Oncol* 11:1858–1865, 1993.
7. Ihde DC, Mulshine JL, Kramer BS et al.: Prospective randomized comparison of high-dose and standard-dose Etoposide and Cisplatin chemotherapy in patients with extensive-stage small cell lung cancer. *J Clin Oncol* 12:2022–2034, 1994.
8. Souhami RL, Rudd R, Ruiz de Elvira MC et al.: Randomized trial comparing weekly versus 3-week chemotherapy in small cell lung cancer: A cancer research campaign trial. *J Clin Oncol* 12:1806–1813, 1994.
9. Norton L, Simon R: Tumor size, sensitivity to therapy, and design of treatment schedules. *Cancer Treat Rep* 61:1307–1317, 1977.
10. Klastersky J, Nicaise C, Longeval E et al.: Cisplatin, Adriamicin and Etoposide (CAV) for remission induction of small cell bronchogenic carcinoma - Evaluation of efficacy and toxicity and pilot study of a "late intensification" with autologous bone-marrow rescue. *Cancer* 50:652–658, 1982.
11. Stewart P, Buckner CD, Thomas ED et al.: Intensive chemotherapy with autologous mar-

row transplantation for small cell carcinoma of the lung. *Cancer Treat Rep* 67:1055–1059, 1983.

12. Ihde DC, Deisseroth AB, Lichter AS et al.: Late intensive combined modality therapy followed by autologous bone marrow infusion in extensive-stage small cell lung cancer. *J Clin Oncol* 4:1443–1454, 1986.
13. Humblet Y, Symann M, Bosly A et al.: Late intensification chemotherapy with autologous bone marrow transplantation in selected small cell carcinoma of the lung: A randomized study. *J Clin Oncol* 5:1864–1873, 1987.
14. Elias DA, Ayash L, Frei E III et al.: Intensive combined modality therapy for limited-stage small cell lung cancer. *J Natl Cancer Inst* 85:559–566, 1993.
15. Marangolo M, Rosti G, Amadori D et al.: High-dose etoposide and autologous bone marrow transplantation as intensification treatment in small cell lung cancer: A pilot study. *Bone Marrow Transplant* 4:405–408, 1989.
16. Jennis A, Levitan N, Pecora AL et al.: Sequential high-dose chemotherapy (HDT) with filgrastim/peripheral stem cell support (PSCS) in extensive stage small cell lung cancer (SCLC). *Proceedings of ASCO*, vol 15 [abstr 1021] May 1996.
17. Gregory WM, Birkhead BG, Souhami RL: A mathematical model of drug resistance applied to treatment for small-cell lung cancer. *J Clin Oncol* 6:457–461, 1988.
18. Arriagada R, Le Chevallier T, Pignon JP et al.: Initial chemotherapeutic doses and survival in patients with limited small cell lung cancer. *New Engl J Med* 329:1848–1852, 1993.
19. Souhami RL, Harper PG, Linch D et al.: High-dose Cyclophosphamide with autologous marrow transplantation as initial treatment of small cell carcinoma of the bronchus. *Cancer Chemother Pharmacol* 8:31–34, 1982.
20. Souhami R.L, Finn G, Gregory WM et al.: High-dose Cyclophosphamide in small cell carcinoma of the lung. *J Clin Oncol* 3:958–963, 1985.
21. Spitzer G, Farha P, Valdivieso M et al.: High-dose intensification therapy with autologous bone marrow support for limited small cell bronchogenic carcinoma. *J Clin Oncol* 4:4–13, 1986.
22. Humblet Y, Bosquée L, Weynants P et al.: No local relapse in limited disease small cell lung cancer patients after 4 cycles of concomitant radiotherapy and high-dose chemotherapy with G-CSF and peripheral blood stem cell rescue. *Proceedings of ASCO*, vol 15 [abstr 1243] May 1996.
23. Leyvraz S, Ketterer N, Perey L et al.: Intensification of chemotherapy for the treatment of solid tumors: Feasibility of a 3-fold increase in dose intensity with peripheral blood progenitor cells and granulocyte colony-stimulating factor. *Br J Cancer* 72:178–182, 1995.
24. Thatcher N, Lind M, Stout R et al.: Carboplatin, Ifosfamide and Etoposide with mid-course Vincristine and thoracic radiotherapy for "limited" stage small cell carcinoma of the bronchus. *Br J Cancer* 60:98–101, 1989.
25. Rosti G, Albertazzi L, Ferrante P et al.: Epirubicine + G-CSF as peripheral blood progenitor cells (PBPC) mobilising agents in breast cancer patients. *Ann Oncol* 6:1045–1047, 1995.

DOSE-INTENSIVE THERAPY IN SMALL CELL LUNG CANCER

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ABSTRACT

Approximately 30,000 new cases of small cell lung cancer (SCLC) are reported each year in the United States. SCLC is 98% associated with heavy smoking and presents at a median age of 60–65 years: thus, the application of dose-intensive therapy of lung cancer patients may be complicated by underlying smoking-related comorbid disease and enhanced risk of secondary smoking-related malignancies. The strategies of intensifying induction therapy, multicycle dose-intensive combination therapies, chest radiotherapy and stem cell purging trials will be discussed.

While excellent immediate palliation from combination chemotherapy is achieved, the long-term progression-free survival (PFS) remains poor. By two years, only 20–40% of limited (LD) and <5% of extensive stage (ED) patients remain alive. Numerous combination regimens constructed from the established agents produced almost identical short-term and long-term results, an observation that strongly suggests that many of our systemic drugs kill off the same tumor subpopulation but fail to eradicate a central core of tumor stem cells, presumably enriched for various in vivo resistance mechanisms. The identification of these residual cancer cells and systematic evaluation of their biologic characteristics may guide strategies to specifically target these cells. Minimal residual tumor (MRT) characterization could then be employed as a determinant for additional treatment such as modification of chemotherapy, tumor vaccination or other form of biological therapy. To this end, the detection of heterogeneity and analysis of patterns of coexpression of various markers forms the thrust of our program in the detection of MRT.

At the DFCI and Beth Israel Hospital, we have over 40 patients with LD and over 25 with ED SCLC responding to conventional dose induction therapy with high-dose combination alkylating agents. Of the original cohort of 36 LD SCLC, 29 were in or near CR prior to treatment with high-dose CBP with marrow support followed by chest and prophylactic cranial radiotherapy, With minimum follow-up of 22 months (out to 10 years) after completion of high-dose chemotherapy, 52% remain disease-free. Of the ED or extrapulmonary patients, approximately 20% have PFS >2 years after high-dose therapy (manuscript in preparation). Local

regional represents about 50% of all relapses. The CALGB & SWOG are perforating a phase II feasibility trial based on the DFCI/BIH experience: patients under age 60 with LD are treated with four cycles of cisplatin and etoposide with concurrent twice-daily chest radiotherapy. Those patients achieving CR or near CR receive high-dose CBP with autologous stem cell support. It is hoped that this will lead to a phase III trial testing the concept of dose intensification. Moreover, studies exploring CD34 selection of stem cells are underway.

High-dose therapy can result in prolonged PFS. It may also produce MRT in an additional cohort of patients. Biologic targets such as replacement of RB, 3p and/or p53 function, interference with autocrine or paracrine growth loops, or immunologic therapy (IL2, IL12, immunotoxins, tumor vaccine) are probably most effective in the setting of MRT; combined approaches may therefore be of additional benefit in such patients.

INTRODUCTION

Rationale for dose-intensive chemotherapy in SCLC

In the United States, 180,000 new cases of lung cancer are diagnosed per year and the disease is currently the leading cause of death from cancer in both men and women, resulting in 163,000 deaths.[1] Of these, approximately 15–25% (about 30,000) are new cases of small cell lung cancer (SCLC). The use of dose-intensive therapy in lung cancer must allow for an older patient population (median age 60–65 years) who have underlying smoking-related, cardiovascular and pulmonary disease, together with an increased risk of secondary smoking-related malignancies.

The principles of dose-response and combination chemotherapy, demonstrated in laboratory models, are basic to the design of regimens for malignancies that are now curable with chemotherapy.[2–6] Moreover, combinations of active noncross-resistant agents have proved critical to decreasing the emergence of drug resistance. Further, agents with different dose-limiting toxicities combined may result in subadditive toxicity. Hematopoietic stem cell support, using marrow or peripheral blood progenitor cells, allows evaluation of dose-response to the limits of organ tolerance.

We will review evidence for the contribution of dose to response and survival in the treatment of SCLC, the clinical trials designed to address these relationships and suggest future directions.

Since SCLC usually presents with disseminated disease, treatment strategies have focused on systemic therapy. A high degree of responsiveness has been observed to multiple cytotoxic drugs both as single agents and in combination. Overall and complete response rates from combination chemotherapy or chemo-radiotherapy for limited stage SCLC (confined to the chest within a single radiation port) are 80–90% and 50%. While excellent immediate palliation is achieved, the

long-term progression-free survival (PFS) remains poor. By two years, only 20–40% of patients remain alive, of whom only half survive five years. For extensive stage disease, expected overall and complete response rates are 60–80% and 15–20%, respectively;[7] however, fewer than 5% of patients survive two years.[8]

Numerous chemotherapeutic agents have major activity against SCLC. The most important of these are etoposide (and teniposide), cisplatin (and carboplatin), ifosfamide, cyclophosphamide, vincristine and doxorubicin. Several new agents appear to have at least equivalent activity compared with these established drugs, including taxanes (paclitaxel and taxotere), gemcitabine and the topoisomerase I inhibitors (topotecan, irinotecan). Numerous combinations constructed from the established agents are effective, but remarkably produce almost identical short-term and long-term results. It remains to be seen whether the newer agents will improve long-term survival.

Log-linear or near linear dose-response curves are obtained for numerous chemotherapeutic agents, particularly alkylating agents and radiation, but not antimetabolites, in preclinical experiments in vitro and in vivo.[3] The clinical correlation between chemotherapy dose and response for SCLC was originally demonstrated by Cohen et al. in 1977[9] using cyclophosphamide, lomustine and methotrexate, and later confirmed by others.[10–14] Higher response rates in patients, both complete and partial, and a modestly longer median survival time in high-dose groups compared with groups given conventional doses of these same agents have been shown in several studies.[9,12]

Klasa et al. performed a meta-analysis using the methodology of Hryniuk and Bush[15] to ascertain whether dose intensity (expressed as drug dose given per m^2 per week) of agents either alone or in combination regimens correlated with response or survival in SCLC trials.[16] In performing this analysis, it was assumed that all drugs were therapeutically equivalent, and that cross resistance (or synergy) between drugs, peak drug concentrations, or schedule and duration of drug exposure have no effect. The CAV, CAE and EP regimens were evaluated over narrow dose intensity (DI) ranges (DI: 0.8–1; 0.6–1.7; 0.75–1.8, respectively). The DI for CAV and CAE were associated with longer median survival in extensive disease patients, but the effects observed were small.

Most randomized trials evaluating DI have been conducted in extensive disease or mixed stage SCLC patients. Arriagada et al. recently reported the results of a trial randomizing limited stage patients to six cycles of conventional dose chemotherapy with modestly intensified chemotherapy for the first cycle only or the same therapy without intensification, with the higher dose arm showing a complete response and survival advantage.[17] It can be argued that those randomized trials showing survival advantage generally compared less than standard to full dose therapy, whereas an incremental DI between 1–2 times greater than conventional dose has not clearly demonstrated response or survival advantage.

Table 1. ABMT for SCLC: Literature (373 patients)

	#	% CR	% DF*	% Toxic deaths
relapsed	49	20	0	12
initial Rx	98	41	7	7
S/P induction therapy:				
1st PR/SD	130	35	5	12
1st CR				
ED	25	100	16	8
LD	71	100	34	1

**Median F/U 3 years with wide variability.*

Currently available cytokines (e.g., GM-CSF and G-CSF) shorten chemotherapy-induced myelosuppression and consequent febrile neutropenia.[18] Cumulative thrombocytopenia remains dose-limiting. Currently, dose and DI can be increased by only 1.5–2 fold with cytokine use; differences, as detailed above, are unlikely to produce survival advantages. The effect of various thrombopoietins on achievable DI remains to be seen.

Studies of ABMT in small-cell lung cancer are reviewed below and summarized in Table 1. Patients in these studies were analyzed according to response status: either relapsed or refractory; untreated; or responding to first-line chemotherapy (partial or complete response), and their extent of disease (limited or extensive stage). They were then pooled for aggregate relapse-free and overall survival characteristics.

Fourteen studies included patients who had either relapsed or refractory disease.[19–32] Of 52 evaluable patients, complete and partial responses were observed in 19% and 37%, respectively. However, the median duration of these responses was roughly two months and median survival was approximately three months. Combination chemotherapy regimens, especially those containing multiple alkylating agents, appeared to be more effective (overall response rate 58%, complete response rate 26%), but more toxic (treatment-related mortality rate 18% versus 6%). Further, these strategies did not appear to improve duration of response or long-term survival. In summary, the observed high overall and complete response rates support a dose-response relationship, but the effects were insufficient to improve survival.

High dose therapy with autologous bone marrow transplant as initial treatment has been reported in 103 patients with SCLC, of whom 71% had limited disease.[33–40] The overall and complete response rates of 84% and 42%, and the relapse-free, 2-year and overall survivals were comparable to treatment with conventional multicycle regimens. However, it should be noted that patients with newly diagnosed SCLC frequently have threatening complications from uncontrolled disease, and

Table 2. ABMT for SCLC

Randomized trial: Conventional versus high dose		
101 given combination chemo & PCI 45 randomized (32 limited disease):		
Limited Disease	*ABMT*	*Conventional*
# randomized	16	16
PR to CR	77%	0%
DF > 2 yrs	19%	0%
chest-only relapse	70%	100%

Humblet et al., *JCO, 1987.*

therefore may not be optimal candidates for high-dose intensification. In addition, untreated autografts have a high rate of potential tumor cell contamination.

Approximately 282 patients responding to first line chemotherapy received high-dose chemotherapy with autologous marrow support as intensification.[41] Conversion to complete response occurred in 50% of partial responders. However, there was no overall survival benefit. It would seem that most promise is demonstrated in patients with limited stage disease in complete response at the time of high-dose therapy. Within this subset (excluding the DFCI/BIH trial described below), about 35% remain disease-free at the time of publication with a median follow-up over 3 years.

It should be emphasized that much of the experience with high-dose therapy for SCLC occurred during the developmental phase of high-dose therapy for solid tumors. Therefore, many of these high-dose trials employed either single chemotherapeutic agents with or without other drugs at low dose and/or chest radiotherapy,[35,36,42–46] single alkylating agents alone with or without chest radiotherapy,[36,38,41,47–50] or combination alkylating agents with or without chest radiotherapy.[23,26,37,51–55] Since high treatment-related morbidity and mortality was encountered, investigators concluded that potential benefits did not justify the risks of the procedure.

Humblet et al. treated 101 SCLC patients with chemotherapy for 5 cycles, and then randomized 45 responding patients to one cycle of either high-dose or conventional dose therapy (Table 2).[53] No chest radiotherapy was given. A clear dose-response was demonstrated with conversion from partial to complete response in about 75% of patients after high-dose therapy compared with none after conventional dose treatment. Disease-free, but not overall, survival was significantly enhanced. However, an 18% toxic death rate on the ABMT arm led the investigators to conclude that dose-intensive therapy should not be considered a standard therapy in SCLC.

As observed with conventional dose therapy, patients will generally relapse in sites of prior tumor involvement following high-dose stem cell supported treatment.[35,49] The high rate of regional relapse may be explained by greater tumor burden in the chest, and host pharmaco-dynamic with local environmental drug delivery or resistance factors also playing a role. Since chest relapse occurs in about 90% of individuals following chemotherapy alone, and in 60% after radiotherapy, radiotherapy to sites of bulk disease is likely to represent an essential component in curative treatment approaches.

At the Dana-Farber Cancer Institute and Beth Israel Hospital, we have treated over 40 patients with limited stage and over 25 patients with extensive stage SCLC using high-dose combination alkylating agents following response to conventional dose induction therapy. Of the original cohort of 36 limited stage IIIA or B SCLC, 29 were in or near complete response before treatment with high-dose cyclophosphamide, carmustine and cisplatin with marrow support followed by chest and prophylactic cranial radiotherapy.[55] With minimum follow-up of 21 months after completion of high-dose chemotherapy (range 21 months to 9 years), 52% remain disease-free. Of the extensive stage patients, approximately 20% remain progression-free greater than two years after high-dose therapy (manuscript in preparation). Local regional recurrence represents about 50% of all relapses.

FUTURE DIRECTIONS

Intensification of involved field radiotherapy

Numerous randomized trials have demonstrated that chest radiotherapy provides a 25% improvement in local-regional control and is associated with a 5% increase in long-term PFS for patients with limited stage SCLC.[56,57] However, even with 4500–5000 cGy thoracic radiotherapy, chest relapse remains unacceptably high (about a 60% actuarial risk of local relapse by 3 years),[58–60] and may be underestimated due to the competing risk of systemic relapse.[61] Further enhancement of local-regional control may increase the proportion of long-term survivors since chest-only relapse is observed in about 40% of patients. If high-dose chemotherapy improves systemic control, initial failure in local-regional sites may become more prevalent.

Few trials have examined the dose intensity of chest radiotherapy. One phase III trial recently reported compared 45 Gy chest radiotherapy given either daily over 5 weeks or twice daily over 3 weeks concurrent with cisplatin and etoposide chemotherapy.[62] Intensified chest radiotherapy increased chest control from 39% to 52% actuarial at 2–3 years. In a phase I trial, cohorts of 5–6 patients with limited stage SCLC were given thoracic radiotherapy concurrently with cisplatin and etoposide either as daily 180 cGy fractions or as twice daily 150 cGy

fractions.[63] The maximum tolerated doses appear to be 45 Gy for twice daily administration and 66–70 Gy when given once daily. Thus marked intensification of radiotherapy dose appears to be possible and should be evaluated in a randomized setting.

The Cancer and Leukemia Group B and Southwest Oncology Group have activated a phase II feasibility trial stemming from the DFCI/BIH experience described above. Patients under age 60 with limited stage are treated with four cycles of cisplatin and etoposide with concurrent twice-daily chest radiotherapy to 45 Gy (150 cGy fractions). Those patients achieving complete or near complete response receive high-dose cyclophosphamide, cisplatin and carmustine (CBP) with autologous stem cell support. Upon recovery, prophylactic cranial irradiation is given. It is hoped that this will lead ultimately to a phase III trial testing the concept of increased radiotherapy dose during intensification.

Intensification of induction

Induction therapy reduces tumor burden and allows selection of patients with chemosensitive disease to undergo intensification. Moreover, most patients present with rapidly progressive systemic and local symptoms from SCLC. With therapy, they can improve performance status dramatically. Conversely, chemoresistance may develop during induction. Several strategies may circumvent such resistance. As suggested by the Arriagada trial,[17] initial intensification of induction may improve overall disease-free and overall survival. Extension of this concept would be to administer multicycle dose-intensive combination therapies such as those supported by cytokines and peripheral blood progenitor cells either using repeated cycles of the same regimen[64] or preferably utilizing a sequence of different agents.[65–67]

Minimal residual tumor/autograft involvements

Since stem cells must be protected from the high-dose therapy to make dose escalation feasible, stem cell contamination with tumor cells surviving induction therapy may be a source of relapse. While there is extremely persuasive evidence to support the fact that residual tumor cells contribute to relapse (for example, in hematologic malignancies and neuroblastoma),[68–70] it is unclear in SCLC whether currently available purging methods are sufficiently effective to impact on survival. Indeed, it is not known whether residual tumor in the autograft directly contributes to relapse or simply reflects a residual burden of chemotherapy-resistant tumor cells that is unlikely to be eradicated by high-dose therapy. Gene marking experiments, which might help clarify this issue in solid tumors, have yet to be informative.[71]

The bone marrow is one of the most common sites for metastases. A number of small trials have demonstrated that 13–54% of limited and 44–77% of extensive stage SCLC with negative marrows histologically had subclinical SCLC

involvement at diagnosis when examined by immunohistochemical techniques with a sensitivity of detection of one in 10^4 cells.[72–76] Less data is available for patients undergoing therapy or in response to therapy. Two small series suggest a high rate of residual contamination after treatment. Hay et al. reported 83% positive screens with no obvious decrement with therapy.[77] Eight of 12 limited stage SCLC patients in response had residual tumor cells in the bone marrow detectable by a panel of monoclonal antibodies in a small series from Leonard et al.[78] Six of these eight patients subsequently relapsed.

In a study of patients with metastatic SCLC or breast cancer undergoing induction, peripheral blood progenitor cells mobilized with G-CSF during the first cycle of chemotherapy had demonstrable circulating tumor cells.[79] Anecdotally, these investigators observed less or absent mobilization of tumor cells after the second cycle of chemotherapy, supporting the contention that in vivo chemotherapy as induction can "purge" the patient and the autologous stem cell source.

The observation that numerous chemotherapeutic agents possess major activity against SCLC, yet the clinical outcomes after numerous different combinations of these agents remain uniformly disappointing, strongly suggests that many systemic drugs kill the same tumor subpopulation, but fail to eradicate a central core of tumor stem cells, presumably enriched for various in vivo resistance mechanisms. The identification of these residual cancer cells and systematic evaluation of their biologic characteristics may generate strategies more specifically targeted against these cells. Minimal residual tumor characterization may then be seen as a determinant for additional treatment such as modification of chemotherapy, tumor vaccination or other forms of biological therapy. To this end, the detection of heterogeneity and analysis of patterns of coexpression of various markers forms the thrust of our own program in the detection of these rare cells.

In summary, limited information is available about the incidence of tumor contamination of hematopoietic tissues or the clinical impact this may have in patients. With the increasing interest and expanding technology in the detection of microscopic residual disease, prospective trials to evaluate the clinical significance of marrow or peripheral blood tumor contamination and the impact of novel stem cell sources to support high-dose therapy are needed.

High-dose therapy can result in prolonged PFS. It may also produce minimal tumor burden in an additional cohort of patients. To the extent that additional targets of residual tumor cells can be identified for novel treatment strategies and modalities, high-dose therapy may be of great value in the treatment of SCLC. Biologic targets such as replacement of RB 3p and/or p53 function, interference with autocrine or paracrine growth loops, or immunologic therapy (including IL2, IL12, immunotoxins and tumor vaccines) are probably most effective in the setting of minimal residual tumor; combined approaches may therefore be of additional benefit in such patients.

REFERENCES

1. Boring CC, Squires TS, Tong TT: Cancer Statistics. *A Cancer Journal for Clinicians.* 44:19–51, 1994.
2. Frei E III: Antitumor agents-dose response curve, clinical and experimental consideration. *Exp Hematol* 7(suppl):262–264, 1979.
3. Teicher BA: Preclinical models for high-dose therapy. In: Armitage JO, Antman KH (eds) High-dose Cancer Therapy: Pharmacology, Hematopoietins, Stem cells. Baltimore: Williams and Wilkins, 1992, pp 14–42.
4. Frei E III: Combination cancer chemotherapy: Presidential address. *Cancer Res* 32:2593–2607, 1972.
5. Frei III E, Canellos GP: Dose, a critical factor in cancer chemotherapy. *Am J Med* 69:585–594, 1980.
6. Frei E III, Antman KH: Combination chemotherapy, dose, and schedule: Section XV, principles of chemotherapy. In: Holland JF, Frei E III, Bast RC Jr, Kufe DW, Morton DL, Weichselbaum RR (eds) Cancer Medicine. Philadelphia: Lea and Febiger, 1993, pp 631–639.
7. Seifter EJ, Ihde DC: Therapy of small cell lung cancer: A perspective on two decades of clinical research. *Semin Oncol* 15:278–299, 1988.
8. Osterlind K, Hansen HH, Hansen M, Dombernowsky P, Andersen PK: Long-term disease-free survival in small-cell carcinoma of the lung: A study of clinical determinants. *J Clin Oncol* 4:1307–1313, 1986.
9. Cohen MH, Creaven PJ, Fossieck BE et al.: Intensive chemotherapy of small cell bronchogenic carcinoma. *Cancer Treat Rep* 61:349–354, 1977.
10. Brower M, Ihde DC, Johnston-Early A et al.: Treatment of extensive stage small cell bronchogenic carcinoma: Effects of variation in intensity of induction chemotherapy. *Am J Med* 75:993–1000, 1983.
11. Johnson DH, Einhorn LH, Birch R et al.: A randomized comparison of high dose versus conventional dose cyclophosphamide, doxorubicin, and vincristine for extensive stage small cell lung cancer: a phase III trial of the Southeastern Cancer Study Group. *J Clin Oncol* 5:1731-1738, 1987.
12. Mehta C, Vogl SE: High-dose cyclophosphamide in the induction therapy of small cell lung cancer: Minor improvements in rate of remission and survival. *Proc AACR* 23:155, 1982.
13. Figueredo AT, Hryniuk WM, Strautmanis I et al.: Co-trimoxazole prophylaxis during high-dose chemotherapy of small-cell lung cancer. *J Clin Oncol* 3:54–64, 1985.
14. Ihde DC, Mulshine JL, Kramer BS, Steinberg SM, Linnoila RI, Gazdar AF, Edison M, Phelps RM, Lesar M, Phares JC, Grayson J, Minna JD, Johnson BE: Prospective randomized comparison of high-dose and standard-dose etoposide and cisplatin chemotherapy in patients with extensive-stage small-cell lung cancer. *J Clin Oncol* 12:2022–2034, 1994.
15. Hryniuk W, Bush H: The importance of dose intensity in chemotherapy of metastatic breast cancer. *J Clin Oncol* 2:1281–1288, 1984.
16. Klasa RJ, Murray N, Coldman AJ: Dose-intensity meta-analysis of chemotherapy regimens in small-cell carcinoma of the lung. *J Clin Oncol* 9:499–508, 1991.
17. Arriagada R, Le Chevalier T, Pignon J-P, Riviere A, Monnet I, Chomy P, Tuchais C,

Tarayre M, Ruffie P: Initial chemotherapeutic doses and survival in patients with limited small-cell lung cancer. *N Engl J Med* 329:1848–1852, 1993.

18. Crawford J, Ozer H, Stoller R et al.: Reduction by granulocyte colony-stimulating factor of fever and neutropenia induced by chemotherapy in patients with small-cell lung cancer. *N Engl J Med* 325:164–170, 1991.
19. Douer D, Champlin RE, Ho WG et al.: High-dose combined-modality therapy and autologous bone marrow transplantation in resistant cancer. *Am J Med* 71:973–976, 1981.
20. Harada M et al.: Combined-modality therapy and autologous bone marrow transplantation in the treatment of advanced non-Hodgkin's lymphoma and solid tumors: The kanawaza experience. *Transplant Proc* 4:733–737, 1982.
21. Lazarus HM, Spitzer TR, Creger RT: Phase I trial of high-dose etoposide, high-dose cisplatin, and reinfusion of autologous bone marrow for lung cancer. *Am J Clin Oncol* 13:107–112, 1990.
22. Phillips GL, Fay JW, Herzig GP et al.: Nitrosourea (BCNU), NSC #4366650 and cryopreserved autologous marrow transplantation for refractory cancer: A phase I-II study. *Cancer* 52:1792–1802, 1983.
23. Stahel RA, Takvorian RW, Skarin AT, Canellos GP: Autologous bone marrow transplantation following high-dose chemotherapy with cyclophosphamide, BCNU, and VP-16 in small cell carcinoma of the lung and a review of current literature. *Eur J Cancer Clin Oncol* 20:1233–1238, 1984.
24. Wolff SW, Fer MF, McKay CM et al.: High-dose VP-16-213 and autologous bone marrow transplantation for refractory malignancies: A phase I study. *J Clin Oncol* 1:701–705, 1983.
25. Pico JL, Beaujean F, Debre M, et al.: High dose chemotherapy (HDC) with autologous bone marrow transplantation (ABMT) in small cell carcinoma of the lung (SCCL) in relapse. *Proc. ASCO* 1983;2:206.
26. Pico JL, Baume D, Ostronoff M et al.: Chimiotherapie à hautes doses suivie d'autogreffe de moelle osseuse dans le traitement du cancer bronchique a petites cellules. *Bull Cancer* 74:587–595, 1987.
27. Postmus PE, Mulder NH, Elema JD: Graft versus host disease after transfusions of non-irradiated blood cells in patients having received autologous bone marrow. *Eur J Cancer* 24:889–894, 1988.
28. Rushing DA, Baldauf MC, Gehlsen JA et al.: High-dose BCNU and autologous bone marrow reinfusion in the treatment of refractory or relapsed small cell carcinoma of the lung (SCCL). *Proc ASCO* 3:217, 1984.
29. Spitzer G, Dicke KA, Verma DS, Zander A, McCredie KB: High-dose BCNU therapy with autologous bone marrow infusion: Preliminary observations. *Cancer Treat Rep* 63:1257–1264, 1979.
30. Spitzer G, Dicke KA, Latam J et al.: High-dose combination chemotherapy with autologous bone marrow transplantation in adult solid tumors. *Cancer* 45:3075–3085, 1980.
31. Eder JP, Antman K, Shea TC, Elias A, Teicher B, Henner WD, Schryber SM, Holden S, Finberg R, Critchlow J, Flaherty M, Mick R, Schnipper LE, Frei E III: Cyclophosphamide and thiotepa with autologous bone marrow transplantation in patients with solid tumors. *J Natl Cancer Inst* 80:1221–1226, 1988.

32. Elias AD, Ayash LJ, Wheeler C, Schwartz G, Tepler I, Gonin R, McCauley M, Mazanet R, Schnipper L, Frei E III, Antman KH: A phase I study of high-dose ifosfamide, carboplatin, and etoposide with autologous hematopoietic stem cell support. *Bone Marrow Transplant* 15: 373–379, 1995.
33. Littlewood TJ, Spragg BP, Bentley DP.: When is autologous bone marrow transplantation safe after high-dose treatment with etoposide. *Clin Lab Haemat* 7:213–218, 1985.
34. Littlewood TJ, Bentley DP, Smith AP: High-dose etoposide with autologous bone marrow transplantation as initial treatment of small cell lung cancer: A negative report. *Eur J Respir Dis* 68:370–374, 1986.
35. Souhami RL, Hajichristou HT, Miles DW, Earl HM, Harper PG, Ash CM, Goldstone AH, Spiro SG, Geddes DM, Tobias JS: Intensive chemotherapy with autologous bone marrow transplantation for small cell lung cancer. *Cancer Chemother Pharmacol* 24:321–325, 1989.
36. Lange A, Kolodziej J, Tomeczko J, Toporski J, Sedzimirska M, Jazwiec B, Bochenska J, Mroz E, Bielecka E, Was A, Glejzer O, Tomaszewska-Toporowska B, Jagas M, Zukowska B, Spaltenstein A, Bieranowska D, Klimczak A: Aggressive chemotherapy with autologous bone marrow transplantation in small cell lung carcinoma. *Archiv Immunol et Therap Exp* 39:431–439, 1991.
37. Nomura F, Shimokata K, Saito H, Watanabe A, Saka H, Sakai S, Kodera Y, Saito H: High dose chemotherapy with autologous bone marrow transplantation for limited small cell lung cancer. *Jpn J Clin Oncol* 20:94–98, 1990.
38. Spitzer G, Farha P, Valdivieso M et al.: High-dose intensification therapy with autologous bone marrow support for limited small-cell bronchogenic carcinoma. *J Clin Oncol* 4:4–13, 1986.
39. Johnson DH, Hande KR, Hainsworth JD, Greco FA: High-dose etoposide as single-agent chemotherapy for small cell carcinoma of the lung. *Cancer Treat Rep* 67:957–958, 1983.
40. Elias A, Cohen BF: Dose intensive therapy in lung cancer. In: Armitage JO, Antman KH (eds) High-Dose Cancer Therapy: Pharmacology, Hematopoietins, Stem Cells, 2nd Edition. Baltimore: Williams and Wilkins, 1995, pp 824–846.
41. Farha P, Spitzer G, Valdivieso M et al.: High-dose chemotherapy and autologous bone marrow transplantation for the treatment of small cell lung carcinoma. *Cancer* 52:1351–1355, 1983.
42. Marangolo M, Rosti G, Ravaioli A et al.: Small cell carcinoma of the lung (SCCL): High-dose (HD) VP-16 and autologous bone marrow transplantation (ABMT) as intensification therapy: Preliminary results. *Int J Cell Cloning* 3:277, 1985.
43. Smith IE, Evans BD, Harland SJ et al.: High-dose cyclophosphamide with autologous bone marrow rescue after conventional chemotherapy in the treatment of small cell lung carcinoma. *Cancer Chemother Pharmacol* 14:120–124, 1985.
44. Banham S, Burnett A, Stevenson R et al.: Pilot study of combination chemotherapy with late dose intensification and autologous bone marrow rescue in small cell bronchogenic carcinoma. *Br J Cancer* 42:486, 1982.
45. Banham S, Loukop M, Burnett A et al.: Treatment of small cell carcinoma of the lung with late dosage intensification programmes containing cyclophosphamide and mesna. *Cancer Treat Rev* 10(suppl A):73–77, 1983.

46. Burnett AK, Tansey P, Hills C et al.: Haematologic reconstitution following high dose and Supralethal Chemoradiotherapy using stored non-cryopreserved autologous bone marrow. *Br J Haematol* 54:309–316, 1983.
47. Ihde DC, Diesseroth AB, Lichter AS et al.: Late intensive combined modality therapy followed by autologous bone marrow infusion in extensive stage small-cell lung cancer. *J Clin Oncol* 4:1443–1454, 1986.
48. Cunningham D, Banham SW, Hutcheon AH et al.: High-dose cyclophosphamide and VP-16 as late dosage intensification therapy for small cell carcinoma of lung. *Cancer Chemother Pharmacol* 15:303–306, 1985.
49. Sculier JP, Klastersky J, Stryckmans P et al.: Late intensification in small-cell lung cancer: A phase I study of high doses of cyclophosphamide and etoposide with autologous bone marrow transplantation. *J Clin Oncol* 3:184–191, 1985.
50. Klastersky J, Nicaise C, Longeval E et al.: Cisplatin, adriamycin and etoposide (CAV) for remission induction of small-cell bronchogenic carcinoma: Evaluation of efficacy and toxicity and pilot study of a "late intensification" with autologous bone marrow rescue. *Cancer* 50:652–658, 1982.
51. Cornbleet M, Gregor A, Allen S, Leonard R, Smyth J: High dose melphalan as consolidation therapy for good prognosis patients with small cell carcinoma of the bronchus (SCCB). *Proc ASCO* 3:210, 1984.
52. Wilson C, Pickering D, Stewart S, Vallis K, Kalofonos H, Cross A, Snook D, Goldman JM, McKenzie CG, Epenetos AA: High dose chemotherapy with autologous bone marrow rescue in small cell lung cancer. *In vivo* 2:331–334, 1988.
53. Humblet Y, Symann M, Bosly A et al.: Late intensification chemotherapy with autologous bone marrow transplantation in selected small-cell carcinoma of the lung: A Randomized Study. *J Clin Oncol* 5:1864–1873, 1987.
54. Stewart P, Buckner CD, Thomas ED et al.: Intensive chemoradiotherapy with autologous marrow transplantation for small cell carcinoma of the lung. *Cancer Treat Rep* 67:1055–1059, 1983.
55. Elias AD, Ayash L, Frei E III, Skarin AT, Hunt M, Wheeler C, Schwartz G, Mazanet R, Tepler I, Eder JP, McCauley M, Herman T, Schnipper L, Antman KH: Intensive combined modality therapy for limited stage small cell lung cancer. *J Natl Cancer Inst* 85:559–566, 1993.
56. Pignon JP, Arriagada R, Ihde DC et al.: A meta-analysis of thoracic radiotherapy for small-cell lung cancer. *N Engl J Med* 327:1618–1624, 1992.
57. Warde P, Payne D: Does thoracic irradiation improve survival and local control in limited-stage small-cell carcinoma of the lung? A meta-analysis. *J Clin Oncol* 10:890–895, 1992.
58. Perry MC, Eaton WL, Propert KJ, Ware JH, Zimmer B, Chahinian AP, Skarin A, Carey RW, Kreisman H, Faulkner C, Comis R, Green MR: Chemotherapy with or without radiation therapy in limited small-cell carcinoma of the lung. *New Engl J Med* 316:912–918, 1987.
59. Bunn PA, Lichter AS, Makuch RW, Cohen MH, Veach SR, Matthews MJ, Anderson AJ, Edison M, Glatstein E, Minna JD, Ihde DC: Chemotherapy alone or chemotherapy with chest radiation therapy in limited stage small cell lung cancer. *Ann Int Med* 106:655–662, 1987.

60. Kies MS, Mira JG, Crowley JJ, Chen TT, Pazdur R, Grozea PN, Rivkin SE, Coltman CA, Ward JH, Livingston RB: Multimodal therapy for limited small-cell lung cancer: A randomized study of induction combination chemotherapy with or without thoracic radiation in complete responders; and with wide-field versus reduced-field radiation in partial responders: A Southwest Oncology Group Study. *J Clin Oncol* 5:592–600, 1987.
61. Arriagada R, Kramar A, Le Chevalier T, De Cremoux H: Competing events determining relapse-free survival in limited small-cell lung carcinoma. *J Clin Oncol* 10:447–451, 1992.
62. Turrisi AT, Kim K, Johnson DH, Komaki R, Sause W, Curran W, Livingston R, Wagner H, Blum R: Daily (qd) v twice-daily (bid) thoracic irradiation (TI) with concurrent cisplatin-etoposide (PE) for limited small cell lung cancer (LSCLC): Preliminary results on 352 randomized eligible patients. *Lung Cancer* 11(suppl 1):172 (667), 1994.
63. Choi NC, verbal communication. CALGB Fall Meeting, Atlanta, GA, 11/94.
64. Tepler I, Cannistra SA, Frei E III, Gonin R, Anderson KC, Demetri G, Niloff J, Goodman H, Muntz H, Muto M, Sheets E, Elias AD, Mazanet R, Wheeler C, Ayash L, Schwartz G, McCauley M, Gaynes L, Harvey S, Schnipper LE, Antman KH: Use of peripheral blood progenitor cells abrogates the myelotoxicity of repetitive outpatient high-dose carboplatin and cyclophosphamide chemotherapy. *J Clin Oncol* 11:1583–1591, 1993.
65. Crown J, Wasserheit C, Hakes T et al.: Rapid delivery of multiple high-dose chemotherapy courses with granulocyte colony-stimulating factor and peripheral blood-derived hematopoietic progenitor cells. *J Natl Cancer Inst* 84:1935–1936, 1992.
66. Gianni AM, Siena S, Bregni M et al.: Prolonged disease-free survival after high-dose sequential chemo-radiotherapy and hemopoietic autologous transplantation in poor prognosis Hodgkin's disease. *Ann Oncol* 2:645–653, 1991.
67. Ayash L, Elias A, Wheeler C, Reich E, Schwartz G, Mazanet R, Tepler I, Warren D, Lynch C, Gonin R, Schnipper L, Frei E III, Antman K: Double dose-intensive chemotherapy with autologous marrow and peripheral blood progenitor cell support for metastatic breast cancer: A feasibility study. *J Clin Oncol* 12:37–44, 1994.
68. Gribben JG, Freedman AS, Neuberg D, Roy DC, Blake KW, Woo SD, Brossbard ML, Rabinowe SN, Coral F, Freeman GJ, Ritz J, Nadler LM: Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B-cell lymphoma. *New Engl J Med* 325:1525–1533, 1991.
69. Brenner MK, Rill DR, Moen RC, Krance RA, Mirro J Jr, Anderson WF, Ihle JN: Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. *Lancet* 1993; 341, i: 85–86. and verbal communication, Stem Cell Conference, San Diego, March, 1993.
70. Brenner MK, Rill DR: Gene marking to improve the outcome of autologous bone marrow transplantation. *J Hematother* 3:33–36, 1994.
71. O'Shaughnessy JA, Cowan KH, Cottler-Fox M, Carter CS, Doren S, Leitman S, Wilson W, Moen R, Nienhuis AW, Dunbar CE: Autologous transplantation of retrovirally-marked CD34-positive bone marrow and peripheral blood cells in patients with multiple myeloma or breast cancer. *Proc ASCO* 13:296 (963), 1994.
72. Stahel RA, Mabry M, Skarin AT, Speak J, Bernal SD: Detection of bone marrow metastasis in small-cell lung cancer by monoclonal antibody. *J Clin Oncol* 3:455–461, 1985.

73. Canon JL, Humblet Y, Lebacq-Verheyden AM, Manouvriez P, Bazin H, Rodhain J, Prignot J, Symann M: Immunodetection of small cell lung cancer metastases in bone marrow using three monoclonal antibodies. *Eur J Cancer Oncol* 24:147–150, 1988.
74. Trillet V, Revel D, Combaret V, Favrot M, Loire R, Tabib A, Pages J, Jacquermet P, Bonmartin A, Mornex JF, Cordier JF, Binet R, Brune J: Bone marrow metastases in small cell lung cancer: Detection with magnetic resonance imaging and monoclonal antibodies. *Br J Cancer* 60:83–88, 1989.
75. Berendsen HH, De Leij L, Postmus PE, Ter Haar JG, Popperna S, The TH: Detection of small cell lung cancer metastases in bone marrow aspirates using monoclonal antibody directed against neuroendocrine differentiation antigen. *J Clin Pathol* 41:273–276, 1988.
76. Beiske K, Myklebust AT, Aamdal S, Langhom R, Jakobsen E, Fodstad O: Detection of bone marrow metastases in small cell lung cancer patients. *Am J Pathol* 141:531–538, 1992.
77. Hay FG, Ford A, Leonard RCF: Clinical applications of immunocytochemistry in the monitoring of the bone marrow in small cell lung cancer (SCLC). *Int J Cancer* (suppl 2):8–10, 1988.
78. Leonard RCF, Duncan LW, Hay FG: Immunocytological detection of residual marrow disease at clinical remission predicts metastatic relapse in small cell lung cancer. *Cancer Res* 50:6545–6548, 1990.
79. Brugger W, Bross KJ, Glatt M, Weber F, Mertelsmann R, Kanz L: Mobilization of tumor cells and hematopoietic progenitor cells into peripheral blood of patients with solid tumors. *Blood* 83:636–640, 1994.

AUTOTRANSPLANTATION IN NEUROBLASTOMA

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ABSTRACT

Outstanding issues in autotransplantation for poor prognosis neuroblastoma (NBL) continue to be addressed in cooperative group and institutional studies. The randomized trial CCG-3891 comparing chemotherapy with autologous purged bone marrow transplantation (autoBMT) completed patient accrual earlier in 1996 and is awaiting analysis. A nontotal body irradiation (TBI) containing regimen consisting of high-dose VP-16, carboplatin and cyclophosphamide followed by purged autoBMT has been evaluated in the POG 9342 trial. Additional studies include the use of differentiating agents and chimeric anti-GD2 CH14.18 monoclonal antibody to target minimal residual disease after stem cell transplantation. Both pediatric cooperative groups are in the process of conducting additional studies involving the use of repetitive stem cell rescue following high-dose chemotherapy, an approach previously tried by Philip et al. in Europe. A number of centers are investigating the role of unpurged peripheral blood stem cell (PBSC) transplantation in neuroblastoma. The issue of tumor cell contamination is being addressed with the use of more sensitive molecular techniques to identify neuroblastoma cells. The Autologous Blood and Marrow Transplant Registry (ABMTR) now has core data on over 540 autotransplants in neuroblastoma. Additional data on these transplants is in the process of being collected so that a retrospective analysis can be performed. Questions that can be addressed from this database include the role of TBI in the preparative regimen, the role of purging in NBL autotransplants and the identification of patient- and disease-related variables associated with poor outcome following autoBMT.

INTRODUCTION

Autologous stem cell rescue (ASCR) after myeloablative chemoradiotherapy continues to be offered as therapy to children with metastatic neuroblastoma and those with stage 3 neuroblastoma with poor-prognosis features such as n-myc amplification. Over the past decade, several institutional and cooperative group clinical trials have been performed to address a number of outstanding issues pertaining to the role of this therapeutic modality in the management of poor-

Table 1. AutoBMT in neuroblastoma

Group	# of patients	Conditioning regimen	TBI	DFS (%) 2 yr	4–5 yr	Ref
POG	94	multiple	yes	40	32	2
CCG	147	multiple	yes	51	40	4
EBMT	500	multiple	yes/no	52*	29*	6

**Survival rates shown are overall survival rates,*
DFS: Disease-free survival.

prognosis neuroblastoma.[1] It is clear that myeloablative chemoradiotherapy followed by autologous stem cell rescue can result in long-term disease-free survival rates of 29–45% when performed in first complete remission or very good partial remission (Table 1).[2–6] A small number of patients with progressive disease can also be salvaged with ASCR following myeloablative chemoradiotherapy.[7]

Controversies in autotransplants for neuroblastoma

Induction regimens with higher chemotherapy dose intensity have improved the outcome for patients with metastatic neuroblastoma.[8–9] This has raised the question of whether ASCR following myeloablative chemotherapy is superior to continued conventional chemotherapy (CT). Nonrandomized trials comparing CT with autologous bone marrow transplantation (autoBMT) conducted by the Pediatric Oncology Group (POG-8340,8441) and Children's Cancer Group (CCG-321P3) have yielded contradictory results. The recently completed CCG-3891 trial should provide data that will specifically address this issue. This prospective, randomized comparative trial calls for an intent-to-treat randomization at 8 weeks after diagnosis to either an autoBMT after myeloablative chemoradiotherapy or to continued high-dose chemotherapy without ASCR. The study has recently closed for patient accrual with over 350 patients having been randomized either to autoBMT or to chemotherapy. Since the outcome data has not been analyzed, no results are currently available. Analysis of outcome data is eagerly awaited. Even though early outcome data between the two arms may turn out to be similar, it will take several years of follow-up before comparisons about long-term DFS can be made.

The Pediatric Oncology Group trial (POG-9342) was designed to evaluate a non-TBI containing regimen consisting of high-dose cyclophosphamide, etoposide and carboplatin followed by autologous monoclonal antibody-purged bone marrow rescue in poor-prognosis neuroblastoma. This trial was recently closed for patient accrual. Preliminary analysis reveals no unusual toxicities or engraftment problems but outcome data are not available at this time. Future cooperative group trials

planned for poor-prognosis neuroblastoma include trials to evaluate the role of high-dose chemotherapy and repetitive stem cell rescue utilizing peripheral blood stem cells (PBSC) and the role of adjuvant modalities in eradicating minimal residual disease after ASCR. Post-ASCR therapies will include the use of differentiating agents such as retinoic acid and the human-mouse chimeric anti-GD2 CH14.18 monoclonal antibody combined with immune stimulation. This recombinant/chimeric antibody is composed of the variable region of the parent murine monoclonal antibody directed at the disialoganglioside antigen GD2 and the human gamma-1 and kappa constant regions. Phase II trials of this monoclonal antibody have thus far shown that it is well tolerated with manageable toxicities of fever, abdominal pain and allergic reactions.[10]

The role of purging of autologous bone marrow or PBSC remains unclear. Prospective, randomized trials comparing ASCR using purged versus nonpurged stem cells are unlikely to be performed. DFS rates comparable with those reported in trials using purged marrow have been reported by investigators using unpurged marrow.[11,12] Most relapses in neuroblastoma post-transplantation occur in sites of bulk disease and/or previously involved sites, suggesting that tumor cell contamination of the autograft is unlikely to be contributing to the relapse.[13] However, gene marking studies by Brenner et al. have conclusively shown that tumor cells contaminating the autograft can contribute to relapse.[14] Unlike acute leukemia, relapse rates following allogeneic BMT are not lower than those seen post-ASCR. These results are based on the outcomes seen in the small numbers of patients who have received allografts.[15,16] This finding may be interpreted to mean that relapse post-ASCR is unrelated to tumor cell contamination of the autograft. Alternatively, it may speak to the persistence of residual neuroblastoma in the recipient or a lack of a discernible graft-versus-neuroblastoma effect. The role of purging will continue to be controversial in neuroblastoma as it is in many other malignancies.

PBSCT in neuroblastoma

PBSCs are being investigated as a source of stem cells for ASCR in neuroblastoma and a number of other childhood solid tumors. The early problems of venous access and fluid and electrolyte shifts have been resolved for the most part. Successful peripheral stem cell harvests can now be performed in children weighing less than 20 kg, making it feasible to harvest PBSC in neuroblastoma patients following chemotherapy and growth factor administration.[17–19] Neuroblastoma cells can be detected in the peripheral blood of patients at diagnosis and at relapse and irrespective of marrow disease.[20] A potential theoretical advantage of using PBSC is that there may be lower tumor cell contamination of PBSC harvests.[21] This remains an unresolved issue requiring further investigation. Laver et al. have looked at the issue of contamination of the autograft utilizing a very sensitive reverse transcriptase-polymerase chain reaction (RT-PCR)-based

Table 2. ABMTR autotransplants: core data for neuroblastoma**

Number of patients=541	
male sex	57%
age, years, median (range)	4 (<1–57)
disease state pretransplant	
no disease evident	54%
disease evident	46%
interval Dx-Tx, months, median (range)	98 (1–133)
in vitro treatment	
no	39%
yes	61%
source of hematopoietic cells*	
bone marrow	84%
peripheral blood	10%
bone marrow and peripheral blood	6%
100 day survival	
all patients	91%
disease state	
no disease evident	93%
disease evident	90%
4-year probability of survival	
all patients	32%
disease state	
no disease evident	35%
disease evident	27%

**382 evaluable.*

***These data were obtained from the Statistical Center of the International Bone Marrow Transplant Registry and the Autologous Blood and Marrow Transplant Registry–North America, August, 1996. The analysis has not been reviewed or approved by the Advisory Committees of the IBMTR and ABMTR.*

method that can identify up to 1 in 10^7 mononuclear cells. This RT-PCR technique utilizes the neuroendocrine protein gene product 9.5 (PGP 9.5) found on neuroblastoma cells but not on mononuclear cells.[22] Their studies show that purged bone marrow samples from neuroblastoma patients still contained tumor cells in higher concentrations than unpurged peripheral blood.[23]

The Autologous Blood and Marrow Transplant Registry (ABMTR) currently has core data on over 540 autografts done in North America since 1988. A project is being initiated to collect and analyze the results of these autotransplants. A summary of the core data available in the ABMTR database is shown in Table 2. Almost 40% of the autografts have been performed using unpurged autologous stem cells and a significant number have utilized non-TBI containing preparative

regimens. The overall survival rates for the 540 patients for whom data are available are comparable to the results of cooperative group trials and large institutional trials. The specific objectives of the ABMTR study will be: 1) comparison of results of ASCR for poor-prognosis neuroblastoma using TBI-containing conditioning regimens with those that do not contain TBI; 2) comparison of results of ASCR in NBL using purged marrow versus nonpurged marrow or PBSC; and 3) identification of patient-, disease- and therapy-related variables correlated with transplant outcome in neuroblastoma. This analysis will thus allow us to address a number of important outstanding questions regarding the role of autotransplantation in poor-prognosis neuroblastoma.

REFERENCES

1. Kamani NR: Autotransplants for neuroblastoma. *Bone Marrow Transplant* 17:301–304, 1996.
2. Graham-Pole J, Casper J, Elfenbein G et al.: High-dose chemoradiotherapy supported by marrow infusions for advanced neuroblastoma: A Pediatric Oncology Group Study. *J Clin Oncol* 9:152–158, 1991.
3. Shuster JL, Cantor AB, McWilliams N et al.: The prognostic significance of autologous bone marrow transplantation in advanced neuroblastoma. *J Clin Oncol* 9:1045, 1991.
4. Stram DO, Matthay KK, O'Leary M et al.: Myeloablative chemoradiotherapy versus continued chemotherapy for high risk neuroblastoma. *Prog Clin Biol Res* 385:287–291, 1994.
5. Kamani N, August CS, Bunin N et al.: A study of thiotepa, etoposide and fractionated total body irradiation as a preparative regimen prior to bone marrow transplantation for poor prognosis patients with neuroblastoma. *Bone Marrow Transplant* 17:911–16, 1996.
6. Philip T, Ladenstein R, Lasset C et al.: Adverse prognostic factors in stage 4 neuroblastoma over one year of age: A report from the European Bone Marrow Transplantation solid tumor registry. *Exp Hematol* 22:766, 1994 [abstr].
7. Evans AE, August CS, Kamani N et al.: Bone marrow transplantation for high risk neuroblastoma at the Children's Hospital of Philadelphia: An update. *Med Pediatr Oncol* 23:323–327, 1994.
8. Cheung NV, Heller G: Chemotherapy dose intensity correlates strongly with response median survival, and median progression-free survival in metastatic neuroblastoma. *J Clin Oncol* 9:1050, 1991.
9. Bowman LC, Hancock ML, Santana VM et al.: Impact of intensified therapy on clinical outcome in infants and children with neuroblastoma: The St. Jude Children's Research Hospital Experience, 1962 to 1988. *J Clin Oncol* 9:1599–1608, 1991.
10. Cheung NKV, Lazarus H, Miraldi FD et al.: Reassessment of patient response to monoclonal antibody 3F8. *J Clin Oncol* 10:671, 1992.
11. De Bernardi B, Carli M, Casale F et al.: Standard-dose and high-dose peptichemio and cisplatin in children with disseminated poor-risk neuroblastoma: Two studies by the Italian Cooperative Group for Neuroblastoma. *J Clin Oncol* 10:1870–1878, 1992.
12. Dini G, Lanino E, Garaventa A et al.: Myeloablative therapy and unpurged autologous

bone marrow transplantation for poor-prognosis neuroblastoma: Report of 34 cases. *J Clin Oncol* 9:962, 1991.

13. Matthay K, Atkinson J, Stram D et al.: Patterns of relapse after autologous purged bone marrow transplantation for neuroblastoma: A Children's Cancer Group Pilot Study. *J Clin Oncol* 11:2226–2233, 1993.
14. Rill DR, Santana VM, Roberts WM et al.: Direct demonstration that autologous bone marrow transplantation for solid tumors can return a multiplicity of tumorigenic cells. *Blood* 84:380–383, 1994.
15. Matthay K, Seeger R, Reynolds C et al.: Allogeneic versus autologous purged bone marrow transplantation for neuroblastoma: A report from the Children's Cancer Group. *J Clin Oncol* 12:2382–2389, 1994.
16. Ladenstein R, Lasset C, Hartmann O et al.: Comparison of auto versus allografting as consolidation of primary treatments in advanced neuroblastoma over one year of age at diagnosis: Report from the European Group for Bone Marrow Transplantation. *Bone Marrow Transplant* 14:37–46, 1994.
17. Lasky L, Fox S, Smith J, Bostrom B: Collection and use of peripheral blood stem cells in very small children. *Bone Marrow Transplant* 7:281–284, 1991.
18. Demeocq F, Kanold J, Chassagne J et al.: Successful blood stem cell collection and transplant in children weighing less than 25 kg. *Bone Marrow Transplant* 13:43–50, 1994.
19. Takaue Y, Kawano Y, Abe T et al.: Collection and transplantation of peripheral blood stem cells in very small children weighing 20 kg or less. *Blood* 86:372–380, 1995.
20. Moss TJ, Sanders G: Detection of neuroblastoma cells in blood. *J Clin Oncol* 8:736–40,1990.
21. Moss TJ, Sanders DG, Lasky LC, Bostrom B: Contamination of peripheral blood cell harvests by circulating neuroblastoma cells. *Blood* 76:1879–83,1990.
22. Mattano LA, Moss TJ, Emerson SG: Sensitive detection of rare circulating neuroblastoma cells by the reverse transcriptase-polymerase chain reaction. *Cancer Research* 52:4701–4705, 1992.
23. Laver J, Klann R, Kletzel M et al.: Studies on the presence of tumor cells following marrow purging versus peripheral blood collection in stem cell grafts for neuroblastoma. Proceedings of American Society of Clinical Oncology 15:334, 1996 [abstr].

HIGH-DOSE CHEMOTHERAPY WITH AUTOLOGOUS STEM CELL RESCUE FOR MEDULLOBLASTOMA AND SUPRATENTORIAL PNET

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ABSTRACT

While many patients with newly diagnosed medulloblastomas or supratentorial primitive neuroectodermal tumors (PNET) are cured with radiation therapy and chemotherapy, those with recurrent tumor or young children in whom radiation therapy is avoided due to neuropsychologic concerns are very likely to die from their disease. We have treated such patients with a strategy that includes high-dose carboplatin (500 mg/m^2/day or AUC=7/day via Calvert formula) for 3 days, followed by thiotepa (300 mg/m^2/day) and etoposide (250 mg/m^2/day) for 3 days, with autologous stem cell rescue. Patients with recurrent disease also received radiation therapy and/or conventional chemotherapy, depending on the constraints imposed by prior therapy. The young children with newly diagnosed disease consistently received five cycles of vincristine, cisplatin, cyclophosphamide and etoposide prior to the high-dose chemotherapy/autologous stem cell rescue. Twenty-three patients with recurrent medulloblastoma were treated. Three (13%) died of treatment-related toxicities. The Kaplan-Meier estimates of overall and progression-free survival (PFS) at 36 months post-stem cell rescue are 41% and 30%, respectively. Sixteen patients with recurrent supratentorial PNET were treated. Four patients have no evidence of disease at a mean follow-up period of 2.7 years (range 3 months to 5.3 years). Twenty-seven young children with newly diagnosed medulloblastoma or supratentorial PNET were treated. Twenty-one successfully completed the initial chemotherapy and received the high-dose chemotherapy/autologous stem cell rescue component. For the 13 patients with medulloblastoma, Kaplan-Meier estimates of overall and event-free survival (EFS) at 2 years are 61% and 51%, respectively, and for the 14 patients with supratentorial PNET, they are 58% and 52%. We conclude that high-dose chemotherapy (HDCT) with autologous stem cell rescue may be a valuable component of a treatment regimen for 1) patients with recurrent medulloblastoma or supratentorial PNET and 2) young children with newly diagnosed medulloblastoma and supratentorial PNET as a way of avoiding radiation therapy.

INTRODUCTION

Patients with newly diagnosed medulloblastoma or supratentorial primitive neuroectodermal tumors (PNET) have a reasonable chance of long-term event-free survival (EFS) with conventional therapy that includes neurosurgical resection, radiation therapy and chemotherapy at standard doses. Multicenter trials performed by the Children's Cancer Group (CCG)[1] and the International Society of Paediatric Oncology, in conjunction with the German Society of Paediatric Oncology,[2] noted a 59% estimated 5-year EFS. Another CCG trial for children with supratentorial PNET demonstrated a 45% estimated 3-year progression-free survival (PFS).[3]

Once these tumors recur, however, they are almost invariably lethal. In a series from the Children's Hospital of Philadelphia, 23 patients treated for medulloblastoma between 1980 and 1991 had their tumor recur.[4] Their median survival was five months, with the longest survivor living for 38 months from recurrence. Similarly, 36 patients treated at the Children's Hospital at Stanford University from 1958 to 1986 suffered a recurrence, and there were no long-term survivors.[5]

Another group of patients with a dismal prognosis are young children with newly diagnosed medulloblastoma or supratentorial PNET. Although radiation therapy is an effective therapy for these tumors, the neuropsychological consequences in this age group are considered intolerable by most contemporary pediatric neuro-oncologists, and these children are treated with neurosurgical resection and chemotherapy in an attempt to delay or avoid radiation therapy. The Pediatric Oncology Group reported 34% and 19% estimated 2-year PFS for infants (less than 3 years old) with medulloblastoma and supratentorial PNET, respectively.[6] The CCG reported 22%, 55% and 0% 3-year estimated PFS for infants (less than 18 months old) with medulloblastoma, nonpineal supratentorial PNET and pineal PNET, respectively.[7]

Since 1986, we have explored the role of high-dose chemotherapy (HDCT) with autologous stem cell rescue in the treatment of patients with malignant brain tumors. Originally, a two-drug regimen with thiotepa and etoposide was used. Subsequently, a three-drug regimen that included carmustine (BCNU) in addition to the thiotepa and etoposide was evaluated but was found to be excessively toxic. Most recently, we have employed a three-drug regimen that uses carboplatin in addition to thiotepa and etoposide. While we have used this strategy to treat patients with a variety of malignant brain tumor histologies, including high grade astrocytic tumors, ependymomas and brain stem tumors, we will restrict our discussion in this chapter to our experience treating patients with medulloblastoma or supratentorial PNET.

METHODS

The original HDCT protocol (designated as the TE regimen) consisted of thiotepa (300 mg/m^2/day) and etoposide (250–500 mg/m^2/day), each administered

over three days, followed by autologous stem cell rescue approximately 72 hours after completion of the chemotherapy.[8] The second regimen (not administered to the patients discussed in this chapter) added six doses of BCNU (100 mg/m^2/dose) q 12 hours over three days, either before or after the thiotepa and etoposide.[9] The regimen that we are currently investigating (designated CTE) utilizes carboplatin for three days, followed by TE (with the etoposide dosed at 250 mg/m^2/day). The carboplatin was originally a fixed daily dose of 500 mg/m^2/day, but more recently has been dosed via the Calvert formula[10,11] to a predicted area under the curve=7/day, with each daily dose of carboplatin being determined by the results of a preceding timed creatinine clearance estimate of the glomerular filtration rate. Patients with recurrent tumors were treated on either of two protocols, CCG-9883 (TE or CTE regimen) or MSKCC 89-173 (CTE regimen).

Prior to enrollment on protocol, patients with recurrent tumors were often treated with neurosurgical resection and conventional chemotherapy. Some patients also received radiation therapy, either before or after the protocol-prescribed therapy, if their prior radiation history allowed.

Young children with newly diagnosed medulloblastoma or supratentorial PNET were treated on MSKCC protocol 92-16. Eligible patients included children under 6 years old with supratentorial PNET and children under 3 years old with medulloblastoma. Children aged three to six with medulloblastoma were eligible only if they had evidence of metastatic disease (stage M-1+). Patients received five planned cycles of "induction" chemotherapy with vincristine, cisplatin, cyclophosphamide and etoposide. Autologous stem cells (from either bone marrow or peripheral blood) were harvested early, usually at recovery from the first cycle. If, after five cycles, disease progression had not occurred, they proceeded to the "consolidation" phase with HDCT using the CTE regimen described above and autologous stem cell rescue. If the patients had no evidence of disease before entering the consolidation phase, they did not receive any radiation therapy on this protocol. If there was evidence of unresectable residual disease, then approximately six weeks after autologous stem cell rescue, the patients commenced involved field radiation therapy.

RESULTS

Toxicity of the CTE regimen

We have employed this regimen in patients with other malignant brain tumor histologies, and to better define its toxicity, we will review the results in 76 patients treated between 1989 and 1996 at MSKCC with all eligible histologies. Twenty-four of these patients were young children (less than six years old) with newly diagnosed brain tumors; their median age was 3 years, with a range from 9 months to 5 years. Fifty-two patients had recurrent malignant brain tumors; their median

age was 13 years, with a range from 1 to 45 years. Sixty-four patients received bone marrow rescues and twelve received peripheral blood stem cells.

Toxic deaths occurred in ten patients (13%). All ten had recurrent tumors, leading to a toxic mortality rate of 19% in that group. None of the 24 young patients with newly diagnosed tumors suffered a toxic death. Among the patients with recurrent tumors, introduction of dosing according to the Calvert formula was associated with a decreased toxic mortality rate: 35% for patients with fixed body-surface-area carboplatin dosing versus 9% for those dosed according to the Calvert formula.

Toxicities that occurred in virtually all patients included severe mucositis that required opioid infusions and total parenteral nutrition, and thiotepa-induced dermatitis. Severe renal and hepatic toxicities were uncommon. Neurotoxicity, seen occasionally, occurred in three chronologically distinct phases. Acute neurological deterioration occurred during the administration of HDCT, transient encephalopathy occurred from day 0 to day 100 following stem cell rescue, and prolonged anorexia with nausea occurred late, after day 101. The post-stem cell infusion neurotoxicities were seen almost exclusively in adult patients who had received prior cranial irradiation.

The median time to neutrophil engraftment (ANC >500/mm^3 for 3 consecutive days) for patients rescued with bone marrow was 11 days (range 9 to 18 days) for the newly diagnosed group and 13 days (range 10 to 32 days) for the recurrent disease group. For patients with recurrent disease rescued with autologous peripheral blood stem cells, it was 10 days (range 8 to 14 days).

The median time to platelet engraftment (platelets >50,000/mm^3 without transfusion for 3 consecutive days) for patients rescued with bone marrow was 30 days (range 21 to 116 days) for the newly diagnosed group and 35 days (range 18 days to >38 months) for the recurrent disease group. For those with recurrent disease rescued with autologous peripheral blood stem cells, it was 30 days (range 14 to 107 days).

Outcome of patients with recurrent medulloblastoma

While responses were noted in some patients with recurrent medulloblastoma or PNET treated with the original TE regimen, no long-term survivors were noted.[8] Their poor outcome was thought to be caused by the frequent presence of bulky disease at the time of treatment on the protocol. Subsequently, more emphasis was placed on achieving a state of minimal disease prior to study entry in patients treated on the CTE regimen.

Twenty-three patients with recurrent medulloblastoma, aged 2 to 44 years old, received the CTE regimen from November, 1990, to May, 1995. Three (13%) died of treatment-related toxicities within 21 days of stem cell rescue: two with multi-organ system failure and one with both aspergillosis and veno-occlusive disease. The Kaplan-Meier estimates of overall and PFS at 36 months post-stem cell rescue are 41% (±12%) and 30% (±11%), respectively.

Outcome of patients with recurrent supratentorial PNET

Sixteen patients with recurrent supratentorial PNET were treated, four with the TE regimen and twelve with the CTE regimen. Twelve patients died of progressive disease or complications of therapy, and four patients are alive with no evidence of disease at a mean follow-up period of 2.7 years (range 3 months to 5.3 years). Three of the four survivors received local field radiation therapy after recovery from the HDCT and stem cell rescue.

Outcome of young children with newly diagnosed medulloblastoma and supratentorial PNET

Thirteen patients with newly diagnosed medulloblastoma (12 less than 3 years old, one aged three to six) and 14 patients with supratentorial PNET (seven less than three years old, seven aged three to six) were treated from June, 1991, to May, 1995. Twenty-one successfully completed the induction chemotherapy and received the consolidation HDCT with stem cell rescue. For the 13 patients with medulloblastoma, the Kaplan-Meier estimates of overall and EFS at 24 months are 61% (±28%) and 51% (±35%), respectively. For the 14 patients with supratentorial PNET, the Kaplan-Meier estimates of overall and EFS at 24 months are 58% (±30%) and 52% (±29%).

DISCUSSION

We believe that HDCT with autologous stem cell rescue utilizing the CTE regimen described above is a promising strategy for patients with recurrent medulloblastoma and supratentorial PNET. In conjunction with neurosurgical resection of the recurrent tumor, conventional chemotherapy and radiation therapy (if tolerable given the constraints of previously administered therapy), this strategy can provide extended periods of EFS to some of these patients. Given the lethality of their disease with other modalities of treatment, we feel that this cohort is remarkable. However, our patients were enrolled on protocol at the time of HDCT, not at relapse, and the true proportion of patients with recurrent disease who can be salvaged with this approach remains to be determined.

HDCT with autologous stem cell rescue is also a promising strategy for avoiding radiation therapy in young children with newly diagnosed medulloblastoma and supratentorial PNET. In conjunction with neurosurgical resection of the tumor and conventional chemotherapy, this strategy can provide EFS without radiation therapy to what appears to be a substantial proportion of these patients. Our numbers are small, though, and therefore the confidence intervals of the Kaplan-Meier estimates are very wide. Accrual of additional patients will provide a more precise estimate of the proportion of young children that this approach can benefit.

Use of the Calvert formula to determine the carboplatin dose, and autologous peripheral blood as the source of stem cells has been associated with a decrease in the treatment-related mortality to an acceptable level in these patients with highly lethal diseases. We advocate further assessment of this strategy in the context of well-designed clinical trials in centers with expertise in both neuro-oncology and autologous stem cell rescue.

REFERENCES

1. Evans AE, Jenkin RDT, Sposto R et al.: The treatment of medulloblastoma: Results of a prospective randomized trial of radiation therapy with and without CCNU, vincristine, and prednisone. *J Neurosurg* 72:572–582, 1990.
2. Bailey CC, Gnekow A, Wellek S et al.: Prospective randomized trial of chemotherapy given before radiotherapy in childhood medulloblastoma. International Society of Paediatric Oncology (SIOP) and the (German) Society of Paediatric Oncology (GPO): SIOP II. *Med Pediatr Oncol* 25:166–178, 1995.
3. Cohen BH, Zeltzer PM, Boyett JM et al.: Prognostic factors and treatment results for supratentorial primitive neuroectodermal tumors in children using radiation and chemotherapy: A Children's Cancer Group randomized trial. *J Clin Oncol* 13:1687–1696, 1995.
4. Torres CF, Rebsamen S, Silber JH et al.: Surveillance scanning of children with medulloblastoma. *N Engl J Med* 330:892–895, 1994.
5. Belza MG, Donaldson SS, Steinberg GK et al: Medulloblastoma: Freedom from relapse longer than 8 years—a therapeutic cure? *J Neurosurg* 75:575–582, 1991.
6. Duffner PK, Horowitz ME, Krischer JP et al.: Postoperative chemotherapy and delayed radiation in children less than three years of age with malignant brain tumors. *N Engl J Med* 328:1725–1731, 1993.
7. Geyer JR, Zeltzer PM, Boyett JM et al.: Survival of infants with primitive neuroectodermal tumors or malignant ependymomas of the CNS treated with eight drugs in 1 day: A report from the Children's Cancer Group. *J Clin Oncol* 12:1607–1615, 1994.
8. Finlay JL, Goldman S, Wong MC et al.: Pilot study of high-dose thiotepa and etoposide with autologous bone marrow rescue in children and young adults with recurrent CNS tumors. *J Clin Oncol* 14:2495–2503, 1996.
9. Finlay JL, August C, Packer R et al.: High-dose multi-agent chemotherapy followed by bone marrow "rescue" for malignant astrocytomas of childhood and adolescence. *J Neurooncol* 9:239–248, 1990.
10. Calvert AH, Newell DR, Gumbrell LA et al.: Carboplatin dosage: Prospective evaluation of a simple formula based on renal function. *J Clin Oncol* 7:1748–1756, 1989.
11. Newell DR, Pearson AD, Balmanno K et al.: Carboplatin pharmacokinetics in children: The development of a pediatric dosing formula. The United Kingdom Children's Cancer Study Group. *J Clin Oncol* 11:2314–2323, 1993.

NOVEL HIGH-DOSE REGIMENS FOR THE TREATMENT OF BREAST CANCER

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ABSTRACT

We have investigated three novel high-dose regimens (ICE, MITT and TNT) followed by autologous stem cell infusion for the treatment of breast cancer. The maximally tolerated doses (MTD) of ICE (ifosfamide, carboplatin and etoposide) are 20.1 g/m^2, 1.8 g/m^2 and 3 g/m^2, respectively. The MTD of MITT (mitoxantrone and thioTEPA) are 90 mg/m^2 and 1200 mg/m^2, respectively. The MTD of TNT (Taxol®, Novantrone® and thioTEPA) are 360 mg/m^2, 75 mg/m^2 and 900 mg/m^2, respectively. Dose limiting toxicities are renal failure and coma for ICE and mucositis/enteritis for MITT and TNT. We have also treated patients with stage II, III and inflammatory breast cancer (IBC) with BUCY (novel for early stage breast cancer—busulfan 16 mg/kg, cyclophosphamide 120 mg/kg) and CTC (standard for breast cancer—cyclophosphamide 6 g/m^2, thioTEPA 500 mg/m^2 and carboplatin 800 mg/m^2). Event free survival (EFS) ± standard error (SE) for stage II breast cancer (n=16) treated with ICE is 64 ± 14% at 3 years and with CTC (n=11) is 91 ± 9% at 1 year EFS for stage III breast cancer (n=15) treated with ICE is 53 ± 13% at 3 years, with MITT (n=6) is 67 ± 18% at 2 years, with (n=10) BUCY is 56 ± 18% at 2 years and with CTC (n=10) is 90 ± 9% at 1 year. For patients with IBC (n=6) treated with ICE the EFS at 3 years is 33 ± 19%. For patients with anthracycline responsive metastatic breast cancer EFS is 25 ± 13% at 3 years for 14 patients treated with ICE, 38 ± 14% at 2 years for 21 patients treated with MITT and 50 ± 22% at 1 year for 6 patients treated with TNT. EFS for 28 patients with metastatic breast cancer failing anthracycline but responding to salvage therapy with abbreviated doses of ICE is 13 ± 8% at 3 years. Finally, for patients with metastatic breast cancer failing all forms of induction therapy, EFS for 31 patients treated with MITT is 3 ± 3% at 2 years and EFS for 28 patients treated with TNT is 21 ± 10% at 1 year . These data suggest that the novel regimens (ICE, MITT and TNT) have excellent activity in breast cancer. TNT shows promising activity in stage IV disease that has failed salvage therapy. BUCY and CTC, which can be administered in the outpatient setting because of somewhat reduced dose intensity and limited toxicity, are both appropriate regimens for early stage disease.

Table 1. Dose ranges

ICE (g/m^2)	*MITT* (g/m^2)	*TNT* (g/m^2)	*BUCY* (*mg/kg*)	*CTC* (g/m^2)
ifosfamide	mitoxantrone	paclitaxel	busulfan	cyclophosphamide
17.1–24.0	0.09–0.105	0.36	16	6
carboplatin	thiotepa	mitoxantrone	cyclophosphamide	thiotepa
1.8	1.2-1.5	0.048-0.075	120	0.5
etoposide		thiotepa		carboplatin
2.1–3.0		0.90		0.8
n=92	n=60	n=34	n=10	n=21

INTRODUCTION

High-dose chemotherapy (HDCT) with autologous stem cell infusion for the treatment of breast cancer was introduced in the 1980s as a result of several factors. Standard dose chemotherapy, while producing objective responses in 40–80% of patients with metastatic disease, does not result in durable remissions in the vast majority of patients. Fewer than 5% of patients maintain a complete remission beyond 5 years.[1] Even in patients with stage II or III breast cancer with more than 10 axillary lymph nodes positive for metastasis, 5-year disease-free survival after conventional adjuvant therapy is approximately 40% varying with the hormonal receptor status and menopausal status of the patient at the time of diagnosis.[2] The relative lack of benefit from standard dose chemotherapy coupled with laboratory data suggesting a steep dose-response relationship for breast cancer models has led to the evaluation of dose-intensive therapy in several clinical trials.[3] Many of these trials have shown a therapeutic advantage with more intensive therapy. Recent improvements in supportive care, including the use of hematopoietic growth factors, has reduced hematologic and related complications associated with dose-intense therapy. Current trials comparing conventional therapy with high-dose therapy in a prospective, randomized fashion in both the adjuvant and metastatic settings are ongoing.

Since the Bone Marrow Transplant Program at the H. Lee Moffitt Cancer Center opened in 1989, we have evaluated several HDCT regimens for efficacy in breast cancer. While other investigators had published results with regimens that have included cisplatin, carmustine, cyclophosphamide, thiotepa and/or melphalan, our program chose to take advantage of agents that had been recently approved as well as others that had documented activity in breast cancer or potential synergy with the investigational agents. In general, regimens that were available in 1989 were efficacious only in chemoresponsive disease with treatment-related mortality reported as high as 25%.[3] Our goals were to broaden the therapeutic index of HDCT (increase efficacy, in both responsive and refractory disease, and reduce treatment-related mortality), define the dose-limiting toxicities and maximally tolerated doses of these

Table 2. Treatment outcomes for patients with breast cancer by stage and regimen

Stage of disease	*ICE (EFS@3yr)*	*MITT (EFS@2yr)*	*TNT (EFS@1yr)*	*BUCY (EFS@2yr)*	*CTC (EFS@1yr)*
II	64 ± 14 (16)	---	---	---	91 ± 9 (11)
III	53 ± 13 (15)	67 ± 18 (6)	---	56 ± 18 (10)	90 ± 9 (10)
IBC	33 ± 19 (6)	---	---	---	---
IV-Adr*	25 ± 13 (14)	38 ± 14 (21)	50 ± 22 (6)	---	---
IV-mICE**	13 ± 8 (28)	---	---	---	---
IV-Ref***	---	3 ± 3 (31)	21 ± 10 (28)	---	---

**IV-Adr=stage IV disease responsive to anthracycline.*
***IV-mICE=stage IV disease refractory to anthracycline but responsive to abbreviated doses of ICE.*
****IV-Ref=stage IV disease refractory to both anthracycline and abbreviated doses of ICE.*

new combinations, and determine the efficacy of these regimens in breast cancer. These novel regimens include ifosfamide, carboplatin, and etoposide (ICE), and mitoxantrone and thiotepa with or without paclitaxel (MITT/TNT). We have also evaluated busulfan and cyclophosphamide (BuCy) and carboplatin, thiotepa and cyclophosphamide (CTC) as potential outpatient regimens in the treatment of breast cancer. Results of these evaluations will be presented here.

PATIENTS AND METHODS

From October, 1989, through June, 1996, we treated 304 breast cancer patients with one of five HDCT regimens. These regimens were incorporated into phase I/II studies that were IRB approved and for which written, informed consent was given. Only data on patients treated at the upper dose levels will be presented. The ranges for these dose levels are shown in Table 1.

ICE is given as a six-day regimen as previously described.[4] Stem cells were reinfused after a 48-hour chemotherapy-free interval. Mitoxantrone and thiotepa are administered as short infusions in both the MITT[5] and TNT[6] regimens; paclitaxel is given as a 72-hour continuous infusion. Stem cells are reinfused after a 7-day chemotherapy-free period in patients receiving the MITT and TNT regimens. BuCy is given in the traditional manner: busulfan 1 mg/kg orally every 6 hours for 16 doses followed by cyclophosphamide 60 mg/kg over 2 hours every day for 2 days with stem cells reinfused 48 hours after cyclophosphamide. CTC is administered as short infusions over 3 days (to facilitate outpatient administration) with stem cells reinfused 48 hours after the last chemotherapy infusion.

Initially, we treated all breast cancer patients on the ICE regimen. This included patients with stage II disease and eight or more involved axillary lymph nodes, stage III disease IBC, and stage IV disease which had demonstrated a response to abbreviated doses of ICE (miniICE).[7] In 1991, we introduced the MITT regimen for stage IV

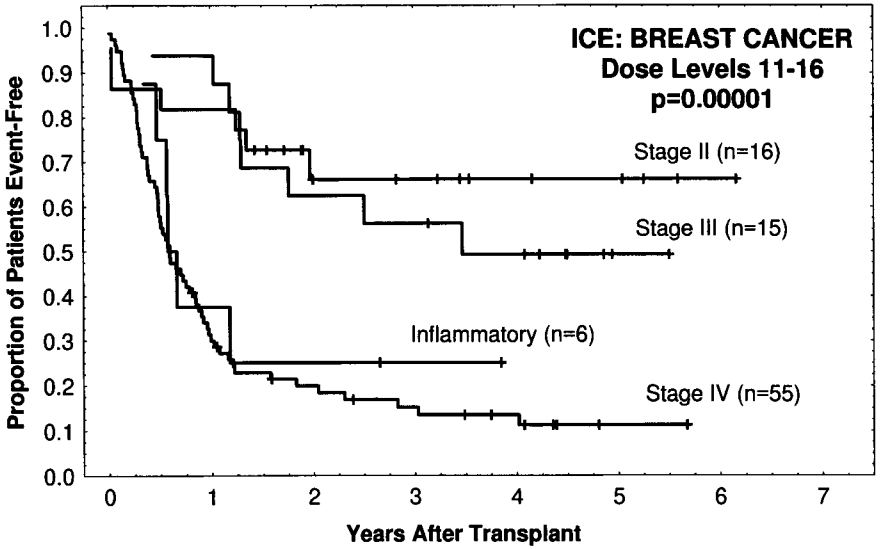

Figure 1. *EFS for patients with breast cancer treated with ICE.*

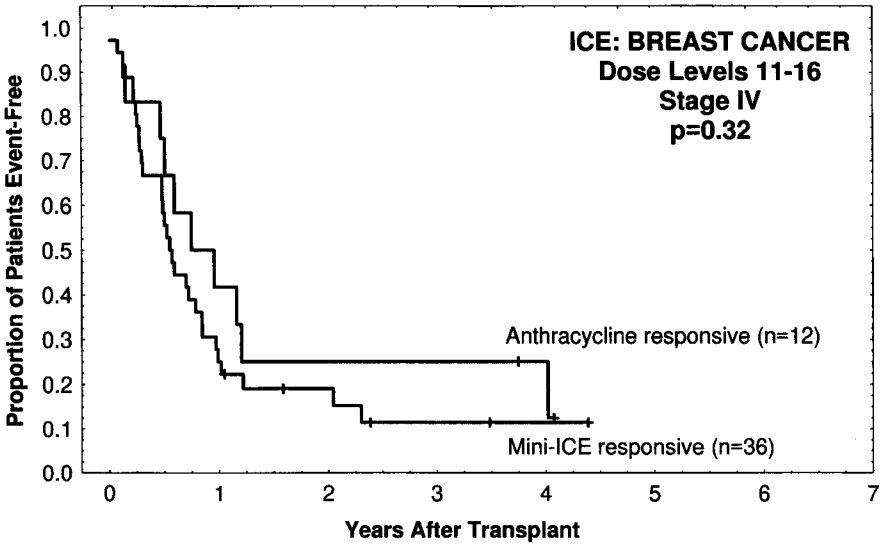

Figure 2. *EFS for patients with stage IV breast cancer treated with ICE based on anthracycline responsiveness.*

patients who did not have a demonstrable response to miniICE but were otherwise candidates for high-dose therapy. Because of the early promising efficacy data of the MITT regimen, in 1993 we began using it in stage III disease and in patients with anthracycline responsive stage IV disease. At that time, we added paclitaxel to MITT creating TNT to treat the stage IV patients with refractory disease. Due to an increased incidence of cardiotoxicity not seen in the phase I portion of the trial, we abandoned the MITT regimen and switched to TNT in the stage IV responsive patients and BuCy in the stage III patients. In an effort to consolidate our treatment approach to breast cancer, in 1995 we began treating all stage II (8 or more involved lymph nodes) and all stage III patients with CTC and all stage IV patients with TNT.

RESULTS

Table 2 illustrates event-free survival (EFS) based on the stage of disease and regimen for all patients treated at the upper dose levels of ICE, MITT and TNT and all patients treated with BuCy and CTC at our institution. Additionally, for patients with metastatic breast cancer, EFS based on responsiveness to induction therapy with an anthracycline-based regimen or, failing that, to 2 cycles of an abbreviated course of ICE is described.

Figure 1 illustrates EFS for patients treated at the upper dose levels of the ICE regimen. Included in this group are patients with stage II and III breast cancer treated in the adjuvant setting, patients with inflammatory breast cancer treated in the adjuvant setting and patients with stage IV disease, both responsive to anthracyclines and refractory to anthracyclines but responsive to miniICE. Figure 2 illustrates EFS for patients treated with ICE at the upper dose levels based on anthracycline responsiveness. As can be seen, there is no significant difference in outcome for patients who are anthracycline refractory but responsive to salvage therapy with miniICE as compared with those who are anthracycline responsive.

Figure 3 illustrates EFS for patients with anthracycline responsive breast cancer treated with ICE, MITT or TNT. As can be seen there are no significant differences between protocols. Figure 4 illustrates EFS in patients with refractory breast cancer treated with MITT or TNT. Although TNT appears to offer an early advantage, these differences are not statistically significant.

Treatment-related mortality for ICE, MITT and TNT has been previously described.[4–6] The treatment-related mortality at the maximum tolerated doses of ICE for all patients with breast cancer (n=60) was 10% with central nervous system toxicity and acute renal failure noted to be dose limiting. For 45 patients with breast cancer treated at the maximum tolerated doses of MITT, treatment-related mortality was 30% with dose-limiting toxicities noted to be protracted mucositis and delayed engraftment in patients treated on the phase I portions of the trial, whereas dose cardiotoxicity was noted for patients treated on the phase II portion

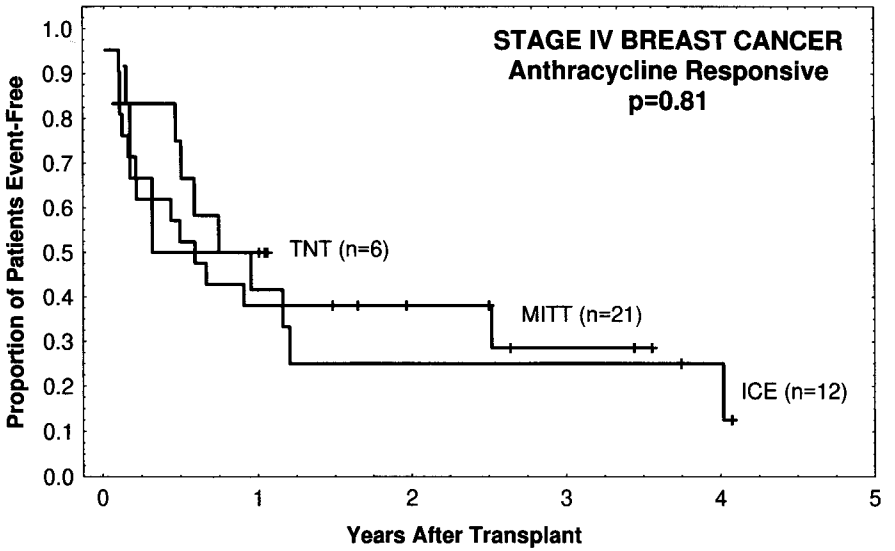

Figure 3. *EFS for patients with anthracycline responsive metastatic breast cancer treated with ICE, MITT or TNT.*

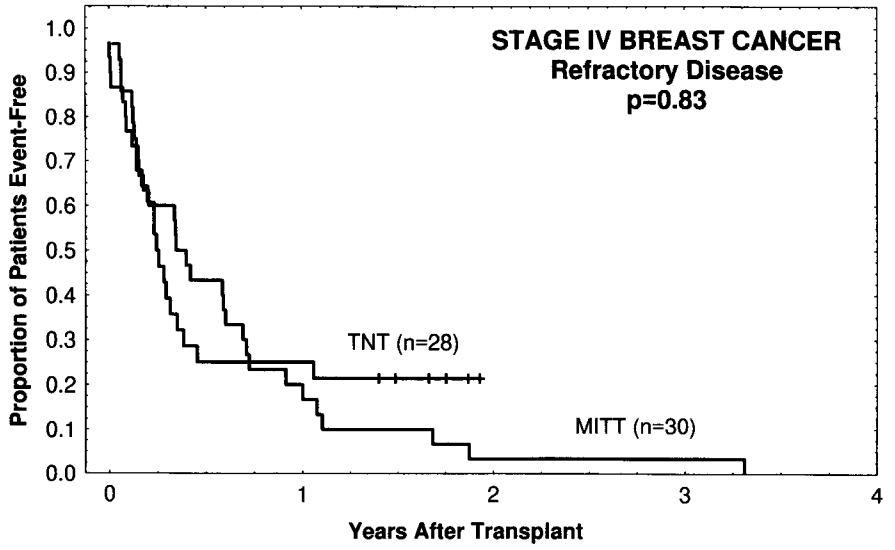

Figure 4. *EFS for patients with refractory metastatic breast cancer treated with MITT or TNT.*

of the trial. For 46 patients with breast cancer treated at the maximum tolerated doses of TNT, treatment-related mortality was 25% with dose limiting toxicities found to be enteritis for the phase I portion of the trial and cardiotoxicity for patients treated on the phase II portion of the trial.

CONCLUSIONS

We have presented results following high-dose therapy and autologous stem cell rescue from several phase I and II clinical trials in patients with breast cancer. These data suggest that the novel regimens (ICE, MITT and TNT) have excellent activity in breast cancer. Among patients with metastatic breast cancer treated on these regimens, responsiveness to induction therapy appears to be the major predictor of outcome. Patients responding to an anthracycline-based regimen treated on all regimens, ICE, MITT and TNT, had superior EFS compared with patients failing anthracyclines. This finding has been described by others; however, the differences are only seen in patients achieving a complete response to conventional therapy prior to high-dose therapy, not in those achieving a partial response.[8] Our data suggest that patients responding to therapy, with either a complete or partial response, benefit from high-dose therapy. It is important to note, as well, that some patients failing anthracyclines but responding to a salvage regimen consisting of an abbreviated course of ICE, demonstrated significant EFS following high-dose ICE. This suggests that this population of patients—anthracycline refractory patients responsive to salvage induction therapy—may also benefit from high-dose therapy. Additionally, regimens combining drugs with more individual activity against breast cancer, such as the TNT regimen, may allow more activity even in refractory patients. In our experience, TNT shows promising activity in stage IV disease that has failed salvage therapy, suggesting that the addition of paclitaxel to mitoxantrone and thiotepa results in superior antitumor activity.

In patients with a lower tumor burden, i.e., early stage breast cancer, regimens with less dose intensity may be appropriate. Our early experience with BuCy and CTC defines and/or confirms the antitumor activity of these regimens in breast cancer. The use of these regimens does not appear to compromise long-term results. Additionally, these regimens have toxicity profiles that lend themselves to administration in the outpatient setting.

Although high-dose therapy and autologous stem cell rescue have been used with increasing frequency for the treatment of breast cancer, it is remarkable that no one standard high-dose regimen exists but that multiple, different regimens are in common use.[3] At least one prospective, randomized trial has demonstrated that high-dose therapy for the treatment of metastatic breast cancer results in improved overall and EFS when compared with standard therapy.[9] However, despite the apparent superiority of high-dose therapy in inducing long-term remissions, the

majority of patients with metastatic breast cancer still relapse following high-dose therapy. The pursuit of newer, more active and less toxic high-dose regimens becomes imperative in the attempt to improve outcomes.

In summary, the development of novel high-dose regimens with specific aims of increasing antitumor activity and decreasing toxicity remains an important aspect of improving the therapy of patients with breast cancer. Patients with early stage breast cancer and chemoresponsive metastatic breast cancer appear to derive the most benefit from this type of therapy but the development of regimens with superior antitumor activity can also result in improved outcomes for patients with advanced refractory disease.

REFERENCES

1. Greenberg PAC, Hortobagyi GN, Smith TL et al: Long-term follow-up of patients with complete remission following combination chemotherapy for metastatic breast cancer. *J Clin Oncol* 14:2197–2205, 1996.
2. Robert NJ, Gray R, Gelber RD, Goldhirsch A et al.: Node positive (N^+) breast cancer: Which patients (pts) are at high risk? *Proc Am Soc Clin Oncol* 10:59A, 1991.
3. Antman K, Corringham R, de Vries E et al.: Dose intensive therapy in breast cancer. *Bone Marrow Transplant* 10:(suppl):67–73, 1992.
4. Fields KK, Elfenbein GJ, Lazarus HM et al.: Maximum tolerated doses of ifosfamide, carboplatin, and etoposide given over six days followed by autologous stem cell rescue: Toxicity profile. *J Clin Oncol* 13:323–332, 1995.
5. Fields KK, Elfenbein GJ, Perkins JB et al.: Two novel high dose treatment regimens for metastatic breast cancer: Ifosfamide, carboplatin plus etoposide (ICE) and mitoxantrone plus thiotepa (MITT): Outcomes and toxicities. *Semin Oncol* 20(suppl 6):59–66, 1993.
6. Fields KK, Perkins JB, Elfenbein GJ et al.: A phase I dose escalation trial of high dose Taxol®, Novantrone®, and thiotepa (TNT) followed by autologous stem cell rescue (ASCR): Toxicity. *Proc Am Clin Oncol* 14:953, 1995 [abstr].
7. Fields KK, Zorsky PE, Hiemenz JW, Kronish LE, Elfenbein GJ: Ifosfamide, carboplatin, and etoposide: A new regimen with a broad spectrum of activity. *J Clin Oncol* 12:544–552, 1994.
8. Rowlings PA, Antman KS, Horowitz MM et al.: Prognostic factors in autotransplants for metastatic breast cancer. *Blood* 86(suppl 1):618a, 1995.
9. Bezwoda WR, Seymour L, Dansey RD: High-dose chemotherapy with hematopoietic rescue as primary treatment for metastatic breast cancer: A randomized trial. *J Clin Oncol* 13:2483–89, 1995.

CHAPTER 8
Options with the Graft

CULTURE EXPANSION AND DIFFERENTIATION OF PERIPHERAL BLOOD CD34+ CELLS IN AUTOLOGOUS STEM CELL TRANSPLANTATION FOR METASTATIC BREAST CANCER

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ABSTRACT

Ex vivo expansion and culture differentiation of $CD34^+$ peripheral blood progenitor cells (PBPC) to neutrophil precursors may shorten the duration of neutropenia after high-dose chemotherapy (HDCT). We report our results in 16 patients with metastatic breast cancer utilizing these ex vivo cultured PBPCs. All underwent mobilization with cyclophosphamide, etoposide and granulocyte colony-stimulating factor (G-CSF). Four to five aphereses were performed at the appropriate time. One product from each patient was prepared using the Isolex 300 Magnetic Cell Separation System (Baxter Immunotherapy, Irvine, CA) to obtain $CD34^+$ cells, which were then cultured in gas permeable bags containing serum-free X-VIVO 10 medium (Bio Whittaker, Walkersville, MD) supplemented with 1% HSA and 10 ng/mL PIXY321. At day 12 of culture the mean fold expansion was 18× (range 6–36×). Cells from three patients did not expand. The final cell product contained an average of 37% $CD15^+$ neutrophil precursors (range 18.5–60%). Patients underwent HDCT with cyclophosphamide, carboplatin and thiotepa. Both cryopreserved nonexpanded and culture-expanded PBPCs were reinfused into 13 patients. The mean number of cultured cells reinfused was 58×10^6 cells/kg (range, $0.8–156\times10^6$). No toxicity was observed related to the reinfusion. Engraftment was, on average, day 9 for absolute neutrophil count (ANC) >500 and day 10 for platelets >20,000. Ex vivo culture expanded and differentiated PBPCs can be obtained and safely reinfused.

INTRODUCTION

High-dose chemotherapy (HDCT) with autologous stem cell rescue (autoSCR) has become an important treatment modality for many patients with breast cancer. In order to shorten the duration of severe myelosuppression after high-dose

therapy, autologous hematopoietic progenitor cells collected from the marrow or mobilized and collected from the blood have been utilized. Several studies have demonstrated the benefits of peripheral blood progenitor cells (PBPC) as a reliable long-term hematopoietic rescue from this myelosuppression.[1] In addition, the use of post-transplant myeloid colony stimulating factors such as G- or GM-CSF has also been shown to hasten neutrophil recovery but not red cell or platelet recovery.[2] Despite the development of these advances there remains a "neutropenic window" of about 10–12 days. This delay or window is hypothesized to be caused by the obligatory time that it takes committed myeloid precursors to differentiate, mature and be released into the circulation. This paper discusses a potential new strategy to reduce this "neutropenic window", while at the same time allowing full long-term hematopoietic recovery after HDCT for breast cancer.

We have previously reported our preclinical experience on the growth and differentiation of selected marrow $CD34^+$ cells cultured in the mixture of IL-3, GM-CSF and G-CSF that resulted in an 18-fold increase in cell number after 10 to 12 days in culture.[3] Based upon these results we developed a protocol to expand selected $CD34^+$ cells from PBPC for 12 days in liquid culture with PIXY321 (the fusion product of IL-3 and GM-CSF) and reinfuse these expanded, differentiated cells as a supplement to 3 cryopreserved PBPC after HDCT in women with metastatic breast cancer. We report here on the feasibility and safety of this approach.

MATERIALS AND METHODS

Patient selection and treatment protocol

Sixteen women with metastatic breast cancer were enrolled onto this feasibility trial after obtaining written informed consent approved by the University of Chicago Institutional Review Board. Patients underwent mobilization with cyclophosphamide 4 gm/m^2, etoposide 1 gm/m^2 and G-CSF 10 $\mu g/kg/d$. Leukapheresis was performed when white blood cell count recovered and was greater than $1000/\mu L$ and platelet count greater than $20{,}000/\mu L$. The HDCT regimen consisted of cyclophosphamide $6gm/m^2$, thiotepa $600mg/m^2$ and carboplatin 800 mg/m^2 in 4 divided doses given continuous IV infusion over 4 days. Table 1 is a summary of the prior therapy for each patient and the results of the mobilization treatment.

PB collection procedure

Patients underwent leukapheresis using the Fenwal CS3000® Plus blood cell separator (Baxter Fenwal, Deerfield, IL). The apheresis product from day 1, 2 or 3 was used for $CD34^+$ selection and culture expansion. The apheresis product was assayed for total $CD34^+$ cell content. The day of selection was chosen based on a minimum $CD34^+$ cell content of 37 million cells/apheresis for patients no.

Table 1. Patient characteristics and mobilization

Pt ID	*Pt age*	*Prior chemo*	*Prior RT*	*Total cryo MNC/kg* $\times 10^8$	*Total cryo CD34/kg* $\times 10^6$
3000	39	CMF, CAF	Yes	2.93	3.73
3001	36	CMF, CTX/Adria	No	4.23	5.08
3002	52	CAF	No	2.32	9.38
3003	32	CMF, CA	No	2.77	5.12
3004	41	CAF, MTX/L-PAM/5FU	No	3.80	48.30
3005	49	CMF, CAF	Yes	1.59	2.67
3006	47	CMF, Tamox	Yes	2.83	8.61
3007	39	CMF, CAF	No	4.06	18.67
3008	51	CMF, Tamox, MVF, Adria	No	5.39	10.09
3009	35	CAF, Tamox	No	2.09	32.35
3010	47	CMF, Tamox	Yes	1.88	9.74
3011	46	CAF	No	1.45	34.23
3012	56	Adria/CTX, Taxol	No	1.91	34.20
3013	33	CMF,CA, Taxol	Yes	3.07	6.54
3014	49	CAF, Tamox, MVF	No	2.75	25.73
3015	48	CMF/Tamox, CAF	No	3.78	28.94
average	44			2.93	17.71

3000–3002 and 100 million cells/apheresis for all subsequent patients. The unselected apheresis products were cryopreserved in the usual fashion.

Enrichment of CD34+ cells by immunomagnetic selection

$CD34^+$ cells were isolated from one of the patient's apheresis collections utilizing the Baxter Isolex 300 Magnetic Cell Separation system (Baxter Immunotherapy, Irvine, CA) as previously described.[4]

Culture of CD34+ Cells

The $CD34^+$ cells were initially seeded at a concentration of $0.5–1 \times 10^5$ cells/mL in the presence of PIXY321 (Immunex, Seattle, WA) in a modified prototype PL732 gas permeable bag containing serum-free X-VIVO 10 (BioWhittaker, Walkersville, MD). The bag(s) were incubated for a total of 12 days in a high-humidity, 37°C incubator containing 5% CO_2 and 95% air. For patients no. 3004–3008, the culture condition was modified to 5% CO_2 and 5% O_2. At day 7 of the culture, an additional volume of culture medium containing PIXY321 at 100ng/mL was supplemented to the culture to return the cell concentration to $<3 \times 10^5$ cells/mL. If no additional culture medium was required only 100 ng/mL of PIXY321 was added to the culture bag(s).

Table 2. Expansion of cultured CD34+ cells

	Day 0		Day 12	(Day 1–12)	Day 12			
Pt ID	CD34+ cell purity (%)	Total cell $\times 10^7$	Total cells $\times 10^7$	Fold expansion	Pooled cells % viable	Pooled cells %CD15+	Cultured cells infused $\times 10^6$/kg	Morph % gran*
3000	98.2	1.8	33.2	44.1	83	24.8	18.7	72
3001+	73.4	3.2						
3002	98.0	0.9	11.6	12	88	29.5	21.4	81
3003	64.0	0.9	5.6	6	86	48.1	0.8	81
3004	98.7	49.8	1066	21	92	33.2	156.6	83
3005	90.6	1.9	70.1	36	83	25.2	6.5	63
3006	98.0	19.3	481.3	25	71	30.8	48.6	55
3007	96.9	17.0	337.2	20	80	24.5	74.7	74
3008	98.2	10.2	282.0	28	54.0	18.5	29.7	49
3009+	98.8	19.5						
3010	98.3	7.6	62.5	8.2	95.0	47.7	6.8	93
3011	97.2	26.2	348.0	13.3	95.0	60.3	77.4	85
3012	97.9	99.3	866.0	8.7	86.6	57.3	99.6	84
3013+	98.3	2.0						
3014	99.8	85.3	658.0	7.7	73.0	46.7	144.6	84
3015^	99.0	56.9	482.0	8.5	81.3		74.1	75
aversge	94.1	25.1	366.0	18.3	82.1	37.2	58.4	75.3

Granulocyte precursors=promyelocytes, myelocytes, metamyelocytes.

+Patient not reinfused with cultured cells.

^No Day 12 CD15 data due to an assay problem.

Phenotype and morphology of the culture expanded CD34+ cells

The percentage of $CD34^+$ cells was determined following selection with the Isolex 300 Magnetic Cell Separation System and, after the 12-day culture, by flow cytometry using a CD34 monoclonal antibody conjugated to phycoerthyrin, HPCA2-PE (Becton Dickinson, San Jose, CA) and a FACScan flow cytometer (Becton Dickinson). In addition, the $CD15^+$ cells were quantitated with FITC-Leu M1 (Becton Dickinson).

Reinfusion of culture-expanded cells

The number of cells administered following expansion did not exceed the total number of cells that was obtained by a single apheresis procedure (approximately 10^{10} cells). At day 12 the cultured cells were procured and washed using the CS3000 Plus Cell Separator and resuspended in 150 to 250 mL of a reinfusion solution consisting of Plasma-Lyte A (Baxter Fenwal, Deerfield, IL) (code 2B2543) supplemented with 1% human serum albumin (Baxter Hyland, Glendale, CA). The cells were then rapidly reinfused into the patient through an indwelling central venous catheter.

RESULTS

Proliferation of CD34+ cells in culture

Table 2 summarizes the results of the proliferation in vitro of these positively selected $CD34^+$ PBPC. Thirteen patients' $CD34^+$ PBPC showed an average proliferation of 18-fold with a range of 6 to 44 fold. The PBPC from three patients did not expand because of technical errors and, subsequently, they did not receive in vitro culture expanded $CD34^+$ PBPC. The mean viability of these procured cultured cells before reinfusion was 82% (range 54 to 95%). The average total number of cells after 12 days of culture was 366×10^7 (range $5.6–1066\times10^7$ cells).

Morphology and flow cytometric phenotype

Morphologically, these expanded cells averaged 75% granulocytic precursors including promyelocytes, myelocytes and metamyelocytes as observed from the cytospin preparations. The mean percentage of $CD15^+$ cells in the final product infused into the patient was 37% (range 18.5 to 60%).

Toxicity and hematologic recovery

Thirteen patients received in vitro culture expanded $CD34^+$ PBPC after infusion of cryopreserved PBPC. There were no adverse events associated with the reinfusion of these cultured cells. Hematopoietic recovery was prompt with a median neutrophil recovery greater than 500/L at day 8 (range 8 to 10 days). Platelets were greater than 20,000/L with transfusion independence at day 10

(range 8 to 12) and platelet recovery greater than 50,000/L was achieved on day 1 (range 9 to 14 days). Platelet recovery could not be determined in one patient because of necrotizing fascitis and need for platelet transfusions because of multiple surgical procedures.

CONCLUSION

In this report we demonstrate that this approach is feasible. In addition, the reinfusion of these expanded myeloid progenitors did not lead to acute toxicity. There were no signs or symptoms of leukagglutination reactions. Hematopoietic recovery, especially neutrophil recovery, was prompt; however, at the dose of cells infused it did not appear to be shortened in this preliminary trial. It should be pointed out, however, that the dose of cultured cells reinfused covered a 2-log range. Interestingly, the higher cultured cell doses were associated with patients showing the most efficient cell recovery, although the database is too small to draw a final conclusion.

Significant large-scale culture expansion and differentiation of $CD34^+$ selected PBPC can be achieved in vitro. These cells can be safely reinfused into patients. Further studies will be needed to determine its full impact upon hematologic recovery after HDCT. In addition, the system described here is amenable to the use of additional growth factors, which may enhance or redirect CD34 cell differentiation. This methodology provides a means of full-scale expansion of CD34 cells to address a variety of problems related to the cytopenias associated with antineoplastic therapy.

REFERENCES

1. Kessinger A, Armitage JO: The evolving role of autologous peripheral stem cell transplantation following high dose chemotherapy for malignancies. *Blood* 77:211, 1991.
2. Brandt SJ, Peters WP, Atwater SK et al.: Effect of recombinant human granulocyte-macrophage colony-stimulating factor on hematopoietic reconstitution after high-dose chemotherapy and autologous bone marrow transplantation. *N Engl J Med* 325:164, 1988.
3. Smith SL, Bender JG, Maples PB et al.: Expansion of neutrophil precursors and progenitors in suspension cultures of $CD34^+$ cells enriched from human bone marrow. *Exp Hematol* 21:870, 1993.
4. Williams SF, Lee WJ, Bender JG et al.: Selection and expansion of peripheral blood $CD34^+$ cells in autologous stem transplantation for breast cancer. *Blood* 87:1687–1691, 1996.

MOBILIZATION OF PERIPHERAL BLOOD PROGENITOR CELLS FOLLOWING G-CSF–SUPPORTED CYTOTOXIC CHEMOTHERAPY FOR AUTOLOGOUS TRANSPLANTATION

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ABSTRACT

Since June 1991, 312 patients with hematological malignancies and solid tumors were enrolled onto a study to receive high-dose therapy with PBSC transplantation. The patients (170 females/142 males) had a median age of 44 years (range, 18–65 years). The spectrum of diagnoses included 126 patients with low- and high-grade non-Hodgkin's lymphoma (NHL), 23 patients with Hodgkin's disease, 67 patients with multiple myeloma, 8 patients with leukemia and 67 patients with primary or metastatic breast cancer. An additional 21 patients had other solid tumors. For the collection of PBSC, the patients received a granulocyte colony-stimulating factor (G-CSF) (filgrastim)-supported cytotoxic chemotherapy which was dependent on the diagnosis. For instance, patients with NHL received high-dose cytarabine and mitoxantrone (HAM), whereas patients with multiple myeloma received high-dose cyclophosphamide. Leukaphereses (LP) were performed during leukocyte recovery on the basis of a daily $CD34^+$ cell measurement. A strong correlation was observed between the number of circulating $CD34^+$ cells and the number of $CD34^+$ cells harvested. With respect to the yield of $CD34^+$ cells, significant differences were observed among the various patient groups. The amount of previous cytotoxic chemotherapy was found to have an adverse effect on the collection efficiency. We were particularly interested in the subset of early $CD34^+/Thy\text{-}1^+$ stem cells, especially in factors influencing its proportion and yield in the LP products. The mean telomere length (MTL) of mononuclear cells from LP products was also assessed in relation to the content of $CD34^+$ cells, since the MTL is thought to reflect the developmental stage of lymphohemopoietic cells on a molecular level. Independent of the diagnosis, PBSC collection could be significantly improved by the introduction of large-volume leukaphereses.

INTRODUCTION

Peripheral blood stem cells (PBSC) are increasingly used to support myeloablative therapy in patients with hematological malignancies and solid tumors. In an attempt to improve mobilization and harvesting of PBSC, we first defined the quantity of PBSC required for engraftment after high-dose therapy, as well as factors that influence mobilization and autografting in patients with malignant lymphoma.[1] It was the purpose of the present study to retrospectively evaluate factors influencing mobilization of PBSC in patients with low- and high-grade non-Hodgkin's lymphoma (NHL), Hodgkin's disease, multiple myeloma, acute leukemia and solid tumors. The questions addressed included monitoring of collection, the search for predictive parameters for the yield of hematopoietic progenitor cells, the collection efficiency in relationship to the patients' disease, the characterization of the hematopoietic progenitor and stem cells on the basis of immunophenotyping and mean telomeric length as well as the introduction of large-volume leukapheresis.

PATIENTS AND METHODS

Patients

The data presented are based on 312 cancer patients who were enrolled onto different mobilization protocols between June, 1991, and August, 1995. The common denominator for their inclusion was that they received a cytotoxic chemotherapy followed by the administration of R-metHuG-CSF (filgrastim: Amgen, Thousand Oaks, CA). There were 142 males and 170 females with a median age of 44 years (range 18 to 65). The choice of cytotoxic chemotherapy was dependent on the diagnosis in order to combine an effective antitumor therapy with a cytokine-supported mobilization of PBSC. One hundred twenty-six patients had NHL and received high-dose cytarabine (2 g/m^2, q12 hours, day 1 and 2) and mitoxantrone (10 mg/m^2 q24 hours, day 2 and 3). Twenty-three patients with Hodgkin's disease were treated with a polychemotherapy consisting of dexamethasone (24 mg/day on days 1-7), BCNU (60 mg/m^2 on day 3), etoposide (75 mg/m^2 on days 4–7), cytosine arabinoside (200 mg/m^2 on days 4–7) and melphalan (30 mg/m^2 on day 3). Sixty-seven patients with multiple myeloma either received 4 g/m^2 or 7g/m^2 cyclophosphamide, while eight patients with acute leukemia underwent PBSC collection following early consolidation therapy with high-dose cytarabine (1 g/m^2 q12 hours, days 1 to 4) and mitoxantrone (10 mg/m^2 q24 hours, days 2 to 5). The 88 patients with solid tumors were treated with a combination of ifosfamide (2500 mg/m^2 on days 1–2) and epirubicine (40 mg/m^2 on days 1–3).

Filgrastim (R-metHuG-CSF; Amgen) was given at a dose of 300 μg/day, subcutaneously. As a result, the daily doses per kg bodyweight varied between 2.66

and 6.67 μg. Treatment with filgrastim began 24 hours after completion of cytotoxic chemotherapy and continued until PBSC harvesting was finished.

The study was conducted under the guidelines of the Ethical Committee of the University of Heidelberg. Informed consent was obtained from each patient.

PBSC collection

PBSC collection was started as soon as a white blood count greater than 1×10^9/L was reached and a distinct population of $CD34^+$ cells was detectable by direct immunofluorescence analysis. Harvesting was performed using a Fenwal CS 3000 (Baxter, Deutschland GmbH, Munich, Germany), or a COBE Spectra (Cobe Laboratories, Lakewood, CO). Two hundred twenty-four patients underwent a total of 767 regular leukaphereses with processing of 10 L of blood at flow rates between 50–70 mL/min. For 88 patients, the collection of PBSC was performed during the course of 154 large-volume leukaphereses by processing 20 liters of blood at flow rates between 75–150 mL/min.

The apheresis product was mixed with the same volume of minimum essential medium containing 20% dimethylsulfoxide (Merck, Darmstadt, Germany). The final 100 mL cell suspension was transferred into freezing bags (Delmed Inc., New Brunswick, NJ) and frozen to –100°C using a computer-controlled cryopreservation device (Cryoson BV-6; Cryoson Deutschland GmbH, Schöllkrippen, Germany). The frozen cells were transferred into the liquid phase of nitrogen and stored at –196°C.

Immunofluorescence staining and flow cytometry

For the immunofluorescence analysis, 1×10^6 mononuclear cells (MNC) or 20–50 μL of whole blood (EDTA) were incubated for 30 minutes at 4°C with the fluorescein (FITC)-conjugated monoclonal antibodies (mAb) HPCA-2 (CD34) and HLe-1 (CD45). CD34 subsets were further analyzed using phycoerythrin (PE)-labeled HPCA-2 (CD34) and the FITC-conjugated mAb CDw90 (Thy-1, clone 5E10).

Isotype identical antibodies served as control (Simultest). Except for Thy-1 (Pharmingen, San Diego, CA), all antibodies were obtained from Becton Dickinson, Heidelberg, Germany. The cells were analyzed using a Becton Dickinson FACScan, as previously described.[2]

Telomere length analysis

DNA extraction from 1×10^7 leukapheresis products-derived MNC was performed as previously described.[3] Genomic DNA (10 μg) was digested using 20 U of HinfI and RsaI (Boehringer Mannheim, Germany) to obtain terminal

restriction fragments (TRF) which contain telomeric TTAGGG repeats and subtelomeric regions. After phenol/chloroform extraction the fragments were precipitated with ethanol and dissolved in 50 µL TE-buffer. Fifteen microliters of the resulting restriction fragments were separated by 0.7% agarose gel electrophoresis at 40 V for 15 hours and transferred to nylon membranes (Gene Screen Plus, Biotechnology Systems, Boston, MA). Hybridization was performed as described[3] using 5'end-labeled $^{[32]}$P-$(CCCTAA)_3$ oligodeoxyribonucleotides (MWG Biotech, Ebersberg, Germany).

The membranes were scanned by a PhosphorImager (Molecular Dynamics) using the IMAGEQUANT software (Molecular Dynamics). Mean TRF lengths were determined as described by Kruk et al.[4]

Statistical analysis

Data were evaluated according to standard statistical methods (Statworks, Cricket Software, Philadelphia, PA). The results are given as mean ± standard error of the mean (SEM) or as median and range. Statistical significance between differences of grouped data was determined using the Student's *t*-test. A significance level of $p<0.05$ was chosen. The relationship between the hematological parameters of the peripheral blood and leukapheresis products was evaluated using linear regression and correlation analysis.

RESULTS

Circulating CD34+ cells and monitoring of PBSC harvesting

PBSC were harvested following G-CSF–supported chemotherapy which was chosen according to the disease. As soon as a white blood count of 1.0×10^9/L was reached following the chemotherapy-induced nadir, the level of circulating $CD34^+$ cells in the peripheral blood was determined. If a distinct population of $CD34^+$ cells was discernible, leukaphereses were performed. There was a strong correlation between the number of circulating $CD34^+$ cells in the peripheral blood and the yield in the respective leukapheresis product ($R=0.72$, $p<0.001$), whereas no relationship was observed between the $CD34^+$ cell yield and the white blood count or mononuclear cell count. As shown in Figure 1A, patients presenting with levels of circulating $CD34^+$ cells above 100/µL were likely to obtain a target quantity of at least 2.5×10^6 $CD34^+$ cells/kg during the course of a single standard volume leukapheresis with processing of 10 liters of blood. In contrast, patients with $CD34^+$ cell levels below 10/µL rarely reached this target quantity. Still, due to the substantial variability in the yield observed in patients with levels of circulating $CD34^+$ cells below 20/µL, at least one leukapheresis was performed in each patient. It turned out that the minimum quantity of

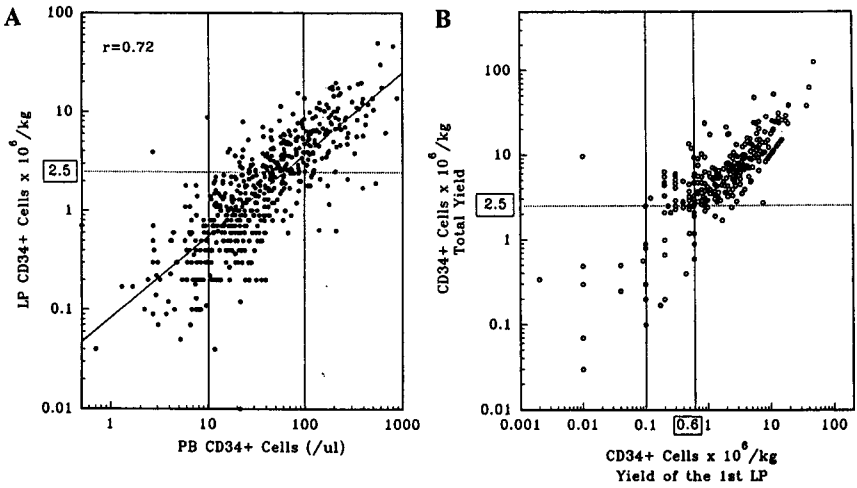

Figure 1. ***(A)*** *Number of CD34⁺ cells/μL in the peripheral blood before leukapheresis and in the corresponding leukapheresis product (per kg bodyweight). The correlation analysis is based on a total of 614 paired samples and performed after logarithmic transformation.* ***(B)*** *Yield of CD34⁺ cells/kg in the first leukapheresis product and total number of CD34⁺ cells/kg harvested. The data were obtained in 224 patients who underwent a total of 767 standard 10-liter leukapheresis procedures.*

2.5×10^6 CD34⁺ cells/kg envisaged for an autograft could not be obtained, if the patients' first harvest contained less than 0.1×10^6 CD34⁺ cells/kg (Figure 1B). Both analyses are based on 224 patients who underwent a median number of 2 standard volume leukaphereses (range 1–10).

Confirming a previous observation in 15 patients,[5] a strong correlation was found between the concentration of CD34⁺ cells determined during steady-state hematopoiesis before mobilization and the peak value observed during G-CSF-stimulated recovery. The measurement of CD34⁺ cells during steady-state hematopoiesis is based on the acquisition of a mean number of 132,000 events. This allows acquisition of a mean number of 76 events in the CD34 gate, while appropriate controls are needed to exclude false positive events. Although the measurement of CD34⁺ cells before mobilization proved to be helpful and reliable in patients with circulating CD34⁺ cells above the detection limit, its value for patients with levels below the threshold is only relative. Of 56 patients with baseline CD34⁺ cell concentrations below detection limit, 46 (82%) still obtained a CD34⁺ cell harvest of at least 2.5×10^6/kg during the course of 2 leukaphereses (median, range 1–8).

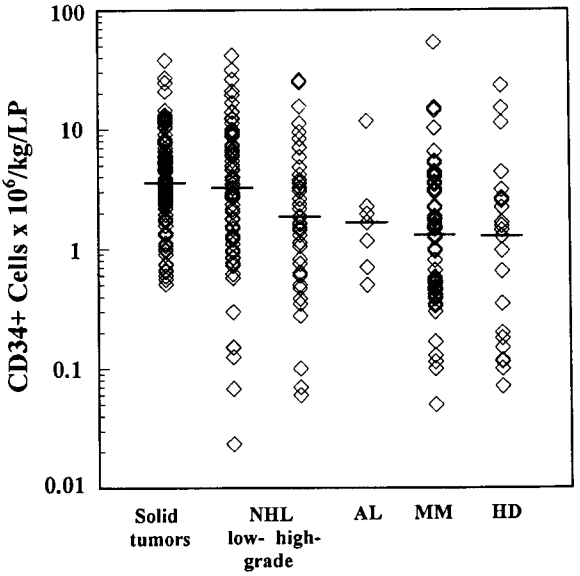

Figure 2. *Yield of CD34⁺ cells per kg bodyweight and leukapheresis. The horizontal bars indicate the median value for each patient group according to the diagnosis. Non-Hodgkin's leukemia: NHL; AL: acute leukemia; MM: multiple myeloma; HD: Hodgkin's disease; LP: leukapheresis.*

Disease-related mobilization efficiency

PBSC mobilization data for the different patient groups are based on a total of 961 measurements. As a result of the diversity in the mobilization regimens, differences in the hematological effects observed between patient groups may reflect not only the nature of the underlying disease, but also the disease-related mobilization chemotherapy. Confirming our previous results in a group of 61 patients,[1] a major factor adversely affecting the mobilization efficiency was the amount of previous cytotoxic chemotherapy which is particularly detrimental when additional radiotherapy had been administered.

In general, patients with solid tumors mobilize very well, since out of 88 patients evaluated, only 7 patients (8%) did not reach the target quantity of 2.5×10^6 $CD34^+$ cells/kg. This includes patients with metastatic disease and previous cytotoxic therapy as well as three patients with breast cancer treated neo-adjuvantly. The results are similar for patients with low-grade NHL, while a significantly smaller yield was observed for patients with high-grade NHL, acute leukemia and multiple myeloma. The smallest number was observed in patients with relapsed Hodgkin's disease, particularly since more than 90% of these patients also received large-field radiotherapy (Figure 2).

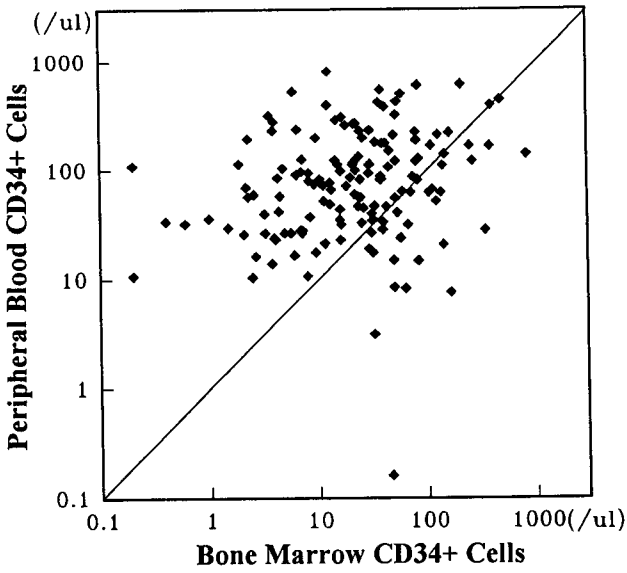

Figure 3. *Intra-individual comparison between the concentration of CD34⁺ cells in steady-state BM and peripheral blood during G-CSF-enhanced marrow recovery in 155 patients. The assessment is based on the individual peak concentration of CD34⁺ cells observed during the rebound period. The mean peak concentration of circulating CD34⁺ cells was 2.3-fold greater compared to BM samples (120.98 ± 11.14/μL versus 51.65 ± 7.60/μL, p<0.001).*

Circulating versus bone marrow (BM) CD34⁺ cells

There are strong arguments for the use of PBSC rather than BM for supporting high-dose therapy that relates to quantitative as well as qualitative aspects. In a group of 155 patients, BM aspirates were obtained before mobilization to compare the concentration of CD34⁺ cells with the peak value of circulating CD34⁺ cells observed during marrow recovery. As shown in Figure 3, the peripheral blood contained a greater number of CD34⁺ cells in 79% of the patients when compared with their respective BM samples. Since PBSC comprise clonogenic cells at various developmental stages with different capacities of self-renewal and differentiation, our evaluation included a subset analysis for the Thy-1 antigen.[6] Co-expression of this antigen on a CD34⁺ cell indicates an early hematopoietic progenitor cell with extensive self-renewal ability as well as long-term repopulating capacity. Comparing both hematopoietic compartments with regard to the proportion of CD34⁺/Thy-1⁺ cells contained therein, a relevant finding was the 2.2-fold greater mean proportion of this particular subset in the blood-derived

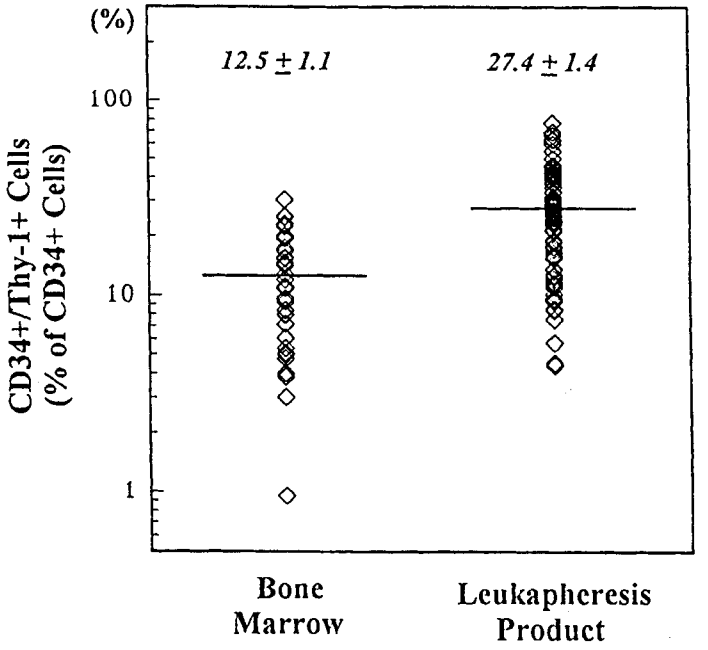

Figure 4. *Proportion of CD34⁺/Thy-1⁺ cells in BM and leukapheresis (LP) products of 40 patients. For the intra-individual comparison, a BM sample was obtained before the start of cytotoxic chemotherapy. The evaluation of blood-derived CD34⁺ cells in these patients is based on 109 LP products collected during G-CSF-enhanced marrow recovery. A 2.2-fold greater mean proportion of this early progenitor cell subset was observed in LP products compared with BM.*

leukapheresis products (27.47 ± 1.43% versus 12.56 ± 1.15%, $p<0.001$). The results are summarized in Figure 4. It was also interesting to note that the proportion of CD34⁺/Thy-1⁺ cells collected during the course of sequential leukaphereses gradually decreased. The results are based on 22 patients who underwent a minimum of at least 3 leukaphereses (Figure 5). This decline in the proportion of more primitive progenitor cells argues for PBSC collection at an early time point during the G-CSF-enhanced recovery period.

Mean TRF length of blood-derived mononuclear cells

An attempt was made to correlate the amount of CD34⁺ cells collected per leukapheresis with the mean telomere length (MTL) of the mononuclear cells in the respective product. Considering the telomere length as a parameter for the replicative capacity of cells,[7,8] we expected that a longer MTL—as a reflection of

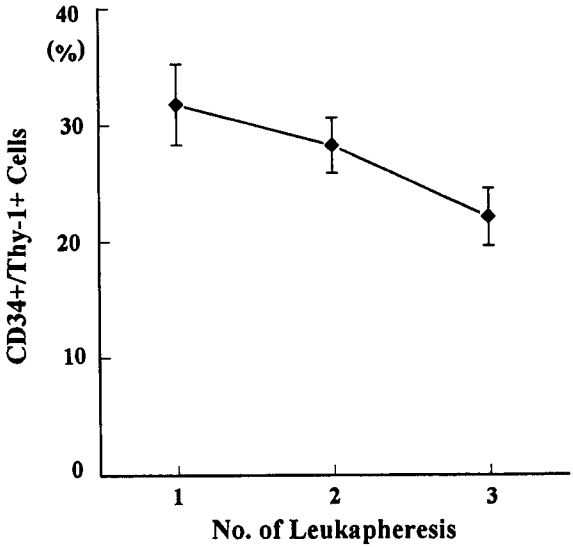

Figure 5. *Proportion (mean ± SEM) of CD34⁺/Thy-1⁺ cells during the course of the first three leukaphereses. The assessment is based on a total of 66 leukapheresis products sequentially harvested in 22 patients.*

a more juvenile population of lymphohematopoietic stem cells—would be associated with greater levels of circulating CD34⁺ cells. The mean terminal restriction fragment (TRF) length served as parameter for the MTL. Evaluating the LP products of 54 patients, there was no relationship between the CD34⁺ cell yield and the TRF length which varied between 7.0 and 9.7 kb (median 8.11 kb).[9] The results suggest that the proliferative capacity of the hematopoietic system is not related to this molecular marker which rather reflects the replicative capacity of cells than the patient's hematopoietic reserve.

Large-volume leukaphereses for PBSC collection

Independent of the diagnosis, PBSC collection could be significantly improved, when large-volume leukaphereses were introduced with processing of 20 liters of blood instead of 10 liters. Clinically well tolerated, the procedure led to an average 2-fold increase in the number of CD34⁺ cells harvested, which is particularly relevant when ex-vivo selection is envisaged. For 35 procedures, the content of CD34⁺ cells was determined after processing of 10 liters and 20 liters, respectively. In all patients studied, the second harvest contained as many or more CD34⁺ progenitor cells when compared with the first product.[10] This finding suggests a

mobilizing effect of the leukapheresis procedure, as we observed a 2-fold increase in the concentration of circulating $CD34^+$ cells following processing of 16 liters (41.6 ± 25.1 versus 20.9 ± 6.0 $CD34^+$ cells/μL). The underlying mechanisms of this mobilization effect are not clear. As expected, large-volume leukaphereses were particularly advantageous for patients with small concentrations of circulating $CD34^+$ cells. The impact of processing large volumes of blood can also be appreciated by looking at the target number of 2.5×10^6 $CD34^+$ cells/kg, which provide rapid and sustained engraftment following high-dose therapy. Single harvests containing at least 2.5×10^6 $CD34^+$ cells/kg were collected in 74% of patients undergoing large-volume leukaphereses compared with a proportion of 32% in patients undergoing regular procedures. As patients stay in hospital during PBSC collection post-chemotherapy, introduction of large-volume leukaphereses resulted in a shorter time of hospitalization.

DISCUSSION

This is a retrospective study involving 312 patients with hematological malignancies and solid tumors who received cytotoxic chemotherapy followed by G-CSF in order to mobilize PBSC for autologous transplantation. The combination of cytotoxic chemotherapy with G-CSF support leads to significant rebound levels of circulating progenitor cells during marrow recovery and permits the collection of PBSC for autografting in the majority of patients. The peak value of $CD34^+$ cells observed during G-CSF-stimulated marrow recovery correlates with the $CD34^+$ cell concentration in peripheral blood before mobilization. Patients presenting without $CD34^+$ cells during steady-state hematopoiesis might be considered for experimental mobilization modalities to increase the yield of $CD34^+$ cells. These approaches include the addition of other cytokines with potential synergism such as c-*kit* ligand (also termed stem cell factor)[11] or interleukin-3.[12–15] Recent data suggest that chemokines like interleukin-8[16] and macrophage-inhibitory protein-1-alpha (MIP-1α)[17,18] effectively mobilize $CD34^+$ cells, while the underlying mechanisms are apparently different from those of the hematopoietic growth factors mentioned earlier. Monitoring of PBSC collection has been greatly facilitated by the $CD34^+$ cell assessment in the PB and LP products. Based on these measurements, a decision can be made when to start and when to finish PBSC collection. It also permits an estimate of whether an ex-vivo selection of $CD34^+$ cells is worthwhile. To ensure a sufficient number of $CD34^+$ cells, harvesting of PBSC should be envisaged as early as possible during the course of the disease, as the hematopoietic reserve declines with each cycle of cytotoxic chemotherapy. Large field radiotherapy has an additional negative effect on the progenitor cell pool. The gradual decrease in the proportion of $CD34^+$/Thy-1^+ cells during the course of the first three leukaphereses indicates that more primitive progenitor cells

are generated and released during an earlier period of marrow recovery. Considering that more primitive progenitor cells are important for long-term reconstitution following high-dose chemotherapy, PBSC harvesting is more effective during the early rebound phase.

The mean TRF length of the blood-derived MNC was not related to the amount of $CD34^+$ cells collected, implying that the molecular marker reflecting the 'mitotic age' and replicative capacity of the cells is not predictive for the proliferative response of the hematopoietic system to G-CSF following cytotoxic chemotherapy. Another point of practical relevance relates to the introduction of large-volume leukaphereses, which permit the collection of an average 2-fold greater number of $CD34^+$ cells when compared with LP products following a regular 10-liter leukapheresis. The additional mobilization effect is particularly beneficial in patients with a history of extensive cytotoxic pretreatment that adversely affects the response to cytokine and/or chemotherapy-based mobilization regimens. Earlier concerns, that cytokine-mobilized $CD34^+$ cells from peripheral blood could be inferior compared with $CD34^+$ cells from BM, appear to be invalid. Based on an intraindividual analysis, in the majority of patients a greater number of $CD34^+$ cells was found in the G-CSF–stimulated PB with an increased proportion of $CD34^+$/Thy-1^+ cells when compared with BM samples obtained before mobilization. PBSC are therefore suitable for the support of dose-escalated polychemotherapy as well as myeloablative high-dose therapy provided that a target quantity of 2.5×10^6 $CD34^+$ cells/kg is reinfused. In the same line, PBSC are apt targets for somatic gene therapy, as they are composed of a substantial proportion of stem cells with great self-renewal and long-term repopulation ability.

REFERENCES

1. Haas R, Moehle R, Fruehauf S et al.: Patient characteristics associated with successful mobilizing and autografting of peripheral blood progenitor cells in malignant lymphoma. *Blood* 83:3787–3794, 1994.
2. Haas R, Moos M, Karcher A et al.: Sequential high-dose therapy with peripheral-blood progenitor-cell support in low-grade non-Hodgkin's lymphoma. *J Clin Oncol* 12:1685–1692, 1994.
3. Ausubel FM: Current Protocols in Molecular Biology. New York, Chichester, Brisbane, Toronto, Singapore: John Wiley & Sons, 1987.
4. Kruk PA, Rampino NJ, Bohr VA: DNA damage and repair in telomeres: Relation to aging. *Proc Natl Acad Sci USA* 92:258–262, 1995.
5. Fruehauf S, Haas R, Conradt C et al.: Peripheral blood progenitor cell (PBPC) counts during steady-state hematopoiesis allow to estimate the yield of mobilized PBPC after filgrastim (R-metHuG-CSF)-supported cytotoxic chemotherapy. *Blood* 85:2619–2626, 1995.
6. Craig W, Kay R, Cutler R, Lansdorp PM: Expression of Thy-1 on human hematopoietic

progenitor cells. *J Exp Med* 177:1331–1342, 1993.
7. Allsopp RC, Vaziri H, Patterson C et al.: Telomere length predicts replicative capacity of human fibroblasts. *Proc Natl Acad Sci USA* 89:10114–10118, 1992.
8. Lansdorp PM: Telomere length and proliferation potential of hematopoietic stem cells. *J Cell Sci* 108:1–6, 1995.
9. Kronenwett R, Murea S, Haas R: Telomere length of blood-derived mononuclear cells from cancer patients during G-CSF-enhanced marrow recovery. *Bone Marrow Transplant* (in press).
10. Murea S, Goldschmidt H, Hahn U et al.: Successful collection and transplantation of peripheral blood stem cells in cancer patients using large-volume leukapheresis. *J Clin Apheresis* (in press).
11. Andrews RG, Briddell RA, Knitter GH et al.: In vivo synergy between recombinant human stem cell factor and recombinant human granulocyte colony-stimulating factor in baboons: Enhanced circulation of progenitor cells: *Blood* 84:800-810, 1994.
12. Haas R, Ehrhardt R, Witt B et al.: Autografting with peripheral blood stem cells mobilized by sequential interleukin-3/granulocyte-macrophage colony-stimulating factor following high-dose chemotherapy in non-Hodgkin's lymphoma. *Bone Marrow Transplant* 12:643–649, 1993.
13. Geissler K, Valent P, Mayer P et al.: Recombinant human interleukin-3 expands the pool of circulating hematopoietic progenitor cells in primates—Synergism with recombinant human granulocyte/macrophage colony-stimulating factor. *Blood* 75:2305–2310, 1990.
14. Brugger W, Bross K, Fritsch J et al.: Mobilization of peripheral blood progenitor cells by sequential administration of interleukin-3 and granulocyte-macrophage colony-stimulating factor following polychemotherapy with etoposide, ifosfamide, and cisplatin. *Blood* 79:1193–1200, 1992.
15. Huhn RD, Yurkow EJ, Tushinski R et al.: Recombinant human interleukin-3 (rhIL-3) enhances the mobilization of peripheral blood progenitor cells by recombinant human granulocyte colony-stimulating factor (rhG-CSF) in normal volunteers. *Exp Hematol* 24:839–847, 1996.
16. Laterveer L, Lindley IJ, Heemskerk DP et al.: Rapid mobilization of hematopoietic progenitor cells in rhesus monkeys by single intravenous injection of interleukin-8. *Blood* 87:781–788, 1996.
17. Hunter MG, Bawden L, Brotherton D et al.: BB-10010: An active variant of human macrophage inflammatory protein-1 alpha with improved pharmaceutical properties. *Blood* 86:4400–4480, 1995.
18. Lord BI, Woolford LB, Wood LM et al.: Mobilization of early hematopoietic progenitor cells with BB-110: A genetically engineered variant of human macrophage inflammatory protein-1. *Blood* 85:3412–3415, 1995.

OPTIMAL TIMING OF LEUKAPHERESIS BASED ON MOBILIZATION OF LONG-TERM CULTURE-INITIATING CELLS

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ABSTRACT

Cumulative myelosuppression expressed in progressive neutropenia and thrombocytopenia occurs during multicycle chemotherapy treatment. Mobilization of hematopoietic progenitor cells decreases during subsequent chemotherapy cycles. The effect of chemotherapy on mobilization and proliferation of long-term culture-initiating cells (LTC-IC) has not been examined extensively and is addressed here.

Sixteen patients with early advanced breast cancer received four cycles of intensive FAC chemotherapy (5-FU, adriamycin, cyclophosphamide) with lenograstim (glycosylated r-Hu-G-CSF) support. Granulocyte colony-stimulating factor (G-CSF) was administered during two additional 10-day periods before the start, and at least three weeks after completion of chemotherapy. Blood samples for assessment of colony-forming cells (CFC) and $CD34^+$ cells and proliferation of LTC-IC were collected during the periods of G-CSF administration, in cycles 1 and 4 and from leukapheresis samples. Mobilization of granulocyte-macrophage (GM)-CFC and burst-forming unit-erythroid (BFU-E) was most optimal in cycle 1 and decreased during chemotherapy. It recovered partially during G-CSF after chemotherapy compared with cycle 4, but to a lower level than during G-CSF before chemotherapy. Mobilization of $CD34^+$ cells was comparable in cycles 1 and 4 and at a lower level during the periods of G-CSF only. The proliferative potential of LTC-IC was best in blood collected within 1–2 days of maximal circulating CFC and $CD34^+$ cell numbers during each particular period and most optimal during G-CSF before chemotherapy and cycle 1.

Timing leukapheresis during G-CSF in chemotherapy naive status or in the first chemotherapy cycle within one day from maximal concentrations of CFC and $CD34^+$ cells ensures the highest collection of LTCIC.

INTRODUCTION

Since the first clinical trials in the late seventies, various studies have reported hematopoietic reconstitution after myeloablative treatment by infusion of peripheral blood progenitor cells (PBPC) collected in steady state[1] or during recovery from chemotherapy.[2–4] Secondary graft failure occurred in some cases.[2,3] Infusion of PBPC, collected during administration of granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF) alone or during growth factor administration after chemotherapy, induced more rapid hematological recovery than infusion of autologous bone marrow.[5–10]

Most reports focus on mobilization of cells important for early post-transplant hematological recovery, and timing of leukapheresis has been based on peak concentrations of CFC and $CD34^+$ cells[11–17] or on the assumption that cells important for durable engraftment are mobilized early during hematological recovery at WBC count >0.5 or 1×10^9/L or unequivocal rise of $CD34^+$ cell numbers.[12,18–21] Specific markers are required to detect putative stem cells by flow cytometry[19,22,23] because $CD34^+$ antibodies may inadequately predict circulation of stem cells. We used long-term cultures[20,24,25] to assess presence of putative stem cells at various times before, during and after chemotherapy treatment and evaluated two mobilization regimens to define the optimal time for collection of primitive cells (LTCIC), which have the capacity to generate CFC.

MATERIALS AND METHODS

Patients and treatment

Sixteen patients with early advanced breast cancer (median age 42 years), of whom six had received prior adjuvant chemotherapy (cyclophosphamide, methotrexate, 5-fluorouracil), received four cycles of standard or intensified FAC chemotherapy at two- or three-week intervals. Lenograstim was administered at a dose of 10 μg/kg/d s.c. for ten days from day two in each chemotherapy cycle. Eight patients received 10 μg/kg/d of G-CSF s.c. for a period of ten days before initiation of chemotherapy and eight patients 10 μg/kg/d of G-CSF s.c. for ten days at least three weeks after completion of chemotherapy treatment when the hematogram had normalized (Figure 1). Treatment according to protocol, approved by the South Ethics Committee, was performed after written informed consent was obtained. A single leukapheresis was planned at WBC recovery $>4\times10^9$/L in cycle 1 as previously described.[12]

Assessment of CFC, $CD34^+$ cells and LTCIC

Blood samples for full blood count, CFC and $CD34^+$ cell assessment were collected daily during the periods of G-CSF before and after chemotherapy treatment and on day 1, 4 and 7 through 13 in cycle 1 and 4. Each sample was

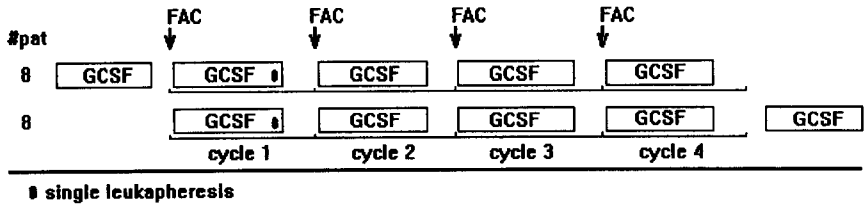

Figure 1. *Treatment schedule.*

collected in 200 U/L preservative-free heparin. Blood MNC were separated using a Ficoll-Hypaque gradient (density 1.077 g/mL, Pharmacia, Germany). MNC were washed twice in phosphate buffered saline supplemented with 2% fetal calf serum (FCS) and counted in a Neubauer hematometer. LTC were initiated on day 5 and 10 during the periods of G-CSF and on day 9 and 11 in cycle 1 and 4. Leukapheresis samples were assessed for mononuclear cells (MNC), CFC, $CD34^+$ cells and LTCIC. Normal bone marrow of allogeneic transplant donors or volunteers was used for control LTC, with informed consent.

Clonogenic assays and flow cytometry

CFC were assessed as previously described.[25] Colonies were counted after 14 days of incubation and classified as granulocyte-macrophage type (GM-CFC), burst-forming units-erythroid (BFU-E) or multipotent (Mix-CFC). MNC were prepared for FACS analysis as previously described[26] and analyzed within 24 hours.

Bone marrow stromal cultures

Bone marrow cultures were prepared as previously described.[25] Confluent stromal layers were used after 3 to 8 weeks. They were irradiated with 15 cG/min to suppress endogenous hematopoiesis. The supernatant was removed and replaced with 10 mL fresh LTC medium containing the test cells.

Two stage long-term cultures

Blood mononuclear cells at a concentration of 1×10^5 cells/mL in a volume of 10 mL LTC medium were seeded onto performed irradiated stromal layers. Half of the supernatant and nonadherent cells were removed weekly during feeding. The cells were counted and assayed for CFC as described above. The cultures were maintained for 8 weeks. Control cultures were seeded with equal numbers of normal bone marrow MNC.

Statistical methods

Data from all patients within a particular period of G-CSF or chemotherapy cycle were pooled for analysis, as no significant difference could be detected

between different chemotherapy dose intensity levels. Data from all cycles were compared by nonparametric (ranking) methods. Statistically significant results were analyzed by the Wilcoxon matched-pairs signed rank test. The relationship between cell counts was calculated using Pearson's product moment correlation. The magnitude of GM-CFC generation in LTC over 8 weeks was expressed by the area under the curve (AUC). The Mann Whitney test was used for comparison of the AUC.

RESULTS

Mobilization of CFC and CD34$^+$ cells

Results of mobilized CFC and CD34$^+$ cell numbers in the peripheral blood and leukapheresis have been published elsewere.[12] Briefly, G-CSF administration before chemotherapy did not influence mobilization of CFC and CD34$^+$ cells at later periods, in the cohort of patients receiving G-CSF prior to chemotherapy compared with the cohort receiving G-CSF after completion of chemotherapy. Mobilization of CFC and CD34$^+$ cells occurred concurrently during all assessment periods. Maximal CFC and CD34$^+$ cell numbers in the circulation were seen on days 6–7 during G-CSF before and days 9–10 during G-CSF after chemotherapy treatment. WBC recovery from nadir on median day 8 (range 8–9) in cycle 1 and day 9 (range 8–9) in cycle 4 occurred simultaneously with mobilization of progenitor cells. WBC recovery $>4\times10^9$/L, the time for leukapheresis, occurred on median day 11 (range day 10–13) in cycle 1. Correlation coefficients for the relationship between WBC count and circulating CFU-GM and CD34$^+$ cells on the day of leukapheresis were r=0.576 and r=0.559, respectively.

Median peak CFC and CD34$^+$ cell numbers during all periods are shown in Figure 2. Mobilization of CFC was optimal in cycle 1 and had decreased by about 50% for CFC in cycle 4 (p=0.28). Higher numbers of CFC were detected during G-CSF after chemotherapy compared to those in cycle 4, but the levels did not reach those achieved during G-CSF before chemotherapy. Circulating CD34$^+$ cell numbers were comparable during cycle 1 and cycle 4. Thus, CFC mobilization appeared compromised by chemotherapy treatment. This was not observed for CD34$^+$ cells.

LTC

Generation of GM-CFC in LTC is shown in Figure 3. The highest generation of GM-CFC was seen in cultures of LTCIC collected in chemotherapy-naive status on day 5 during G-CSF before chemotherapy. Generation of GM-CFC at the end of the fifth week of culture was comparable in LTC from blood collected on D11 of cycle 1 and from leukapheresis samples also collected during cycle 1. LTC initiated

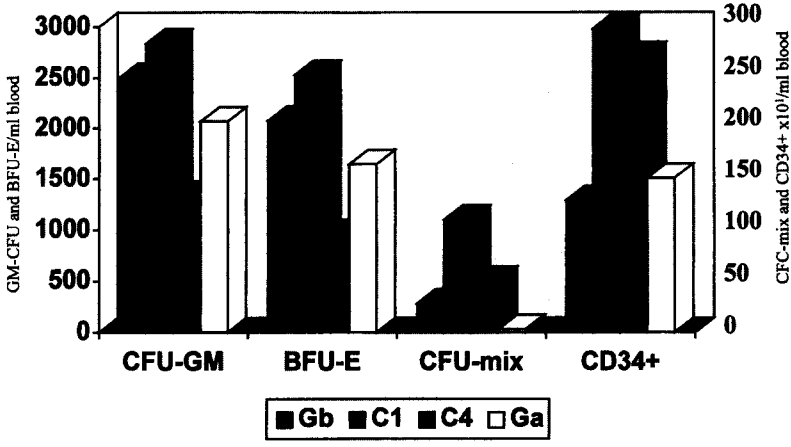

Figure 2. *Median peak CFC and CD34⁺ cell numbers per mL blood during G-CSF before (Gb) and after (Ga) chemotherapy and in cycle 1 and 4.*

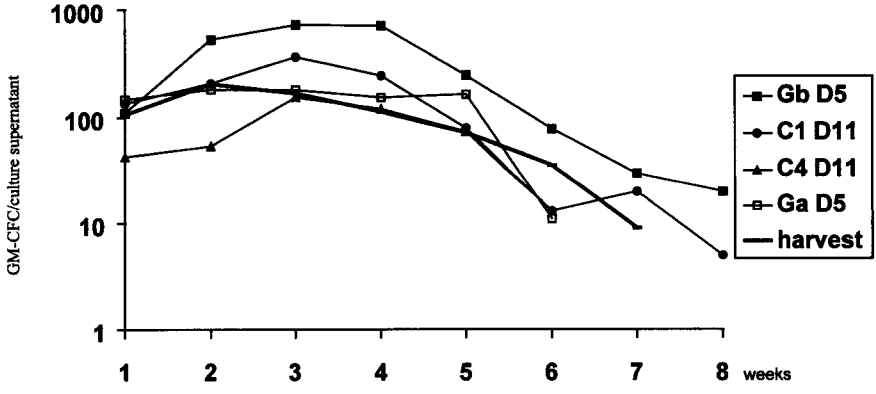

Figure 3. *Generation of GM-CFC in LTC initiated on day 5 during G-CSF before (Gb) and after (Ga) chemotherapy, day 11 in cycle 1 and 4 and from leukapheresis samples, expressed as the median of numbers of GM-CFC per culture supernatant per week.*

from blood samples collected on day 10 during the periods of G-CSF and day 9 in cycle 1 and 4 showed poor capacity to generate GM-CFC (data not shown).

Figure 4 displays the area under the curve (AUC) for LTC initiated on day 5 of G-CSF administration before and after chemotherapy, on day 11 of cycle 1 and 4 and for LTC from normal bone marrow samples. The highest values were observed

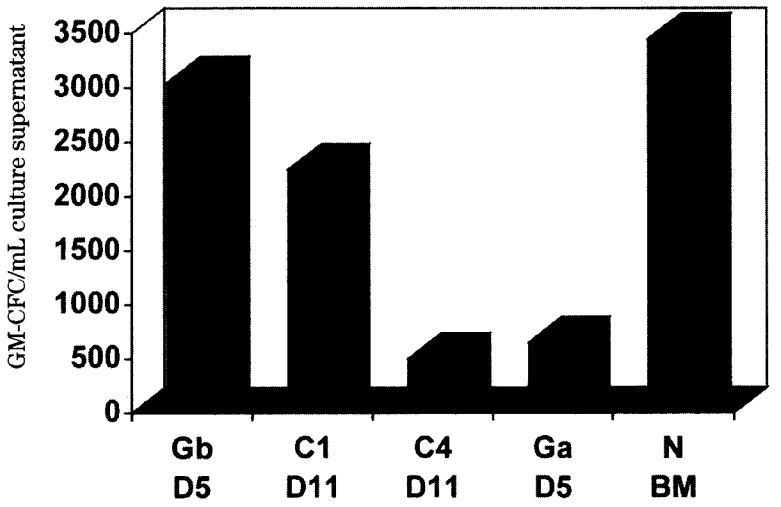

Figure 4. *AUC for generation of GM-CFC in 8 week LTC initiated on day 5 of G-CSF before (Gb) and after (Ga) chemotherapy and on day 11 in cycle 1 and 4 and in LTC from normal bone marrow.*

during G-CSF before chemotherapy. This capacity was slightly reduced after the first chemotherapy administration, decreased further during chemotherapy to a minimal after the fourth chemotherapy administration and recovered slightly during G-CSF after completion of chemotherapy treatment. No significant difference ($p>0.05$) in proliferative potential between LTCIC collected on day 5 during G-CSF before chemotherapy or day 11 of cycle 1 and LTCIC of normal bone marrow was found. Generation of GM-CFC by LTCIC collected on day 11 in cycle 1 compared with day 11 in cycle 4 was significantly different ($p=0.002$).

DISCUSSION

Our data suggest that initiation of leukapheresis on day 5 during G-CSF in chemotherapy naive status is optimal for collection of committed progenitor cells.[8,12] Even the first chemotherapy compromises mobilized LTCIC in their capacity to generate GM-CFC. Adding G-CSF to chemotherapy could not overcome either this effect or further deterioration during chemotherapy treatment. Breems[27] reported a negative relationship between the number of chemotherapy cycles and the number and proliferative potential of collected LTCIC. Deterioration during the first four cycles was exponential with flattening of the curve during later cycles.

Various studies[16,21,28–30] compared LTCIC mobilization by different regimens, mostly in blood samples of patients previously treated with chemotherapy or in samples of leukapheresis collected at WBC recovery >0.5-1$\times 10^9$/L after chemotherapy + growth factor administration. Bender et al.[29] reported superior contents of primitive and lineage restricted cells in leukapheresis performed after mobilization by CY plus G-CSF compared to CY alone. To[21] reported characterization of primitive cells during four mobilization regimens (Cy versus Cy plus G-CSF versus CY plus GM-CSF versus G-CSF). Frequencies of circulating $CD34^+CD38^-$ cells were higher following mobilization by G-CSF than by any of the other regimens. This observation agrees with our results which suggest that primitive cell mobilization becomes compromised by chemotherapy. Sutherland[16] reported higher frequencies of LTCIC in blood samples drawn after mobilization by CY or G-CSF compared with Cy plus GM-CSF. Benboubker[28] also reported lower LTCIC frequencies after mobilization by CY plus GM-CSF compared with CY and attributed this to differentiation induced by GM-CSF. This, however, neglected the possibility that the first chemotherapy cycle (CY only) may have been cytotoxic for primitive cells assessed during the second mobilization cycle consisting of CY plus GM-CSF. Chen et al.[30] characterized primitive cells by expression of CD34 and Thy-1 and absence of Lin markers and reported higher frequencies of $CD34^+Thy\text{-}1^+Lin^-$ cells after mobilization by GM-CSF than G-CSF. Contrasting observations may be due to differences in subsets of primitive cells characterized by LTC compared with flow cytometry, differences in prior exposure to cytotoxic treatment, type of previous chemotherapy and interval since prior cytotoxic treatment, and differences in timing of sampling.

Our data indicate that exposure to cytotoxic treatment compromises mobilization of cells with LTC-initiating potential during a particular mobilization regimen and suggest that collection during G-CSF in chemotherapy naive status or during the first chemotherapy cycle, within one day from peak circulating levels of committed progenitor cells and $CD34^+$ cells at WBC recovery >4$\times 10^9$/L, results in a high yield of LTCIC. GM-CFC mobilization late during chemotherapy treatment or during G-CSF after chemotherapy correlates with mobilization of LTCIC. Upfront planning of future treatment including optimal timing of leukapheresis for collection of LTCIC with capacity to generate high numbers of GM-CFC may be important for the success of new treatment modalities.

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REFERENCES

1. Kessinger A, Armitage JO, Landmark JD et al.: Autologous peripheral blood hematopoietic stem cell transplantation restores hematopoietic function following marrow ablative therapy. *Blood* 71:723–727, 1988.
2. Richman CM, Weiner RS, Yankee RA et al.: Increase in circulating stem cells following chemotherapy in man. *Blood* 47:1031, 1977.
3. Juttner CA, To LB, Haylock DN et al.: Circulating autologous stem cells collected in very early remission from acute non-lymphoblastic leukemia prompt but incomplete hematopoietic reconstitution after high-dose melphalan or supralethal chemo-radiotherapy. *Br J Haematol* 61:739–745, 1985.
4. Bell AJ, Figes A, Oscier GD et al.: Peripheral blood stem cell autografts in the treatment of lymphoid malignancies: Initial experience in three patients. *Br J Haematol* 66:63–68, 1987.
5. Socinski MA, Elias A, Schnipper L et al.: Granulocyte-macrophage colony stimulating factor expands the circulating haematopoietic progenitor cell compartment in men. *Lancet* 1;1194–1199, 1988.
6. Siena S, Bregni M, Brando B et al.: Circulation of $CD34^+$ hematopoietic cells in the peripheral blood of high-dose cyclophosphamide treated patients: enhancement by intravenous recombinant human granulocyte-macrophage colony stimulating factor. *Blood* 74:1905–1914, 1989.
7. Gianni AM, Siena S, Bregni M et al.: Granulocyte-macrophage colony stimulating factor to harvest circulating haematopoietic stem cells for autotransplantation. *Lancet* 2:580, 1989.
8. Sheridan WP, Begley CG, Juttner CA et al.: Effect of peripheral-blood progenitor cells mobilized by filgrastim (G-CSF) on platelet recovery after high-dose chemotherapy. *Lancet* 339:640, 1992.
9. Bensinger W, Singer J, Appelbaum F et al.: Autologous transplantation with peripheral blood mononuclear cells collected after administration of rG-CSF. *Blood* 11:3158–3163, 1993.
10. Van Hoef MEHM. The effect of the mobilization regimen on hematological recovery after peripheral blood progenitor cell transplantation. (submitted)
11. Ho AD, Gluck S, Germond C et al.: Optimal timing for collections of blood progenitor cells following induction chemotherapy and granulocyte-macrophage colony stimulating factor for autologous transplantation in advanced breast cancer. *Leukemia* 7:1738–1746, 1993.
12. Van Hoef MEHM, Baumann I, Lange C et al.: Dose-escalating chemotherapy supported by lenograstim preceding high-dose consolidation chemotherapy for advanced breast cancer. *Ann Oncol* 5:217–224, 1994.

13. Pettengell R, Testa NG, Swindell R et al.: Transplantation potential of haematopoietic cells released into the circulation during routine chemotherapy for non-Hodgekin's lymphoma. *Blood* 82:2239–2248, 1993.
14. Brugger W, Bross K, Frisch J et al.: Mobilization of PBPC by sequential administration of interleukin-3 and granulocyte-macrophage colony-stimulating factor following polychemotherapy with etoposide, ifosfamide and cisplatin. *Blood* 79:1193–1200, 1992.
15. Dreger P, Marquaedt P, Haferlach T et al.: Effective mobilization of PBPC with dexa-BEAM and G-CSF: Timing of harvesting and composition of the leukapheresis product. *Br J Cancer* 68:950–957, 1993.
16. Sutherland HJ, Eaves CJ, Lansdorp PM et al.: Kinetics of committed and primitive progenitor mobilization after chemotherapy and growth factor treatment and their use in autotransplantation. *Blood* 83:3808–3814, 1994.
17. Van Hoef MEHM.: Guidelines for efficient peripheral blood progenitor cell collection. *Neth J Med* 48:29–37, 1996.
18. Ellior C, Samson DM, Armitage S et al.: When to harvest peripheral blood stem cells after mobilization therapy: Prediction of $CD34^+$ cell yield by predicting day $CD34^+$ concentration in peripheral blood.
19. Murray L, Chen B, Galy A et al.: Enrichment of human hematopoietic stem cell activity in the $CD34^+Thy\text{-}1^+Lin^-$ subpopulation from mobilized peripheral blood. *Blood* 85:368–378, 1995.
20. Demunck H, Pettengell R, de Campos E et al.: The capacity of peripheral blood stem cells mobilized with chemotherapy plus G-CSF to repopulate irradiated bone marrow stroma in vitro is similar to that of bone marrow. *Eur J Cancer* 28:381–386, 1992.
21. To LB, Haylock DN, Dowse T et al.: A comparative study of the phenotype and proliferative capacity of peripheral blood $CD34^+$ cells mobilized by four different protocols and those of steady-phase PB and bone marrow $CD34^+$ cells. *Blood* 84:2930–2939, 1994.
22. Baum CM, Weissman IL, Tsukamoto AS et al.: Isolation of a candidate human hematopoietic stem cell population. *Proc Natl Acad Sci* 89:2804–2808, 1992.
23. Murray L, DiGiusto D, Chen B et al.: Analysis of human hematopoietic stem cell populations. *Blood Cells* 20:364–370, 1994.
24. Sutherland HJ, Lansdorp PM, Henkelman DH et al.: Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. Proc Natl Acad Sci USA 87:3584–3588, 1990.
25. Couthino LH, Gilleece MH, De Wynter EA et al.: Clonal and long term cultures using human bone marrow, in Testa NG, Molineux G, (eds): Haematology: A practical approach. Oxford, UK: Oxford University, 1993, p 75.
26. Nadali G, De Wynter EA, Testa NG et al.: $CD34^+$ cell separation from basic research to clinical applications. *Int J Clin Lab Res* 25:121–127, 1995.
27. Breems DA, Hennik PB, Kusadasi N et al.: Individual stem cell quality in leukapheresis products is related to the number of mobilized stem cells. *Blood* 87:5370-5378, 1996.
28. Benboubker L, Domenech J, Linassier C et al.: Long-term cultures to evaluate engraftment potential of $CD34^+$ cells from peripheral blood after mobilization by chemotherapy with and without GM-CSF. *Exp Hematol* 23:1568–1573, 1995.
29. Bender JG, Lum L, Unverzagt KL et al.: Correlation of colony-forming cells, long-term

culture initiating cells and $CD34^+$ cells in apheresis products from patients mobilized for peripheral blood progenitors with different regimens. *Bone Marrow Transplant* 13:479–485, 1994.

30. Chen BP, Fraser C, Reading C et al.: Cytokine-mobilized peripheral blood $CD34^+Thy\text{-}1^+Lin^-$ human hematopoietic stem cells as target cells for transplantation-based gene therapy. *Leukemia* 9(suppl 1):S17–S25, 1995.

ROLE OF CD34+38- CELLS IN POST-TRANSPLANT HEMATOPOIETIC RECOVERY

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ABSTRACT

Defining which subpopulation of circulating hematopoietic progenitor cells is predictive of both an optimal performance of the graft harvest and a further short-term trilineage engraftment is still under debate. In an attempt to evaluate autograft engraftment potential, we prospectively studied the relationships between the amounts of blood mononuclear cells (MNC), colony-forming unit granulocyte-macrophage (CFU-GM), $CD34^+$ cells and their $33^+/33^-$ and $38^+/38^-$ subsets contained in graft products, and the kinetics of trilineage engraftment in patients with various malignant diseases. The individual amounts of each subgroup of reinfused cells were plotted against each engraftment kinetics parameter, defined by the number of days to reach absolute neutrophil count (ANC) of 0.5, 1 and 2×10^9/L, unmaintained platelet counts of 20 and 50×10^9/L, and absolute reticulocyte counts of 30 and 50×10^9/L on two consecutive days. Correlations were analyzed using three different statistical tests in parallel (the Spearman correlation test, a linear regression test and a log-inverse model). Whatever the statistical test, there was no significant correlation between the number of days to reach the successive ANC, platelet and reticulocyte counts and the infused numbers of MNC, $CD33^+$ and $CD33^-$ subsets (except ANC 2×10^9/L for the latter). Moreover, the infused numbers of CFU-GM, $CD34^+$ and $CD38^+$ cells only correlated inconstantly with hematopoietic recovery kinetics parameters. In fact, the strongest and the most constant significant correlations within the three statistical models were observed between the number of infused $CD34^+38^-$ cells and each engraftment parameter. More particularly, this cell subset was the only one to significantly correlate with reticulocyte recovery kinetics. Interestingly, the log-inverse model enabled a plateau of the engraftment kinetics correlation curves to be determined, starting from an infused dose of $0.1/0.2\times10^6$ $CD34^+38^-$ cells/kg for the 3 cell lineages. Under this threshold value, the lower the number of cells infused, the slower the engraftment kinetics. This study suggests that the $CD34^+CD38^-$ subpopulation, although essentially participating in late,

complete hematopoietic recovery, also comprises committed progenitor cells involved in early trilineage engraftment. Daily assessment of the $CD34^+CD38^-$ leukapheresis content should also identify when to optimally cease the leukapheresis procedure after the total harvested amounts of these cells reach at least 3×10^5/kg b.w.

INTRODUCTION

Defining which subpopulation of circulating hematopoietic progenitors is optimally predictive of early and/or sustained post-autologous blood cell transplant (ABCT) multilineage recovery is still under debate. There are conflicting reports about the correlation of clinical engraftment with either clonogenic assays or $CD34^+$ cell counts.

Previous studies have demonstrated a great heterogeneity in the stages of differentiation of $CD34^+$ subpopulations that may influence the rapidity and the quality of post-transplant hematopoietic recovery.[1,2] Maturation of $CD34^+$ cells is accompanied by changes in cell surface marker expression. Terstappen et al. have shown that expression of the CD38 antigen on $CD34^+$ cells indicates a commitment of a more primitive cell to myeloid, erythroid or lymphoid lineages.[3] In turn, $CD38^-$ cells contain multilineage hematopoietic cells with extensive proliferative capacities in fetal and adult bone marrow (BM).[4,5] In parallel, the $CD34^+CD33$ subsets were suggested to relate to the mobilization regimens and also to indicate the maturation/differentiation cell stages.[6] Using long-term BM culture, Andrews et al. demonstrated that colony-forming cells were derived from $CD34^+CD33^-$ rather than from $CD34^+CD33^+$ cells.[7]

We have prospectively studied the relationships between the amounts of blood mononuclear cells (MNC), colony-forming unit granulocyte-macrophage CFU-GM, $CD34^+$ cells and their $33^+/33^-$ and $38^+/38^-$ subsets contained in graft products, and the kinetics of trilineage engraftment in patients suffering from various malignant diseases.

MATERIALS AND METHODS

Patients

Twenty-five consecutive patients (15 males, 10 females) were enrolled in this study. Their median age was 43 (range: 21–54). Eleven patients had non-Hodgkin's lymphoma (NHL) and six had multiple myeloma (MM). Four patients had acute leukemia: three acute lymphoblastic leukemia (ALL), and one acute phase (AP) of chronic myeloid leukemia (CML). Two patients had Hodgkin's disease (HD) and two others had breast cancer (BCa).

All patients had received various chemotherapy courses according to their disease,

prior to the mobilization regimen. The latter always comprised chemotherapy, adapted to the type of disease. Additionally, nine of the eleven patients with NHL, the six patients with MM, the two patients with BCa and the two patients with HD, received sequential administration of either granulocyte-macrophage colony-stimulating factor (GM-CSF, n=2) or granulocyte colony-stimulating factor (G-CSF), alone (n=11) or combined with IL-3 (n=6). Daily subcutaneous administration of G- or GM-CSF was started 24 hours after chemotherapy at a dose of 5 μg/kg/day (whatever the cytokine) until completion of leukaphereses (LK), except for patients with MM, who were administered a particular protocol combining IL-3, 5 μg/kg/day, from day 1 to day 7, and G-CSF from day 4.

Evaluation of the graft content in CD34$^+$ cells and subsets

CD34$^+$ cells were identified using the staining method and flow cytometry assay we previously described.[8] In short, nucleated cells were adjusted at a concentration of 1×10^7/mL in Dulbecco's phosphate buffer saline (D-PBS, Gibco, Cergy Pontoise, France), subsequently divided into 200 μL aliquots, and incubated for 30 minutes in the dark at room temperature with 20 μL of phycoerythrin (PE)-conjugated 8G12 monoclonal antibody (MoAb) (HPCA-2, Becton Dickinson, Le Pont de Claix, France) and 25 μL of PE-IgG1 conjugated MoAb (Becton Dickinson). Then, cells were incubated for 7 minutes with a lysing reagent (Ortho, Roissy, France) and washed twice in D-PBS with 2% fetal calf serum (FCS).

Cell aliquots were stained with 8G12 MoAB as described above, for detection of CD34$^+$CD33 and CD34$^+$CD38 subsets, then incubated with 20 μL of fluorescein isothiocyanate (FITC)-conjugated anti-CD33 MoAb or 20 μL of FITC-conjugated anti-CD38 MoAb (Immunotech, Marseille, France). In parallel, both unstained cells and cells stained with a control isotype applied as a combination of nonspecific FITC-IgG1 with PE-IgG1 (Simultest Control, Becton Dickinson) were used as negative controls. After a 30-minute incubation, the cells were mixed with 4 mL of lysing reagent (Ortho) and washed twice in D-PBS with 2% FCS. They were then immediately analyzed with a FACStar (Becton Dickinson, Mountain View, CA) equipped with a 5 W argon laser. Fifty thousand cells were acquired in mode LIST and the data were analyzed with the Consort 30 program. Cell aggregates and debris were excluded by gating in acquisition. CD34$^+$ cells were obtained by side scatter cytogram (SSC) versus PE-red fluorescence. Filters (530 nm and 570 nm) were used for selection of green and red fluorescence attributable to FITC and PE-labeled antibodies (Abs). Compensation levels were set to limit the superposition of fluorochrome emission spectrum. CD34$^+$ cell subpopulations were determined by electronic gating on the CD34$^+$ population identified in a two-color display of PE-8G12 versus side scatter. These gated CD34$^+$ events were studied with FITC-labeled Ab. Subpopulations of CD34$^+$ cells were identified on the cytogram of PE versus FITC fluorescence. The limit between negative and positive

expression of the CD33 or CD38 markers was set according to negative control-Ab (PE versus FITC fluorescence) in the lymphocyte region.

Finally, absolute quantification of $CD34^+$ cells and subsets per mL was evaluated in LK product (LKP) samples by multiplying the MNC count determined with a STKS Cell Counter Coulter (Coultronics, Margency, France) with the percentage of $CD34^+$ cells determined by flow cytometry. Results were finally expressed as the numbers of total $CD34^+$ cells or subsets per kg body weight (b.w.).

CFU-GM colony assay

Fresh MNC from each LKP sample were adjusted and plated at a concentration of 2×10^5 viable cells *n* duplicate in a culture medium consisting of 0.8% methylcellulose supplemented with 20% human placental conditioned medium (standardized against Medium H431 [Stem Cell Technologies, Vancouver, Canada]) and 20% fetal calf serum (FCS). Plates were incubated at 37°C and 5% CO^2, in a humidified atmosphere. Colonies containing more than 50 cells were scored on day 12, using an inverted microscope.

Myeloablative conditioning therapy and ABCT

Pretransplant conditioning regimens depended on the disease. Fractionated total body irradiation (total-body irradiation [TBI]=10–12 Gy delivered in 5–6 fractions) was combined with high-dose chemotherapy (HDCT) in 14 patients, while the others underwent HDCT only. Twenty-four hours after completion of high-dose therapy, cryopreserved blood cells were thawed at the patient's bedside and immediately reinfused to the patient. No patient received cytokines post-transplant. All patients were nursed in a laminar air flow room and routinely received gut decontamination, as well as broad spectrum antibiotics and irradiated cytomegalovirus-negative blood products, as required. The kinetics of engraftment was defined by the number of days from graft infusion to reach an absolute neutrophil count (ANC) of 0.5, 1 and 2×10^9/L, a platelet count of 20 and 50×10^9/L without transfusion support, and an absolute reticulocyte count of 30 and 50×10^9/L, on two consecutive days.

Statistical analysis

The nonparametric Spearman rank correlation test was used for examining the relationships between the individual amounts of different subvarieties of cells reinfused and the number of days required for hematopoietic recovery after ABCT for each patient. When dependent and predictive variables were related, the parameters of a linear regression model ($Y=b_o + b_1 x\ cells$) and a log-inverse model ($Y=427e^{(^bo + {}^b1\ x\ cells)}$) were estimated, and the goodness-of-fit of the models was evaluated with the coefficient determination (R2). Patients who died before engraftment of either lineage were censored at the date of death.

Table 1. Summary of relationships between the kind of infused cells and days required for hematopoietic recovery depending on the statistical model

Type of reinfused cells		*Days to:*						
		ANC			*Platelets*		*Reticulocytes*	
		≥0.5 G/L	*≥1 G/L*	*≥2 G/L*	*≥20 G/L*	*≥50 G/L*	*≥30 G/L*	*≥50 G/L*
MNC	1)	–	–	–	–	–	–	–
	2)	–	–	–	–	–	–	–
	3)	–	–	–	–	–	–	–
CFU-GM	1)	+	–	–	+	+	–	–
	2)	+	+	–	+	+	–	–
	3)	+++	+++	–	++	++	–	+
34^+	1)	–	–	+++	++	+	–	–
	2)	–	–	++	–	–	–	–
	3)	–	–	+++	+	+	–	–
34^+33^+	1)	–	–	–	–	–	–	–
	2)	–	–	–	–	–	–	–
	3)	–	–	–	–	–	–	–
34^+33^-	1)	–	–	++	–	–	–	–
	2)	–	–	++	–	–	–	–
	3)	–	–	++	–	–	++	++
34^+38^+	1)	–	–	+++	++	+	–	–
	2)	–	–	++	–	–	–	–
	3)	–	–	+++	+	+	–	–
34^+38^-	1)	++	+	+++	+++	+++	+++	+++
	2)	–	–	++	+	+	+	+
	3)	+	+	+++	+++	+++	+++	++

1) Spearman's test		Spearman's correlation coefficient	R square (L and S models)	p values
2) Linear regression model (L): *Y=bo + b1 ×cells*				
	+++	r ≥ –0.65	$R^2 \geq 0.45$	≤ 0.001
3) Log-inverse model (S):	++	r ≥ –0.50	$R^2 \geq 0.30$	≤ 0.01
*Y=e**(bo + b1/x)*	+	r ≥ –0.40	$R^2 \geq 0.18$	< 0.05
		r < –0.40	$R^2 < 0.18$	> 0.05

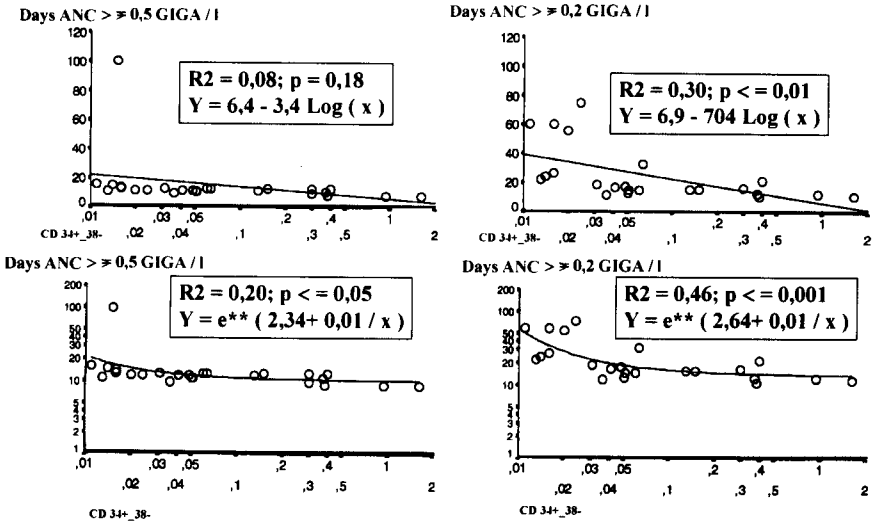

Figure 1A. *Neutrophil recovery kinetics.*

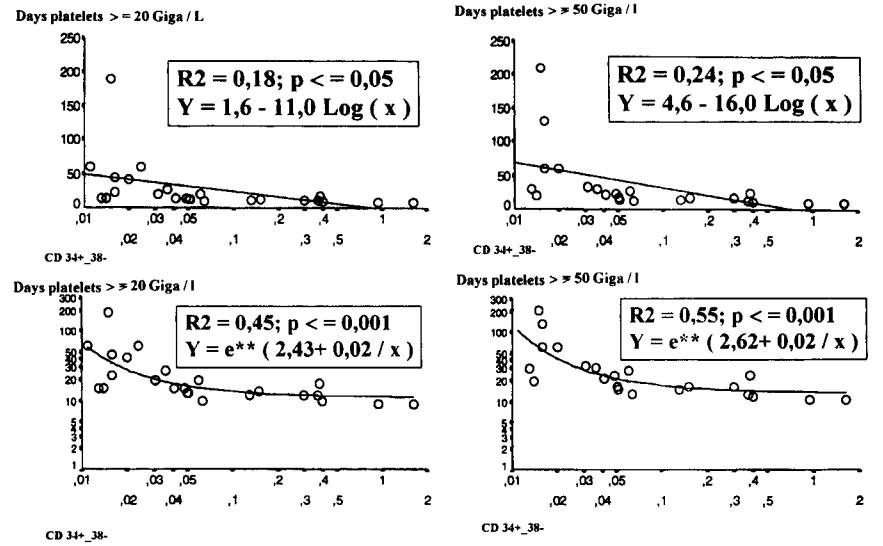

Figure 1B. *Platelet recovery kinetics.*

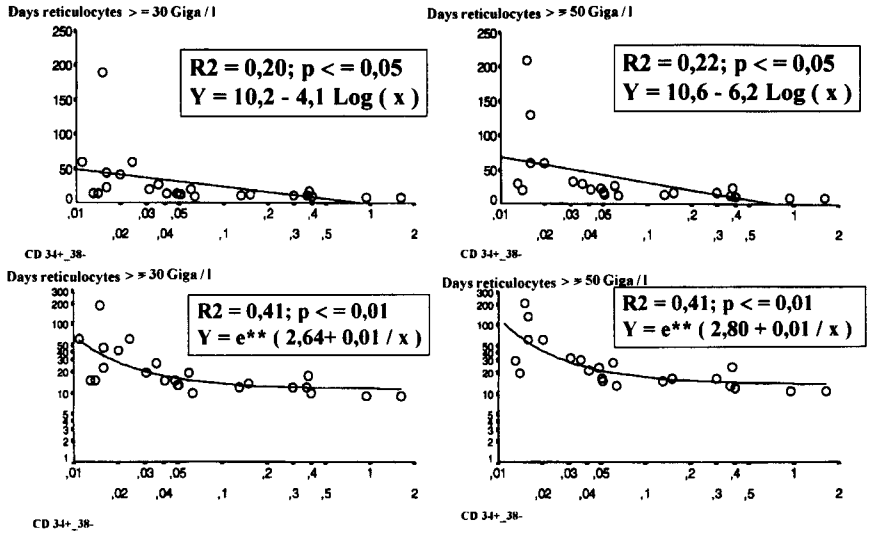

Figure 1C. *Reticulocyte recovery kinetics. Plots of linear fit and log-inverse fit (with 95% confidence intervals of the mean predicted responses) expressing the relationship between the numbers of reinfused CD34$^+$CD38$^-$ cells and a) Neutrophil recovery kinetics, b) Platelet recovery kinetics, c) Reticulocyte recovery kinetics, using either the linear regression test (top) or the log-inverse test (bottom). When considering the latter, the curves always reach a plateau starting from similarly infused CD34$^+$CD38$^-$ doses of 0.1/0.2×10^6 cells/kg for the three cell lineages.*

Data analysis was performed with SPSS® 6.1.2. for Windows™ softwares. The two-tailed significance was determined by using the Student's or the F statistical tests. Results were considered as significant for p value <0.05.

RESULTS

Post-transplant hematopoietic recovery

On average, 1.6×10^8 MNC/kg (range 0.1–4.65), 15.5×10^6 CFU-GM/kg (range 2.5–57.7) and 2.2×10^6 CD34$^+$ cells/kg (range 0.3–10.2), 1.3×10^6 CD34$^+$33$^+$ cells/kg (range 0.07–10.1), 0.77×10^5 CD34$^+$33$^-$ cells/kg (range 0.02–7.7), 1.9×10^6 CD34$^+$38$^+$ cells/kg (range 0.27–6.33), and 0.15×10^6 CD34$^+$38$^-$ cells/kg (range 0.011–1.62) were reinfused.

Table 2. Evidence of a faster trilineage engraftment in patients receiving $CD34^+$ cell amounts $>2\times10^6$/kg compared to those receiving less than 2×10^6/kg $CD34^+$ cells

	Median no. of days to: (range values)						
No. of infused	*ANC*			*Platelets*		*Reticulocytes*	
$CD34^+$ cells	*>0.5*	*≥1*	*≥2*	*≥20*	*≥50*	*≥30*	*≥ 50*
$\geq2\times10^6$/kg	10.5 (9–13)	12.5 (11–17)	15 (12–22)	14 (9–27)	17 (11–31)	18 (12–22)	21 (14–33)
$\leq2\times10^6$/kg	12.5 (9–16)	15 (12–22)	30 (11–75)	27 (10–60)	38 (13–130)	24 (14–42)	30 (15–90)
p value	<0.05	<0.05	<0.001	<0.001	<0.001	=0.01	<0.01

The average times to reach ANC of 0.5, 1 and 2×10^9/L were 16, 20 and 32 days, respectively. The highest day values were related to the AP-CML patient, whose hematopoietic recovery was extremely delayed, as is frequent in this disease. When excluding this patient's values, the average times decreased to 11, 14 and 23 days, respectively. The median times to reach 20 and 50×10^9/L platelets were 28 and 37 days, respectively, or 21 and 29 days after exclusion of values from patient N° 21. However, three patients never reached 50×10^9/L platelets before they died on day 14, 120 and 550, respectively. Absolute reticulocyte counts reached 30 and 50×10^9/L within 21 and 27 days (or 20 and 25), respectively.

Correlation analysis of the numbers of infused cells with the engraftment kinetic

The individual amounts of the different cell subvarieties reinfused for transplant were plotted against the number of days to reach the different neutrophil, platelet and reticulocyte counts defining the trilineage engraftment kinetics for each patient. Whatever the test, there was no significant correlation between the infused numbers of MNC, $CD33^+$ and $CD33^-$ subsets and the number of days to reach the successive ANC (with the exception of ANC 2×10^9/L for $CD33^-$), platelet and reticulocyte counts. The number of infused CFU-GM weakly correlated with the time to ANC ≥0.5 and 1×10^9/L, correlated significantly better with the kinetics of platelet engraftment, but not with reticulocyte recovery. The correlation of the total $CD34^+$ cell numbers with engraftment kinetics was inconstant in the 3 statistical models: it was not

significant with the time to low ANC and reticulocyte count recovery, but good enough with the time to ANC $\geq 2\times10^9$/l and weak with the two platelet values. The $CD38^+$ subset showed a good correlation with ANC $\geq 2\times10^9$/l, but only a weak to no correlation with all the other parameters. In fact, the strongest and the most constant significant correlations within the 3 statistical models were observed between the number of infused $CD38^-$ cells and each of the engraftment parameters. More particularly, this cell subset was the only one to significantly correlate with reticulocyte recovery kinetics. All correlative values are summarized in Table 1.

Interestingly, the log-inverse model allowed a plateau of the engraftment kinetics correlation curves to be determined, similarly starting from an infused dose of $0.1/0.2\times10^6$ $CD38^-$ cells/kg for the 3 cell lineages. Under this threshold value, the lower the number of cells infused, the slower the engraftment kinetics, while in contrast, infusion of $>0.3\times10^6$/kg $CD38^-$ cells did not shorten the time of hematopoietic recovery.

DISCUSSION

While in recent years several studies have attempted to define which cell subpopulation could best predict the kinetics of further engraftment, results have been inconsistent. Although a recent study recommended the assessment of MNC dose alone as reliably predicting early granulocyte and platelet recovery[9], this is not confirmed by most other studies,[10,12] including ours. Despite the correlations we found, like others, between CFU-GM and $CD34^+$ cells in LKP, CFU-GM assessment finally appears not to be much more convincing.

Most investigators now insist on the powerful predictive role of graft $CD34^+$ cell values in optimally predicting the rapidity of further engraftment kinetics.[10,11,14–16] However, in our study, the transplanted $CD34^+$ cell values only correlated with the later stage of neutrophil recovery (but not earlier) and platelet recovery kinetics, regardless of which correlation test was applied. When attempting to determine, as other investigators have, threshold $CD34^+$ cell values above those where prompt engraftment occurs with increasing confidence, we observed that patients receiving more than 2×10^6 $CD34^+$ cells/kg significantly experienced a faster trilineage engraftment than those receiving fewer than 2×10^6 $CD34^+$ cells/kg. In the latter situation, the time to achieve 0.5×10^9 neutrophils/L never exceeded 16 days, even when reinfusing numbers of $CD34^+$ cells as low as 0.5×10^6/kg. However, the patients receiving such low $CD34^+$ cell doses reached totally normal blood cell counts significantly later, or never. It is therefore suggested that infusion of low total $CD34^+$ cell doses consistently has less impact on short-term rather than long-term hematopoietic recovery.

Additionally, as both immature stem cells and committed progenitors express the $CD34^+$ antigen, it might be clinically interesting to better refine stem cell measurement by evaluating $CD34^+$ subsets, which could avoid overestimating the true stem cell content of the graft.

In contradiction with others,[11,17] in our present study we did not find any correlation between the number of transplanted $CD34^+CD33^+$ cells and any of the early trilineage engraftment parameters, whatever the statistical test applied, whereas the transplanted $CD34^+CD33^-$ cells only correlated well with the later stage of neutrophil engraftment. The number of infused $CD34^+CD38^+$ cells were not more predictive, since they did not convincingly correlate in our study with any stage of trilineage engraftment kinetics—except for the later stage of neutrophil recovery—and only weakly with platelet recovery kinetics.

Conversely, the strong correlation we observed between the number of transplanted $CD34^+CD38^-$ cells and almost all stages of trilineage engraftment, including the earlier ones, might be considered somewhat surprising. They seem to suggest a functional heterogeneity of the $CD38^-$ subpopulation, initially supposed to participate solely in late, complete hematopoietic reconstitution. Surprisingly, it also appears to have a correlation with early trilineage engraftment.

In a short study, Buscemi et al. recently reported data similar to ours.[18] They also found that the total number of grafted $CD34^+$ cells correlated with platelet recovery but not with early neutrophil recovery. They did not find any correlation between hematopoietic recovery and either the $CD34^+CD33^+$ and $CD34^+CD38^+$ subsets, or the infused numbers of MNC and CFU-GM. But, overall, they found a very strong correlation between platelet recovery and the $CD34^+CD38^-$ subset. Interestingly, using three-color flow cytometry analysis, they also showed that the grafted $CD34^+CD38^-$ cells were either HLA-DR^+ or HLA-DR^-. Therefore, they suggested a lineage commitment, implying that $CD34^+$ cells negative for CD38 also comprise committed progenitors. Further evaluation using three- or four-color flow cytometry together with clonogenic assays, would require purification of both $CD34^+CD38^-$HLA-DR^+ and HLA-DR^- subpopulations to subsequently try to determine the exact role of these two minuscule cell subpopulations with regard to short- and long-term engraftment.

In conclusion, careful assessment of the $CD34^+CD38^-$ subpopulation in graft products might be the best predictive parameter of both short- and long-term trilineage engraftment, much more reliable than the $CD34^+$ assessment. Furthermore, for us, the optimal threshold value seems to be between 1 and 2×10^5 $CD38^-$ cells/kg, and numbers $>3\times10^5$/kg will certainly not reduce time to hematopoietic recovery. The daily assessment of the $CD34^+CD38^-$ content in LKP should additionally identify when to optimally cease the leukapheresis procedure after the total harvested number of these cells reaches such values. Since this can be done without major technical difficulty using just two-color flow cytometry, we suggest its general practice.

REFERENCES

1. Bender JG, Williams SF, Myers S et al.: Characterization of chemotherapy mobilized peripheral blood progenitor cells for use in autologous stem cell transplantation. *Bone Marrow Transplant* 10:281, 1992.
2. Sovalat H, Liang H, Wunder E et al.: Flow cytometry characterization of $CD34^+$ cells in bone marrow, cytapheresis products and cord blood at birth. *Cell Cloning* 10(suppl 1):20, 1992.
3. Terstappen L, Muang S, Safford M et al.: Sequential generation of hematopoietic colonies derived from single nonlineage-committed $CD34^+CD38^-$ progenitor cells. *Blood* 77:1218, 1991.
4. Sutherland HJ, Eaves CJ, Eaves AC et al.: Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. *Blood* 74:1563, 1989.
5. Rusten LS, Jacobsen SEW, Kaalhus O et al.: Functional differences between $CD38^-$ and DR-subfractions of $CD34^+$ bone marrow cells. *Blood* 84:1473, 1994.
6. Siena S, Bregni M, Brando B et al.: Flow cytometry for clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. *Blood* 77:400, 1991.
7. Andrews RG, Singer JW, Bernstein ID. Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties. *J Exp Med* 169:1721, 1989.
8. Herbein G., Sovalat H, Wunder E et al.: Isolation and identification of two $CD34^+$ cell subpopulations from normal human peripheral blood. *Stem Cells* 12:187, 1994.
9. Smith RJ, Sweetenham JW: A mononuclear cell dose of 3×10^8/kg predicts early multilineage recovery in patients with malignant lymphoma treated with carmustine, etoposide, Ara-C and melphalan (BEAM) and peripheral blood progenitor cell transplantation. *Exp Hematol* 23:1581, 1995.
10. Bensinger WI, Longin K, Appelbaum F et al.: Peripheral blood stem cells (PBSCs) collected after recombinant granulocyte colony stimulating factor (rhG-CSF): An analysis of factors correlating with the tempo of engraftment after transplantation. *Br J Haematol* 87:825, 1994.
11. Urashima M, Ohkawara J, Hoshi Y et al.: Peripheral blood progenitor cell transplantation estimated by three-colour (CD34, HLA-DR, CD 33) flow cytometry. *Acta Haematol* 92:23, 1994.
12. Takamatsu Y, Harada M, Teshima T et al.: Relationship of infused CFU-GM and CFU-Mk mobilized with chemotherapy with or without G-CSF to platelet recovery after autologous blood stem cell transplantation. *Exp Hematol* 23:8, 1995.
13. Henon PR, Liang H, Beck-Wirth G et al.: Comparison of hematopoietic and immune recovery after autologous bone marrow or blood stem cell transplants. *Bone Marrow Transplant* 9:285, 1992.
14 Bender JG, To LB, Williams S et al.: Defining a therapeutic dose of peripheral blood stem cells. *J Hematother* 1:329, 1992.
15. Tricot G, Jagannath S, Vesole D et al.: Peripheral blood stem cell transplants for MM: Identification of favorable variables for rapid engraftment in 225 patients. *Blood* 85:588,

1995.

16. Weaver CH, Hazelton B, Birch R et al.: An analysis of engraftment kinetics as a function of the CD34 content of peripheral blood progenitor cell collections in 692 patients after the administration of myeloablative chemotherapy. *Blood* 86:3961, 1995.
17. Siena S, Bregni M, Brando B et al.: Flow cytometry for clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. *Blood* 77:400, 1991.
18. Buscemi F, Indovina A, Scime R et al.: $CD34^+$ cell subsets and platelet recovery after PBSC autograft. *Bone Marrow Transplant* 16:855, 1995 (Correspondence).

PHASE I TRIAL OF CYCLOSPORINE-INDUCED AUTOLOGOUS GRAFT-VERSUS-HOST DISEASE IN PATIENTS WITH MULTIPLE MYELOMA UNDERGOING HIGH-DOSE CHEMOTHERAPY WITH AUTOLOGOUS STEM CELL RESCUE

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INTRODUCTION

Myeloma relapse is the single most important cause of treatment failure for patients undergoing high-dose chemoradiotherapy and autologous progenitor cell support.[1–4] Innovative strategies are needed to prevent relapse of myeloma after autologous transplant. The induction of autologous graft-versus-host disease (GVHD) has been studied for breast cancer,[5,6] lymphoma,[7] and acute myelogenous leukemia,[8] and could also be useful in myeloma, since a graft-versus-myeloma effect exists.[9]

Study schema

Patients with intermediate or high tumor mass multiple myeloma were eligible for this study. The study was approved by the institutional review board and all patients signed a written informed consent according to institutional guidelines.

All patients had autologous stem cells collected after chemotherapy and growth factor mobilization. Three patients required bone marrow harvests to supplement the peripheral blood stem cell collections.

Patients received combination chemotherapy with thiotepa 750 mg/m^2, busulfan 12 mg/kg and cyclophosphamide 120 mg/kg, as previously described.[12] Filgrastim was administered to all patients at a dose of 5 μg/kg daily beginning on the day of stem cell infusion until the neutrophil count was $>5.0\times10^9$/L.

Cyclosporine was begun on the day of stem cell infusion at an initial dose of 1 to 2 mg/kg given as a continuous intravenous infusion over 24 hours. Cyclosporine

levels were measured two to three times a week until the patient stopped taking the drug. The cyclosporine dose was adjusted to maintain whole blood levels between 50–150 ng/dL in the first seven patients (low level group) and between 150–300 ng/dL in the following patients (high level group). Patients were allowed to switch to oral cyclosporine once they were able to tolerate oral medications. Cyclosporine was given until day 28 after stem cell infusion and was discontinued for any grade 3 or greater toxicity, or with a doubling of the baseline creatinine level. Cyclosporine was resumed once toxicity resolved. Six patients also received gamma interferon at a dose of 0.02 mg/m^2 subcutaneously every other day from day 7 to day 28 post transplant. All patients were treated with alpha-interferon at a dose of $1–2\times10^6$ units three times a week from the time of platelet recovery until disease progression.

The primary end points of this study were toxicity and induction of GVHD. Patients were monitored daily while hospitalized and two to three times weekly after discharge until day 28. All patients underwent blind skin biopsies on day 28 to assess for subclinical GVHD. Skin biopsies were independently reviewed and scored by one of the investigators (MC) in a blinded fashion. Established toxicity, engraftment and GVHD criteria were used for study evaluation.[13–15] Survival was determined as of August 1, 1996, and rates were calculated according to the method of Kaplan and Meier.[16]

RESULTS

From November, 1994, until May, 1995, 22 patients were enrolled. Patient and disease characteristics at the time of transplant are summarized in Table 1. All patients were evaluable for toxicity. Fourteen patients developed mucositis requiring narcotic analgesia, 9 patients developed ≥grade 2 nephrotoxicity, and 7 patients developed ≥grade 2 hepatic toxicity. Major toxicity according to cyclosporine group is summarized in Table 2. Two treatment-related deaths were observed: one patient developed progressive renal and liver failure and refused dialysis, and another patient died from veno-occlusive disease of the liver in association with a CMV infection. No significant differences were observed in the incidence of grade 3–4 organ toxicities when compared with 37 historical controls treated with the same preparative regimen (Table 2).

All patients recovered a neutrophil count of 0.5×10^9/L a median of 11 days post transplant (range 6–15). Nineteen patients achieved platelet transfusion independence at a median of 21 days (range 7–209). In the historical control group, the time to neutrophil recovery was 9 days, and the time to platelet transfusion independence was 12 days.

Patients received cyclosporine for a median of 26 days (range 4–28). Fourteen patients had cyclosporine held for 2 to 24 days (median 6 days) secondary to renal and liver dysfunction. Cyclosporine levels according to treatment group are summarized in Table 3.

Table 1. Patient and disease characteristics at time of transplant

Variable	
N	22
age in years, median (range)	50 (39–52)
sex	female: 3 male: 19
globulin type	IgG: 11/nonsecretory: 3 IgA: 4/Bence Jones: 3 IgD: 1
time to transplant in days, median (range)	590 (84–3354)
number of prior therapies, median (range)	3 (1–5)
disease status at transplant	primary refractory: 14 sensitive relapse: 5 refractory relapse: 1 first CR: 2
pre-transplant LDH IU/dL, median (range)	475 (264–734)
pre-transplant β-2M mg/L, median (range)	3.3 (1.3–13.4)
cyclosporine group	low dose: 7 high dose: 9 high dose and γ-interferon: 6

Three patients developed clinical skin GVHD that responded to steroid therapy. Eight patients developed histologic evidence of GVHD without clinical signs of GVHD (subclinical GVHD). The incidence of clinical and subclinical GVHD was four of seven patients in the low cyclosporine level group and three of seven in the high cyclosporine level group.

Seven of 18 patients with measurable disease pretransplant achieved remissions. Seven patients have progressed 84–354 days post transplant (median 160 days). Seven patients died between 28 and 426 days post transplant (median 194 days). Two died from regimen-related toxicity, the other five from complications of their disease. Fifteen patients are alive between 45 and 664 days post transplant. Overall survival is 73 ± 10% at 18 months.

Table 2. Regimen-related toxicity

	Low-dose CSA	*High-dose CSA*	*High CSA + γ-IFN*	*Historical controls*
N	7	9	6	37
>grade 3 kidney	1/7	0/9	1/6	1/37
>grade 3 liver	1/7	0/9	1/6	2/37
peak creatinine median (range)	1.6 (0.6–5.1)	1.5 (0.9-5.4)	1.9 (1.6–5.0)	1.1 (0.5–3.2)
peak bilirubin median (range)	2.4 (0.8–5.3)	1.4 (0.8–5.3)	2.5 (0.6–28.5)	0.8 (0.4–16.3)
100 day TRM	1/7	0/9	1/6	3/37

Table 3. Patient outcomes

	Low-dose CSA	*High-dose CSA*	*High-dose CSA + γ-IFN*
N	7	9	6
median CSA level	145 (80–221)	198 (153–282)	209 (47–240)
clinical GVHD	1/7	0/9	2/6
subclinical GVHD	4/7	3/9	1/6
response	4/7	1/6	2/6

SUMMARY AND CONCLUSIONS

In this report, we show that induction of autologous GVHD is feasible in patients with multiple myeloma undergoing high dose chemotherapy and autologous stem cell transplantation. Several observations made during this study merit further comment.

The addition of cyclosporine early after transplant increased the incidence of mild nephrotoxicity. Many patients required discontinuation of cyclosporine, with one patient developing renal failure requiring dialysis. Further phase II trials will continue with a recommended target cyclosporine level between 150 and 300 ng/dL.

The rate of induction of clinically evident autologous GVHD in this study seemed to be lower than that reported for breast cancer or lymphoma. The lower rate could be related to an underlying T cell dysfunction and immune deficiency associated with myeloma.[16] We continue to explore the feasibility and efficacy of autologous GVHD induction in conjunction with gamma-interferon and interleukin-2, in efforts to harness an antimyeloma effect.

REFERENCES

1. Barlogie B, Alexanian R, Smallwood L, Cheson B, Dixon D, Dicke K, Cabanillas F: Prognostic factors with high dose melphalan for refractory multiple myeloma. *Blood* 72:2015–2019, 1988.
2. Harousseau J, Milpied N, Laporte L et al.: Double intensive therapy in high-risk multiple myeloma. *Blood* 79:2827–2833, 1992.
3. Jagannath S, Vesole D, Glenn L, Crowley J, Barlogie B: Low risk intensive therapy for multiple myeloma with combined autologous bone marrow and blood stem cell support. *Blood* 80:1666–1672, 1992.
4. Attal M, Harousseau J, Stoppa A et al.: High dose therapy in multiple myeloma: Final analysis of a prospective randomized study of the "Intergroupe Français du Myelome" (IFM-90): *Blood* 86(suppl 1):124a, 1995.
5. Kennedy J, Vogelsang G, Beveridge R et al.: Phase I trial of intravenous cyclosporine to induce graft-versus-host disease in women undergoing autologous bone marrow transplantation for breast cancer. *J Clin Oncol* 11:478–484, 1993.
6. Kennedy M, Vogelsang G, Jones R: Phase I trial of interferon gamma to potentiate cyclosporine induced graft-versus-host disease in women undergoing autologous bone marrow transplantation for breast cancer. *J Clin Oncol* 12:249–257, 1994.
7. Jones R, Vogelsang G, Hess A et al.: Induction of graft-versus-host disease after autologous bone marrow transplantation. *Lancet* 1:754–757, 1989.
8. Yeager A, Vogelsang G, Jones R et al.: Induction of cutaneous graft versus host disease by administration of cyclosporine to patients undergoing autologous bone marrow transplantation for acute myeloid leukemia. *Blood* 11:3031–3035, 1992.
9. Tricot G, Vesole D, Jagannath S, Hilton J, Munshi N, Barlogie B: Graft-versus-myeloma effect: Proof of principle. *Blood* 87:1196–1198, 1996.
10. Alyea E, Soiffer R, Murray C et al.: Adoptive immunotherapy following allogeneic bone

marrow transplantation (BMT) with donor lymphocytes depleted of $CD8^+$ T cells. *Blood* 86(suppl 1):293a, 1995.

11. Geller R, Esa A, Beschorner W, Frondoza C, Santos G, Hess A: Successful in vitro graft versus tumor effect against Ia-bearing tumor using cyclosporine-induced syngeneic graft-versus-host disease in the rat. *Blood* 74:1165–1171, 1989.
12. Dimopoulos M, Alexanian R, Przepiorka D et al.: Thiotepa, busulfan, and cyclophosphamide: A new preparative regimen for autologous or blood stem cell transplantation in high risk multiple myeloma. *Blood* 82:2324–2328, 1993.
13. Bearman S, Appelbaum F, Buckner C et al.: Regimen related toxicities in patients undergoing bone marrow transplantation. *J Clin Oncol* 6:1562–1568, 1988.
14. Glucksberg H, Storb R, Fefer A et al.: Clinical manifestations of graft versus host disease in human recipients of marrow from HLA matched sibling donors. *Transplantation* 18:295–304, 1974.
15. Sale G, Lerner K, Barker E, Shulman H, Thomas E: The skin biopsy in the diagnosis of acute graft-versus-host disease in man. *Am J Pathol* 89:621–625, 1977.
16. Kaplan E, Meier P: Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 53:457–481, 1958.

MATHEMATICAL MODELING OF HUMAN HEMATOPOIESIS: LESSONS LEARNED FROM THE BEDSIDE FOLLOWING AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTS

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INTRODUCTION

Hematopoiesis has been a subject of intense investigation during the past four decades since the seminal in vivo studies of Till and McCulloch in the late 1950s.[1] Their studies demonstrated the existence of pluripotent hematopoietic stem cells in an animal model system. The importance of resident, marrow microenvironment in nurturing hematopoiesis was elegantly demonstrated by Dexter et al.[2,3] Their in vitro studies were also conducted in an animal model system. Finally, cloning hematopoietic growth factors[4–6] has enabled us to understand, in detail, the critical role that "marrow hormones" have in regulating hematopoiesis both in vitro and in vivo and in both animal model systems and humans. We now believe that the process that we call hematopoiesis is a carefully orchestrated interaction of the hematopoietic microenvironment, hematopoietic stem cells and hematopoietic growth factors.

The clinical utility of this wealth of fundamental knowledge about hematopoiesis has manifested itself in the field of bone marrow transplantation (BMT). The modern era of BMT began in the late 1960s when the clinical significance of genotypic human leukocyte antigen (HLA) matching was appreciated. The term "bone marrow transplant" has come, now, to mean, in its broadest sense, a therapeutic procedure in which myeloablative/immunosuppressive cytotoxic therapy is given with curative intent for malignant and nonmalignant disorders after which reconstitution of hematopoiesis and lymphopoiesis is required and accomplished by the infusion of hematopoietic stem cells, which may be collected from a variety of donors and from a variety of anatomic sites. The recovery of hematopoiesis after high-dose therapy (HDT) and stem cell transplantation provides us with a unique opportunity to study the rate limiting steps in human hematopoiesis that has been profoundly perturbed.

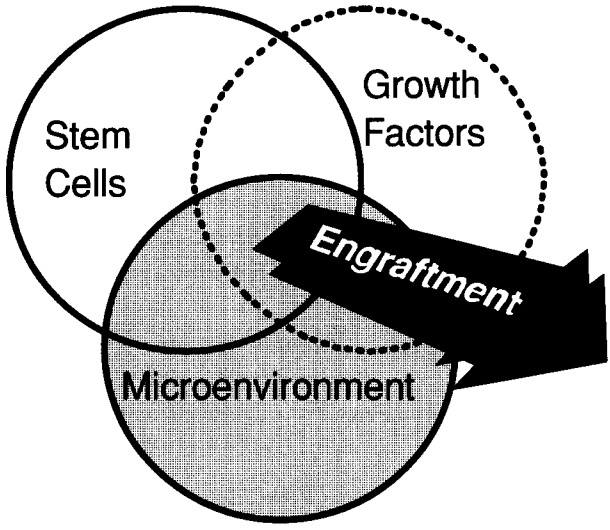

Figure 1. *Interactions of hematopoietic microenvironment, stem cells and growth factors in recovering hematopoiesis (engraftment) following PBSCT as rescue from HDT induced myeloablation. Reproduced with permission of the publisher from the cover of Volume 770 of the Annals of the New York Academy of Sciences.*[23]

Autologous stem cell transplants after HDT for a variety of malignancies have become very commonplace.[7] After allogeneic stem cell transplantation, principally for lymphohematopoietic malignancies, we know that both the initial, recovery phase of hematopoiesis (termed early engraftment) and the ongoing, sustained process of long term hematopoiesis (termed permanent engraftment) are almost exclusively the province of the allogeneic donor cells. In autologous transplants, because of the kinetics (rapidity) of recovery of blood counts, we strongly suspect that early engraftment is due to the previously collected, stored and subsequently reinfused autologous "donor" cells but we are unsure as to the relative contributions of these reinfused cells and the residual, surviving, endogenous cells in sustained hematopoiesis. By genetic marking of donor cells we can demonstrate the participation of reinfused cells in permanent engraftment.[8] But, without the ability to genetically mark endogenous cells, we cannot establish the degree of participation of residual endogenous cells in sustained hematopoiesis. In a recent review of our experience,[9] we demonstrated that early engraftment after autologous transplants is the consequence of the same carefully orchestrated process involving microenvironment, stem cells and growth factors as described above. This interaction is illustrated in Figure 1.

We can visualize, then, two functional "roles" of hematopoiesis; one being recovery from a perturbed state (of which early engraftment is an example) and the other being maintenance of a steady state (of which permanent engraftment is an example). The purpose of this communication is to explore what we have learned at the bedside from patients receiving HDT and autologous peripheral blood stem cell (PBSC) transplants in order to model mathematically how human hematopoiesis responds to a massive insult to the steady state, i.e., recovering from a severely perturbed state. There is no doubt that HDT produces a major insult to steady state hematopoiesis. The autologous PBSC transplant provides the cells necessary to recover from the markedly perturbed state so produced and they are responsible for the phenomenon of early engraftment. This communication will not deal with the homeostatic mechanisms of steady state hematopoiesis, a much more difficult subject to study in humans.

MATERIALS AND METHODS

Patients

All patients whose data will be presented herein were eligible for, enrolled in and gave written informed consent for treatment protocols that were reviewed and approved by the Scientific Review Committee of the H. Lee Moffitt Cancer Center and Research Institute and reviewed and approved annually by the Institutional Review Board of the University of South Florida. The principal diagnoses were breast cancer and lymphoproliferative malignancies, as described previously.[9]

High-dose regimens

All patients for whom hematopoietic recovery was observed were treated with either one of four different HDT regimens (ICE, MITT, TNT and BUCY2) that we have previously reported[9] or with our modification of the CTC protocol in which cyclophosphamide was given at 6000 mg/m^2, thioTEPA at 500 mg/m^2 and carboplatin at 800 mg/m^2 in divided doses as brief intravenous infusions daily for 3 days.[10] ICE is the acronym of the regimen containing ifosfamide, carboplatin and etoposide; MITT represents the mitoxantrone and thioTEPA protocol; TNT stands for the Taxol® (paclitaxel), Novantrone® (mitoxantrone) and thioTEPA regimen; and BUCY2 stands for (version number 2 of) the busulfan and cyclophosphamide protocol.[11]

Growth factors

Throughout the studies from which data will be presented, the only hematopoietic growth factor used was granulocyte-colony stimulating factor (G-CSF; filgrastim, or Neupogen®, Amgen, Thousand Oaks, CA). G-CSF was used in two circumstances: 1) for mobilization of autologous PBSC for transplantation (T) either alone or

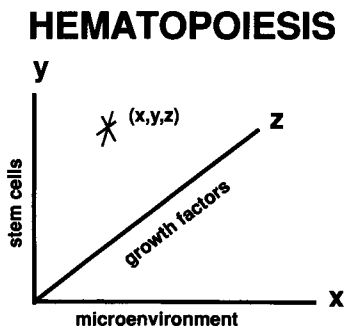

Figure 2. *Three-dimensional spatial representation of the concept of the three participants in the process of hematopoiesis: microenvironment, stem cells and growth factors.*

following chemotherapy (see below) and 2) after HDT and PBSCT at 10 μg/kg/day starting on the day after PBSCT and continuing until the absolute granulocyte count (AGC) exceeded 1000/μL for three consecutive days.

Mobilization regimens

The concept of mathematical modeling was developed from studies involving only PBSC. Autologous PBSC were collected after 1) G-CSF use alone (16 μg/kg/day for 9 days with collection beginning on day 7 and continuing daily thereafter through day 10) termed G-CSF priming or 2) G-CSF (5 μg/kg/day until completion of PBSC collection) given after chemotherapy with cyclophosphamide (CY; 50 mg/kg/day by brief intravenous infusion for 2 days) with or without etoposide (VP16; 600 mg/m^2 continuous intravenous infusion over 48 hours) termed CYVPG mobilization. Details about these PBSC priming/mobilizing regimens have been previously reported.[12]

Statistical analyses

Where given, all values of p are from two-tailed tests derived from appropriate logrank analyses of two or more patient groups. End points for analyses were days after PBSCT to recovery of AGC to more than 500/μL or platelet count (PLT) to more than 20,000/μL, self sustained.

RESULTS

Dose-response relationships

To derive a mathematical model of hematopoiesis recovering from a major insult, we need to change our thinking about HDT and autologous stem cell transplantation. We must visualize this aspect of hematopoiesis differently. We must convert our thinking from the concept of interactions of microenvironment, stem

cells and growth factors to biological dose response relationships for each "participant" in hematopoiesis, albeit complicated by interactions of the participants. We must think of quantifying the presence or activity of the participants as well as the outcome of their presence, activity and/or interactions. We must replace, in our thinking, then, Figure 1 with Figure 2.

Quantification of parameters

Figure 2 illustrates the concept that early engraftment is a result of quantifiable "characteristics" of stem cells, growth factors and the microenvironment. For each participant (or, alternatively termed, variable or parameter), there will be a dose-response relationship that can best be seen if the other two quantifiable parameters are held constant (see below for examples). The form (or shape) of the dose-response relationship is not necessarily predictable. The dose-response relationship may be simply linear. Or it may be complicated as in a sigmoid curve where 1) below a certain "quantity" of the parameter (often termed the threshold or minimum dose), there is no response, 2) above a certain quantity of the parameter (often termed the ceiling or optimum dose), there is no increase in response despite increase in the parameter and 3) between these "limits" of the parameter, the response increases as the parameter increases but this response is not restricted to being linear.

For a specific patient then, Figure 2 illustrates that there will be, after HDT and autologous PBSCT, a specific "quantity" of stem cells (reinfused and endogenous), a specific quantity of growth factors (from exogenous sources and produced endogenously) and a specific quantity of "microenvironmental influence" that will determine the pace of early engraftment. On the one hand, it is quite easy to visualize quantification of stem cells, although we cannot easily determine the "quantity" of residual, endogenous stem cells after HDT. Further, it is also easy to visualize the quantification of growth factors in terms of choice of growth factor(s) and dose, route and schedule variables; however, we cannot easily determine what growth factor(s) are produced endogenously after HDT, let alone determine their "quantity." On the other hand, it is difficult to visualize the quantification of microenvironmental influence. For this, we propose visualizing this quantifiable parameter as the percent of normal. For this exposition, we define the microenvironment broadly to include the cellular elements of the marrow stroma (and the molecules on their cell surface) and the noncellular elements of the stroma (insoluble and soluble molecules produced by the cellular elements). Further, we define microenvironmental influence as the functional ability of the marrow microenvironment to support hematopoiesis. This ability may be good or poor depending upon all of the clinical events that preceded the autologous PBSCT (including, but not limited to, the neoplastic disease process, metastases to the bone marrow, prior chemotherapy and HDT itself, that may have individually or collectively altered the physiology of or produced damage in the microenvironment).

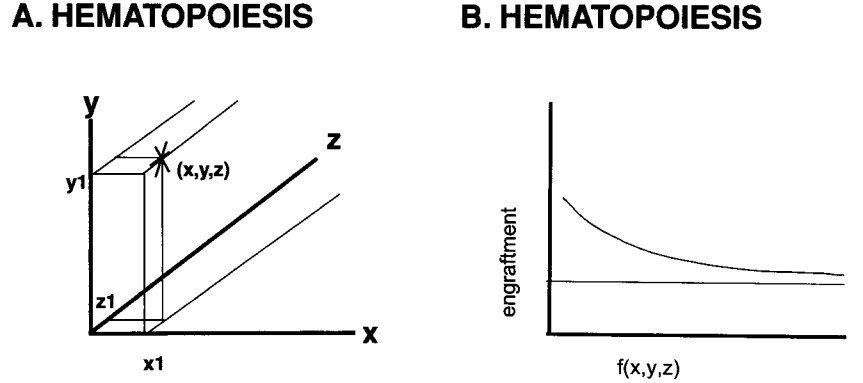

Figure 3. *(A) Every patient who receives a PBSCT after HDT will have quantifiable "amounts" of microenvironmental influence, stem cells and growth factors as shown by the point x1,y1,z1. Summing up the values of xn,yn,zn for many patients describes a surface in three-dimensional space.* ***(B)*** *Engraftment measured as the day that the AGC exceeds 500/μL is a hyperbolic function of the quantitative values of x,y,z, which displays the characteristics of having an asymptote.*

Three-dimensional space

Returning to Figure 2, we propose that for each patient we can, at least theoretically, establish the quantity of each of the three participants in the process of hematopoiesis recovering from a major insult. This gives us a single point (x1,y1,z1) in three-dimensional space for each patient (see Figure 3A). Summing up the individual points (xn,yn,zn) for many patients will describe a surface in three-dimensional space. (It is worthy of parenthetical note that there may be one or two other "independent" variables in addition to the three we have chosen, in which case we would be dealing with, for instance, four- or five-dimensional space when we model hematopoiesis mathematically. This, however, is difficult to visualize and, fortunately, is not necessary for understanding the major thrust of this exegesis.)

Saturation kinetics

Next, we propose that the pace of engraftment can be quantified as the day of recovery of granulocytes or platelets to specific levels (e.g., AGC of 500/μL and PLT of 20,000/μL, self sustained). Further, the pace of engraftment is the result of a mathematical function of the three hematopoietic variables, stem cells, growth factors and microenvironment, as shown in Figure 3B. This mathematical function may contain both independent and interactive terms. Consequences of these variables may be additive or synergistic. In any event, the relationship between this function and

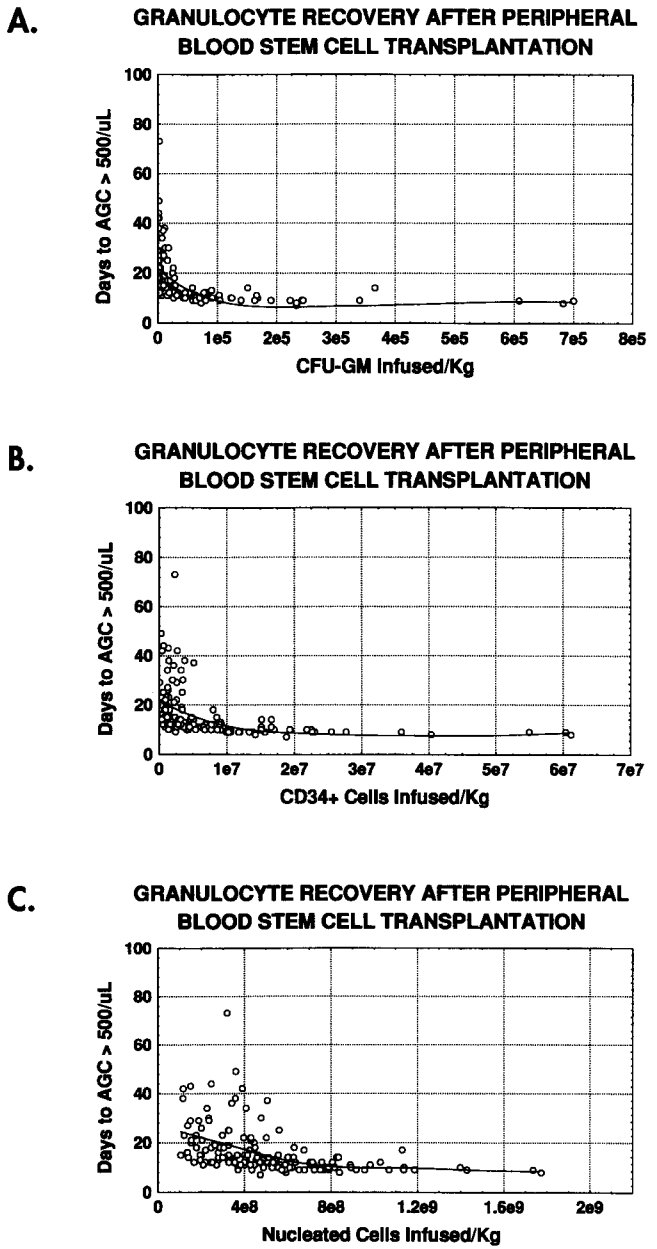

Figure 4. *Demonstration of the hyperbolic relationship for PBSC between engraftment (day to AGC > 500/μL) and the number of nucleated cells infused per kg* ***(A)****, the number of CD34⁺ cells infused per kg* ***(B)*** *and the number of CFU-GM infused per kg* ***(C)****. At high doses there is no faster engraftment; at low doses there may be delayed engraftment.*

engraftment is hyperbolic. At low values of the function, engraftment is late (slow) or fails to occur at all and, at high values of this function, engraftment is prompt (fast) or early but there is a limit to just how fast recovery can occur (the asymptote). The existence of an asymptote (see the horizontal line in Figure 3B) is highly suggestive of saturation kinetics. The biological significance of saturation kinetics should not be underestimated. For instance, it is altogether possible that there are a maximum number of microenvironmental "nurturing" niches available to which stem cells can bind, thus limiting the number of "starting" cells for the process of recovery of hematopoiesis and, thereby establishing a minimum time requirement between the transplant and the signs of engraftment described above due to the constraints of cell cycle times (see below). More stem cells infused than the available niches can handle cannot alter the pace of engraftment. Clinically, if two of the hematopoietic variables were kept fixed (held constant), such as the choice, dose and schedule of exogenous growth factor(s) (e.g., G-CSF at 10 μg/kg/day) and the marrow microenvironment (e.g., in a patient receiving a PBSCT for high risk stage II or III breast cancer after precisely four courses of one single type of adjuvant chemotherapy and following precisely one single type of HDT regimen), then a hyperbolic curve would be seen, as indicated in Figure 4 for the relationship between pace of engraftment and the third hematopoietic variable (whether it be nucleated cells, $CD34^+$ cells or granulocyte-macrophage colony-forming units (CFU-GM) infused per kilogram recipient body weight).[11,13] Unfortunately, we cannot quantitate microenvironmental influence, clinically, even on the percent of normal scale.

Clinically defined variables

However, we can identify, clinically, at least three variables of HDT and PBSCT that do condition the pace of engraftment. The importance of these variables has come to light over the past several years as our group has explored different PBSC priming/mobilizing regimens and different HDT regimens while not changing any aspect of the growth factor we were using after PBSCT and while quantitating, in the laboratory, the number of $CD34^+$ cells and CFU-GM in our PBSC collections. The three conditioning variables are 1) the HDT regimen (a surrogate for microenvironmental influence), 2) the PBSC mobilizing regimen and 3) the $CD34^+$ cell or CFU-GM content of the PBSC collection. The latter two appear to be independent measures of stem cell numbers and function as will be demonstrated below. This is seen in Figure 5A.

Engraftment results

In our clinical trials,[14–16] we have observed that engraftment is faster after the ICE regimen than after the TNT regimen and that CYVPG mobilized PBSC give faster engraftment than do G-CSF primed PBSC (see Figure 6). These observations have been made even though similar numbers of $CD34^+$ cells have been reinfused in all

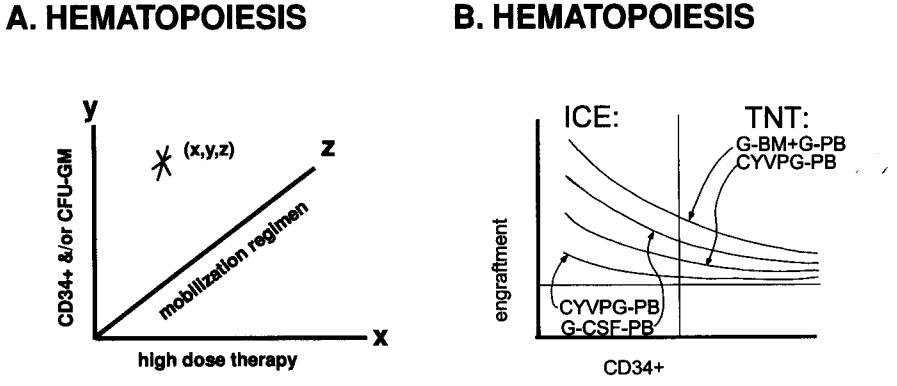

Figure 5. ***(A)*** *Three-dimensional spatial representation of three independent variables: stem cells, mobilization method and HDT regimen.* ***(B)*** *Day of engraftment as a function of CD34⁺ cell count after two different HDT regimens (ICE and TNT) and with two different PBSC preparations (CYVPG mobilized PBSC and G-CSF primed PBSC) illustrating the hyperbolic relationship of one variable (CD34⁺ cells) with respect to the day of engraftment when the other two independent variables (PBSC mobilization and HDT regimen) are fixed (held constant).*

cases. Notice that earliest engraftment times are identical for all four combinations of HDT and PBSC mobilizing regimens, supporting the concept of an asymptote, i.e., a minimum recovery time for hematopoiesis that cannot be significantly reduced further because of saturation kinetics for some rate-limiting parameter, which is likely to be the number of available, functional and nurturing microenvironmental niches.

Hyperbolic functions

Turn your attention now to Figure 5B which shows four hyperbolic curves. When CD34⁺ cells are at levels below that which would saturate the niches (see the vertical line in Figure 5B), we see that the number of CD34⁺ cells are rate-limiting. As described above, we also see that CYVPG mobilized PBSC give speedier engraftment than do G-CSF primed PBSC at a given level of CD34⁺ cells both for ICE and TNT. Further, we see for a given PBSC mobilizing regimen that there is faster engraftment for patients treated with the ICE HDT regimen than for the TNT HDT regimen at a given level of CD34⁺ cells. Thus, by fixing two variables at a time, we can see the contribution of the third variable emerging. But how can different paces of engraftment result from the same number of CD34⁺ cells?

Subsets of CD34⁺ cells

The four panels of Figure 7 may provide an answer. It is important to remember that, when we quantitate the number of CD34⁺ cells in a PBSC collection, we are

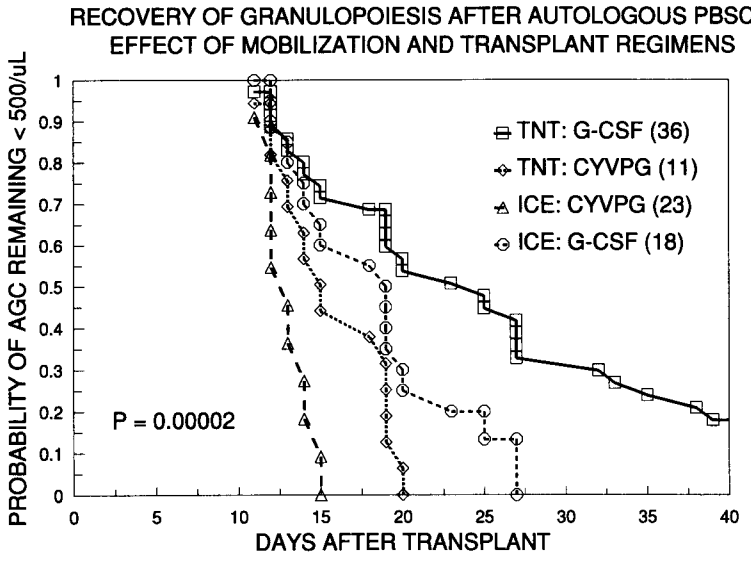

Figure 6. *Time to engraftment curves for four populations of patients: 1) those treated with ICE and received CYVPG mobilized PBSC, 2) those treated with TNT and received CYVPG mobilized PBSC, 3) those treated with ICE and received G-CSF primed PBSC and 4) those treated with TNT and received G-CSF primed PBSC.*

enumerating a heterogeneous population of hematopoietic progenitor cells[17] not all of which are rate limiting in determining the pace of recovery of hematopoiesis. In fact, at present we don't know to which $CD34^+$ progenitor cell subpopulation we can attribute responsibility for the phenomenon of early engraftment. But, for the sake of this portion of the exposition, we shall assume that it is the population of CFU-GM among the $CD34^+$ cells that is directly correlated with the pace of recovery of granulocytes. Figure 7 shows four possible scenarios for enrichment of peripheral blood content of hematopoietic progenitor cells suitable for transplant. Estimated numbers of progenitor cells of different hematopoietic lineages are shown for illustrative purposes.

Insofar as the numbers of $CD34^+$ cells are concerned, the four possible scenarios are: 1) nonmobilization (no increase in the number of $CD34^+$ cells but increases in the absolute numbers of progenitor cells, see Figure 7A), 2) ideal mobilization (increases in the number of $CD34^+$ cells along with increases in the proportions [and absolute numbers] of all measured progenitor cells, Figure 7B), 3) acceptable mobilization (increases in the number of $CD34^+$ cells and of the proportion [and, therefore, numbers] of CFU-GM but no increases in the absolute numbers of other progenitor cells, Figure 7C) and 4) poor mobilization (increases in $CD34^+$ cells

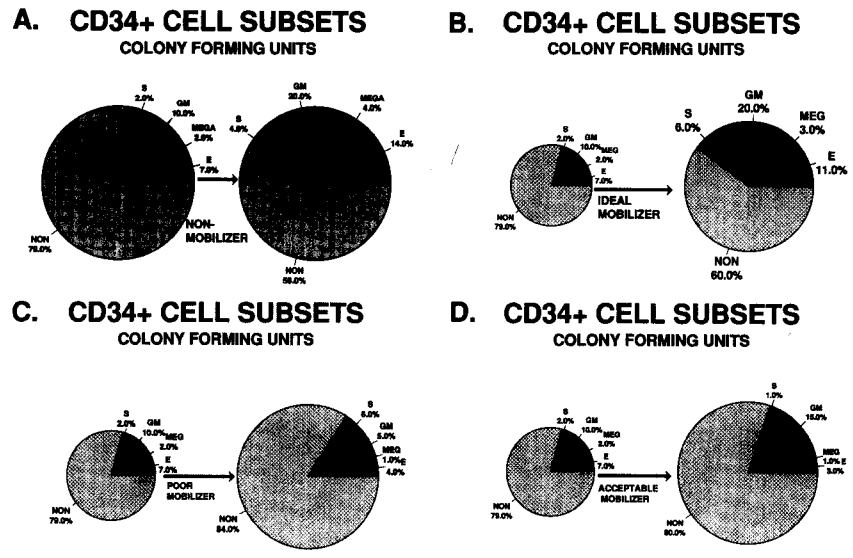

Figure 7. ***(A)*** *Illustration of "non"-mobilization of CD34⁺ cells but with changes in the composition of the collected CD34⁺ cells with respect to clonigenic hematopoietic progenitor cells.* ***(B)*** *Illustration of the effects of an "ideal" mobilizer.* ***(C)*** *Illustration of an "acceptable" mobilizer.* ***(D)*** *Illustration of a "poor" mobilizer.*

with increases in CFU-S [the putative pluripotent hematopoietic stem cell as described by Till and McCulloch] but no increases in the absolute numbers of CFU-GM and other progenitor cells, Figure 7D). If the pace of engraftment were merely a function of the number of CD34⁺ cells, then we would expect identical engraftment times for unmobilized PBSC and nonmobilized PBSC that would be different from the mobilization times for the other three types of PBSC. Further, the engraftment times for ideally mobilized, acceptably mobilized and poorly mobilized PBSC would be identical and faster than for the other two types of PBSC. Intuitively and experientially, we know that this is not the case. It is more likely and supported by our experience that the pace of engraftment is related to a subset of CD34⁺ cells, which we propose to be CFU-GM for the sake of this part of the argument. In that case, the order of engraftment from fastest to slowest for the five possible types of collections would be ideally > (faster than) acceptably > non- > poorly = unmobilized PBSC (see Figure 8A).

Subsets of CFU-GM

At this point, it is important to recognize that there is also heterogeneity in the population of CFU-GM as well as in the population of CD34⁺ cells. This heterogeneity is principally in the cell cycle status and state of activation. (There may also

be other characteristics of CFU-GM that introduce even more heterogeneity.) Cell cycle status is the time honored classification of G_0, G_1, S, G_2 and M. The state of activation for CFU-GM may be viewed as the state of "readiness" to begin proliferation and differentiation to produce mature cells for the circulation without significant delay. After all, we must consider the minimum period of time before engraftment can be detected, the asymptote discussed above, as the time during which there are sufficient rounds of cell division and sufficient time for terminal differentiation of the formed elements of the blood. We propose that "readiness" can reside only in the subpopulation of CFU-GM that are beyond G_1, which we have termed $G_{>1}$ in five of the panels of Figure 8 (see B–F). CFU-GM in the G_1 phase of the cell cycle will more rapidly enter S phase upon appropriate stimulation once in nurturing niches of the marrow than will CFU-GM in G_0. However, the entry of G_1 CFU-GM into S phase may still require a period of time that is genetically predetermined. Our hypothesis is that it is the most "ready" cells that are the ones responsible for the earliest signs of engraftment followed, thereafter, by cells that have been stimulated but had to go through a series of intracellular events before they could proliferate and produce progeny that could terminally differentiate. Five panels of Figure 8 (B–F) show theoretical distributions of CFU-GM subpopulations according to the cell cycle status with the putative activated (ready) CFU-GM residing in the cell cycle phase $G_{>1}$. These distributions are shown side by side in Figure 9A. If the $G_{>1}$ subset were entirely or predominantly responsible for the day of engraftment, it is obvious that there may be quite different days of engraftment for the different categories of mobilized $CD34^+$ cells. We must acknowledge here the fact that we cannot state with certainty that the "ready" cells responsible for early engraftment are, in fact, a subset of CFU-GM even though CFU-GM proliferate and differentiate in vitro into granulocytes and macrophages.

Cytokinetics

No discussion of proliferating cells should fail to include a discussion of cytokinetics. We have introduced the concept of cell cycle status above. Now we wish to discuss the implications of the time it takes a progenitor cell to go completely through the cell cycle insofar as cell number doublings are concerned. If we assume, in the most extreme scenario, that all hematopoietic progenitor cells, that determine the time of early engraftment, produce progeny that are both viable and committed to differentiation (i.e., neither of the daughter cells are committed to self-renewal and neither undergo apoptosis), then simple exponential mathematics will reveal how many doublings can take place in, say, 10 days to produce evidence in the blood of early engraftment (e.g., AGC > 500/μL or PLT > 20,000/μL).

To obtain the answer we must only know the cell cycle time. Most estimates today of cell cycle time for hematopoietic cells are in the vicinity of 24 hours.[18] If that were true, then only 10 doublings could take place in 10 days and the

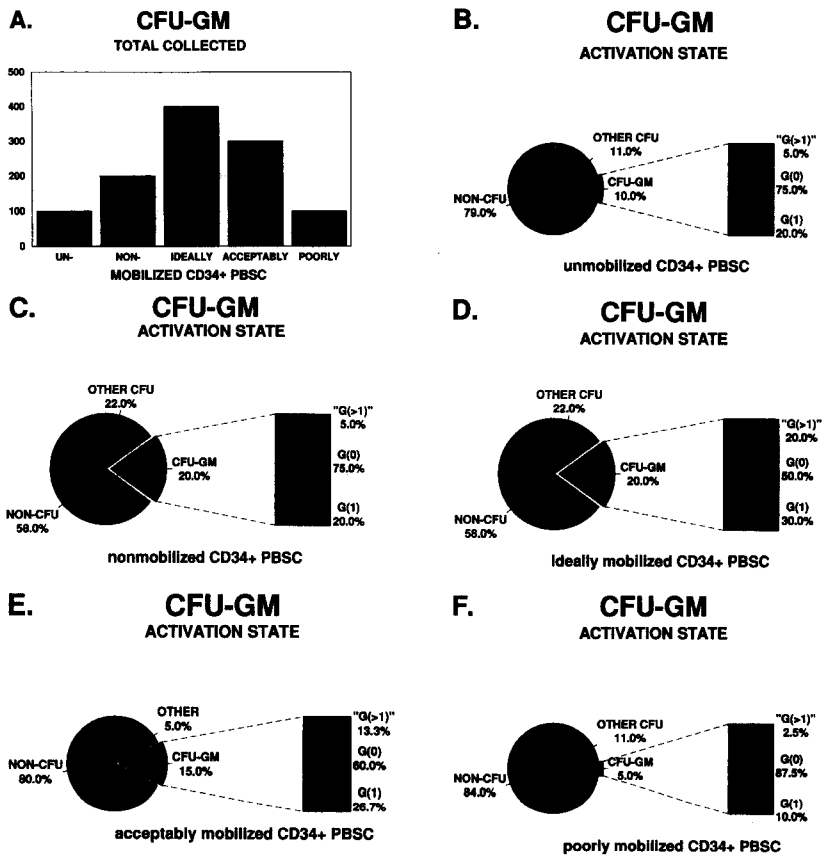

Figure 8. ***(A)*** *Total CFU-GM content of PBSC collections after four types of mobilizers versus "un"-mobilized (control) blood (derived from Figure 7, Panels A through D).* ***(B)*** *CFU-GM subsets by cell cycle status in PBSC collection of "un"-mobilized blood.* ***(C)*** *Subsets after "non"-mobilizer.* ***(D)*** *Subsets after "ideal" mobilizer.* ***(E)*** *Subsets after "acceptable" mobilizer.* ***(F)*** *Subsets after "poor" mobilizer.*

maximum cell production from a single "ready" progenitor cell would be 2^{10} or 1024 cells. With a 24 hour generation (cell cycle) time for hematopoietic cells at peak efficiency and with no dropout, it is obvious that, if $CD34^+$ cell content varied by a factor of 10 between two types of PBSC products and was solely responsible for the pace of engraftment, then the difference in engraftment times for two products would only be approximately 3 days (as 10 is approximately equal to 2^3 [=8]). The same is obviously true for CFU-GM. Rarely do two types of PBSC products differ by 10-fold but not uncommonly do engraftment times differ by 7 or

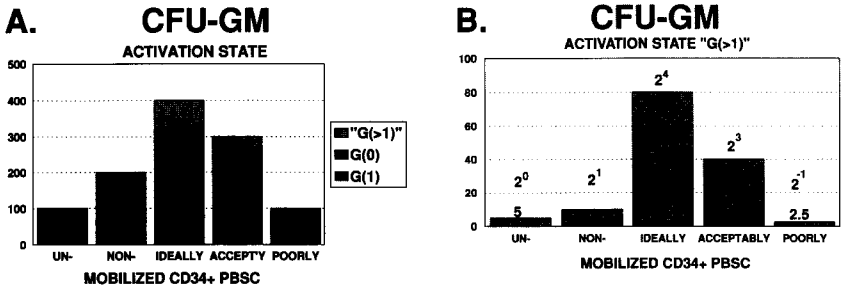

Figure 9. ***(A)*** *Summary of CFU-GM subsets by cell cycle status in PBSC collections after four different types of mobilizers as compared to controls (unmobilized blood).* ***(B)*** *Evaluation of CFU-GM in the most "active" phase of the cell cycle ($G_{>1}$) with respect to the number of doublings (exponent of 2) that it would take to "expand" unmobilized peripheral blood CFU-GM to reach the level in the blood for each of the four types of mobilizers. If, for example, the cell cycle time for CFU-GM were 24 hours, then it would take 4 doublings for the "ready" CFU-GM of "unmobilized" blood to reach the level found in "ideally" mobilized blood which would predict only a 4-day difference in time to engraftment with the two different types of PBSC products.*

more days (Figure 6 above and Figure 10). Figure 9B shows the number of doublings that it would take for "unmobilized" blood to reach the levels in "mobilized" blood with respect to activated CFU-GM ($G_{>1}$). If the cell cycle time were 24 hours and CFU-GM ($G_{>1}$) were the "pivotal" progenitor cell, then we may deduce the number of days of difference in engraftment times for the different PBSC products from the exponent of 2 that describes the relative heights of the columns in Figure 9B.

Further, if the generation time were reduced to 18 hours from 24 hours then 10 doublings (to produce 1024 cells) would take place in 7.5 days. Even more extreme, if a PBSC mobilization regimen were to reduce the generation time to 12 hours, then 1024 cells would be produced in 5 days or half the time would be required to produce enough cells to meet the criterion for demonstrating early engraftment. Numerically, this latter calculation might explain some of the differences we have observed between PBSC preparations; however, there are no data at present to support generation times of less than 18 hours. Finally, when we look at Figures 10A and 10B, we see differences in engraftment times of up to 10 days using a single type of PBSC mobilization but different HDT regimens. If engraftment times were solely due to the number of $CD34^+$ cells and the generation time were 24 hours, then there should be a 2^{10}=1024-fold difference in the number of $CD34^+$ cells to explain a 10-day difference in early engraftment. Further, if the generation time were less than 24 hours, an even greater difference in the number of $CD34^+$ cells must be present to explain the difference in the pace of engraftment.

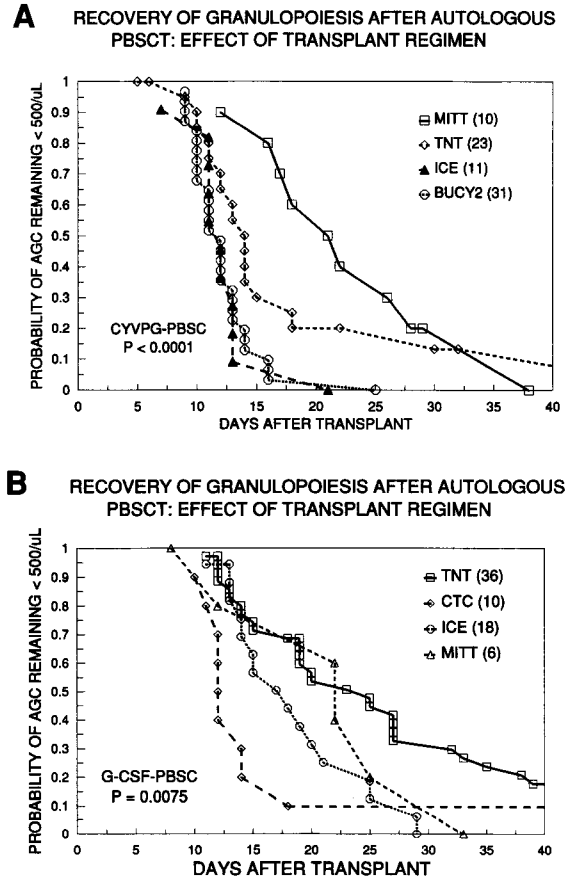

Figure 10. ***(A)*** *Time to engraftment for CYVPG mobilized PBSC after four different HDT regimens: BUCY2, ICE, TNT and MITT.* ***(B)*** *Time to engraftment for G-CSF primed PBSC after four different HDT regimens: CTC, ICE, TNT and MITT.*

Thus, for cytokinetic reasons as well, the clinical and laboratory data we have collected support the mathematical model we propose that the readily quantifiable characteristics of stem cells (i.e., the numbers of $CD34^+$ cells and CFU-GM) are but one of the determinants of the ultimate pace of engraftment.

Clinical examples

It is appropriate, now, to return to our clinical studies to see two examples of pace of engraftment and the variability produced by different HDT regimens. Figure 10A shows the patterns of engraftment for four HDT regimens with the source of stem cells being CYVPG mobilized PBSC. Figure 10B shows the

patterns of engraftment for four HDT regimens with the source of stem cells being G-CSF primed PBSC. (Please note that Figure 6 took two curves each from Figures 10A and 10B.) The two panels of Figure 10 (A and B) once again demonstrate the minimum period required before early engraftment occurs and the major impact of different HDT regimens on the pace of engraftment. An inverse relationship may exist; the more damaging the HDT regimen is to the microenvironment the lower the percent of normal of the microenvironmental influence. However, remember that the HDT regimen was considered above as only a surrogate of the percent of normal of the microenvironmental influence on the pace of engraftment. HDT could be solely responsible for altering the microenvironment or it may just be a covariate representing the cumulative effects of all the damaging events that went on before the PBSCT (see above). It is our opinion, as we have tried to unravel this Gordian knot, that it is more likely to be the HDT that is responsible for the different engraftment times observed than the other marrow microenvironmental, potentially damaging events that may have occurred prior to HDT.

In addition, it is worth listing examples of PBSC-mobilizing regimens that may fit at least three of the theoretical classes of mobilizers listed above. From our data, we consider CYVPG a member of the category of "ideal" mobilizers, G-CSF a member of the category of "acceptable" mobilizers and GM-CSF (as we used it; 9 days of 250 $\mu g/m^2$ with PBSC collections on days 7 through 10[19]) a member of the category of "poor" mobilizers. If a potential PBSC mobilizer didn't increase the $CD34^+$ cell content of the harvest (a "non"-mobilizer), we did not use the collected PBSC for transplantation purposes (hematopoietic rescue).

Mathematical model

Finally, we are prepared to propose two equations that may describe the clinical phenomena we have observed. The equations are derived from the algebraic formula (#1) for a hyperbola:

1) $y = c + 1/x,$

where "x" is the independent variable representative of the participants in hematopoiesis, "y" is the dependent variable representing the outcome of recovering hematopoiesis (the day of engraftment) and "c" is a constant that represents the asymptote value on the y-axis or the minimum time before engraftment can be observed. For the purposes of this discussion, we may replace formula 1 with a modified version shown in formula 2 that may be more "clinically" relevant:

2) $D(AGC>500) = k_{AGC} + 1/F(x,y,z)$

where "D" is the day that the AGC exceeds 500/μL, "k_{AGC}" is the minimum time before engraftment may be detected and "F(x,y,z)" is a function of the three quantifiable participants in early engraftment, i.e., "x" represents either the total number of $CD34^+$ cells or CFU-GM, "y" represents the proportion of "x" that are

activated (ready) as determined by the PBSC mobilization regimen and "z" is percent of normal of microenvironmental influence as represented, in surrogate or covariate fashion, by HDT regimen. We will let $k_{AGC} = 8$ days as median time to engraftment has rarely been reported to be earlier than 9 days. We assert that $0 < F(x,y,z) <$ or $=$ 1 are appropriate upper and lower limits. At these limits for F(x,y,z), we will find that the limits: oo > D(AGC>500) > or = 9 days are reasonable limits for D(AGC>500).

There are, at least, two candidate expressions for F(x,y,z) that would meet the criteria of 1) identifying three independent variables and 2) reflecting interactions of the three variables, as well. These two expressions are shown in formulas 3 and 6 below. The first potential solution is:

3) $F(x,y,z) = f(x) + f(y) + f(z) + f(x,y) + f(y,z) + f(x,z) + f(x,y,z)$

where:

4) $f(\text{variable}) = c_{\text{variable}}(1 - e^{-\text{variable/optimum}})$

for which the variable (v) may range from zero to the optimum (o) "dose" of the variable (i.e., dose which produces the optimum response) and the value of f(variable) ranges from zero to 0.6321 calculated from (1 – 1/e). Further, interaction terms have the form:

5) $f(v1,v2) = l_{v1}m_{v2}(1 - e^{-v1/o1})(1 - e^{-v2/o2})$

where l and m are constants.

The second potential solution is:

6) $F(x,y,z) = d_{AGC}(1 - e^{-x/a})(1 - e^{-y/b})(1 - e^{-z/c})$

where "a," "b" and c are the values of "x," "y" and z at which the optimum response is obtained. The limits of each variable is zero to the optimum dose for that variable which means, again, that the limits for each factor in the equation of the form $(1 - e^{-v/o})$ are zero to 0.6321. If we substitute the expression for F(x,y,z) from formula 6 into formula 2, we get:

7) $D(AGC>500) = k_{AGC} + 1/[d_{AGC}(1 - e^{-x/c})(1 - e^{-y/b})(1 - e^{-z/c})]$.

Setting x, y and z at a, b and c, we can solve for the constant d_{AGC}, which we find to be 4.

The two expressions (formula 3 and formula 6) for F(x,y,z) have reasonable power (when substituted into formula 2 yielding, for instance, formula 7) to describe the clinical results that we have observed. Between the two expressions, we are, however, more attracted to formula 6 because of its conceptual simplicity and because both constants (k_{AGC} and d_{AGC}) are already known for formula 7. Clearly, however, each potential version of F(x,y,z) must be tested thoroughly before acceptance is warranted.

DISCUSSION

From many years of delivering a number of different HDT regimens requiring autologous hematopoietic stem cell rescue, we have been impressed with the lack

of predictability of the duration of aplasia after stem cells have been reinfused and, particularly, with the lack of correlation of $CD34^+$ cell and of CFU-GM levels in the stem cell collection and the duration of aplasia when we looked at different PBSC mobilizing regimens and different HDT regimens. We did not find the anticipated first order kinetics with early engraftment associated only with the number of progenitor cells quantifiable in the laboratory. We did, however, find that the probability of late engraftment or failure to engraft was associated with low numbers of $CD34^+$ cells and with low numbers of CFU-GM infused. Further, we found major differences in engraftment times between different HDT regimens and major differences in engraftment times between different PBSC mobilization regimens. Why have we made these observations?

We have made these observations because we have explored several, novel HDT regimens at truly high-dose intensity and we have explored several PBSC mobilization regimens in virtually all of the HDT regimens. This has given us a unique vantage point from which to begin thinking about engraftment in a different light other than something to agonize over (because the aplasia was too long and patients were susceptible to potentially fatal opportunistic infections) as patients recovered from the aplasia that HDT produces for which the PBSCT is required to optimize survival potential. In other words, clinical observations have made us rethink the fundamental biology of the hematopoietic system. This has been, truly, a bedside-to-bench-to-bedside exercise, a paradigm of translational research. From our evaluation of our clinical observations evolved our mathematic model of human hematopoiesis.

During the process of rethinking about human hematopoiesis, we realized that we were looking at recovery from a profoundly perturbed state and not merely adjustments in steady state hematopoiesis. We realized that we needed to understand recovery of blood counts in the context of biologically meaningful, dose-response relationships between quantifiable independent variables (while including the potential of interaction of these "independent" variables) and quantifiable dependent variables (in this case, the outcome of early engraftment). We recognized that the time to engraftment was a function of at least three independent variables (which mathematically describe a surface in three-dimensional space), that the dose response curve was hyperbolic and that there appeared to be saturation kinetics involved (the asymptote implied a minimum necessary period of time before engraftment, as measured by blood counts, could be observed). Finally, we realized that, when two variables were held constant, we could demonstrate the hyperbolic dose-response to the third variable relatively easily. We explored further the relationship of $CD34^+$ cell and CFU-GM content of PBSC collections and time to engraftment and realized that the cell type responsible for pace of engraftment was in a subset of $CD34^+$ cells and, likely to be, further, in a subset of CFU-GM, as well. At present, we have no way to identify

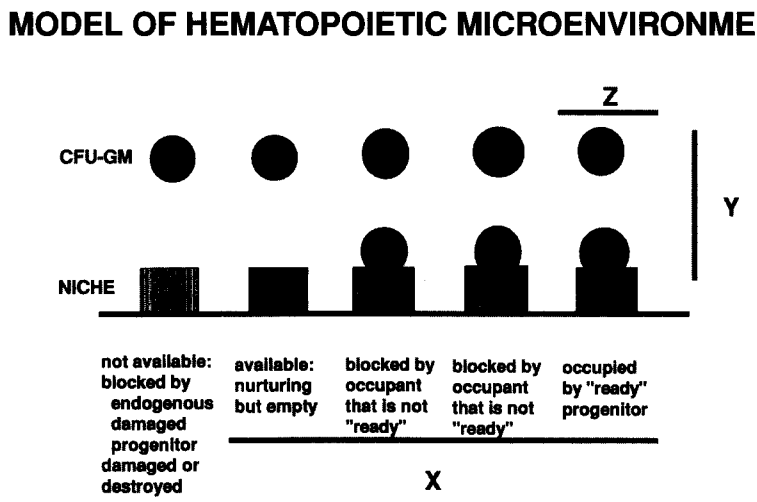

Figure 11. *Schematic representation at the marrow microenvironmental level of the roles of nurturing microenvironmental niches, CFU-GM capable of binding to these niches and the fraction of these CFU-GM which are "ready" (activated) to begin proliferation and differentiation. From this diagram, we can see where the values of x,y,z are derived for formula 7, the mathematical model of recovering hematopoiesis (early engraftment).*

and quantitate this pivotal subpopulation of hematopoietic progenitors. Last, we explored cell cycle phases and cytokinetics and realized that recovery of blood counts after PBSCT could not simply be ascribed to characteristics of stem cells alone without major reductions in generation times and a total commitment to differentiation of all progeny of the pivotal subpopulation that we allude to above.

One of the most difficult parts of this endeavor was accepting the fact that we could not easily quantitate the "microenvironmental influence" on recovery of blood counts even though we know that it exists from animal model studies[20] and in humans, as well. This means that, for any new HDT regimen to treat better and with curative intent, any specific malignancy, we will not know how fast engraftment will be even with our best, "ideal" PBSC mobilizer until we give the HDT regimen and observe the pace of engraftment for a significant number of patients. We cannot drop empiric observations and use only theoretical considerations as we plan new therapies and determine their benefits as well as their drawbacks.

Similarly, with respect to PBSC mobilization regimens, because we cannot quantitate the "pivotal" hematopoietic progenitor cell for the process of hematopoietic recovery (early engraftment), as we develop new mobilizing regimens, we must still determine the repopulating potential of the new type of PBSC with clinical trials. Here, too, empiric observations will be required as new mobilizing regimens are developed.

Our last comments deal with the mathematical model for recovering hematopoiesis. The major thrust of this communication was to develop a mathematical equation for hematopoiesis recovering from the massive insult of HDT with the PBSCT providing the progenitor cells. This was done to "demystify" the process of hematopoiesis and to establish the relationships of the participants in recovering hematopoiesis. If we could understand the mathematical relationships of and the limits placed upon the individual participants established by the biological constraints associated with each participant, then we would not only understand the process better, but we would also be able to begin making certain predictions with respect to the behavior of recovering hematopoiesis as we change our HDT therapies and PBSC mobilization regimens. Formula 7 is, we believe, an excellent beginning effort to achieve the goal we seek. Figure 11 illustrates schematically from where values of x, y and z may be derived in vivo for formula 7. Its true worth will surface with the passage of time, especially if it has real predictive value.

Figure 11 also illustrates the probability that some "nurturing" microenvironmental niches will remain empty despite the presence of "excess" unbound CFU-GM. This follows from the concept that binding of CFU-GM to microenvironmental niches is due, in all likelihood, to reversible ligand-receptor molecular interactions which have a binding constant which is defined as:

$K_{binding}$ = [ligand-receptor complex]/[free ligand][free receptor].

(In this case, the binding constant is, obviously, the reciprocal of a dissociation constant so familiar to all chemists.) If there were but one species of ligand and receptor, then we would find that $K_{binding} = K_{affinity}$ $(= 1/K_{dissociation})$. If there were more than one species of ligand and receptor responsible for binding of CFU-GM to microenvironmental niches (as is likely), then we would find that $K_{binding} = K_{avidity}$ which expresses a "weighted" average of $K_{affinity}$ for individual species. In any event, the consequence of these types of molecular interactions is that not all microenvironmental binding sites will be occupied nor will all available CFU-GM be bound even if below saturating numbers. Depending upon how powerful $K_{binding}$ is, there will be more or fewer free (unbound) sites and CFU-GM. This is independent of the consequences of steric hindrance which most assuredly must exist as well.

We wish to conclude by saying that, at this point in time, we believe there are three rate-limiting steps in recovery from the highly perturbed state produced by HDT and they are: 1) the number of available, nurturing microenvironmental niches a) left after departure of former occupants, b) not damaged by the malignant disease process and c) surviving all of the prior chemotherapy including the HDT regimen; 2) the number of the subset of CFU-GM which are "ready" (activated) to proliferate and differentiate immediately upon entering the circulation but can "perform" only after arriving in the marrow space and successfully competing with non-ready (but capable of binding) CFU-GM for attachment to the marrow

microenvironmental, nurturing niches; and 3) the generation time of the pivotal (ready) hematopoietic progenitor cell, i.e., the time necessary for this cell to go through one complete cell cycle.

The challenges that lie ahead for us are 1) to devise methods to quantitate microenvironmental influence for human marrow stroma and 2) to develop a system for quantitating the pivotal hematopoietic progenitor cell responsible for early engraftment. Shortening cell cycle time may very well be desirable but will probably be very difficult to accomplish. At present, we do not believe that changes in growth factors (numbers, doses and schedules) given after PBSCT will likely make a big difference unless they somehow increase the number of nurturing marrow stromal niches, which may increase the maximum response because the level (number) of "starting" progenitor cells in nurturing niches will be higher as the process of recovery of hematopoiesis from the massive insult of HDT begins.

Finally, one interesting interaction possibility among hematopoietic participants has recently surfaced. It is possible that different mobilizing regimens may not only generate different numbers of "ready," pivotal progenitor cells but also have an effect on the microenvironment, as well, in at least two potential ways: 1) improving the microenvironment in situ and/or 2) mobilizing a microenvironmental precursor cell that is infused along with the hematopoietic progenitor cell.[21,22]

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REFERENCES

1. Till JE and McCulloch EA: A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* 14:213–222, 1961.
2. Dexter TM, Allen TD, Lajtha LG: Conditions controlling the proliferation of hematopoietic cells in vitro. *J Cell Physiol* 91:335–344, 1977.
3. Roberts RA, Spooncer E, Parkinson EK et al.: Metabolically inactive 3T3 cells can substitute for marrow stromal cells to promote the proliferation and development of multipotent haemopoietic stem cells. *J Cell Physiol* 132:203–214, 1987.
4. Nagota S, Tsuchiya M, Asano S et al.: The chromosomal gene structure and two mRNAs

for human granulocyte colony-stimulating factor. *EMBO J* 5:575–591, 1986.

5. Gough NM, Gough J, Metcalf D et al.: Molecular cloning of CHNA encoding a murine haematopoietic growth regulator, granulocyte-macrophage colony stimulating factor. *Nature* 309:763–767, 1984.
6. Schrader JW: The panspecific hemopoietin of activated T lymphocytes (interleukin-3). *Annu Rev Immunol* 4:205–230, 1986.
7. Passweg JR, Rowlings PA, Armitage JO: Report from the International Bone Marrow Transplant Registry and Autologous Blood and Marrow Transplant Registry—North America. In: Cecka and Teraski (eds): Clinical Transplants. Los Angeles: UCLA Tissue Typing Laboratory, 1995, pp 117–127.
8. Brenner MK, Rill DR, Moen RC et al.: Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. *Lancet* 341:85, 1993.
9. Elfenbein GJ, Janssen WE, Perkins JB: Relative contributions of marrow microenvironment, growth factors and stem cells to hematopoiesis in vivo in man: Review of results from autologous stem cell transplant trials and laboratory studies at the Moffitt Cancer Center. In: Sackstein R, Janssen WE, Elfenbein GJ (eds). Bone Marrow Transplantation: Foundations for the 21st Century; Annals of the New York Academy of Sciences, Vol. 770. New York: The New York Academy of Sciences, 1995, pp 315–338.
10. Elfenbein G, Perkins J, Janssen W, Fields K: Variability in engraftment potential of different peripheral blood stem cell preparations given after cyclophosphamide, thiotepa and carboplatin for stage II/III breast cancer. *Exp Hematol* (in press).
11. Ballester OF, Agaliotis DP, Hiemenz JW et al.: Phase I-II study of high dose busulfan and cyclophosphamide followed by autologous peripheral blood stem cell transplantation for hematological malignancies: Toxicities and hematopoietic recovery. *Bone Marrow Transplant* 18:9–14, 1996.
12. Janssen WJ: Mobilization of peripheral blood stem cells for autologous transplantation. Methods, mechanisms and role in accelerating hematopoietic recovery. In: Sackstein R, Janssen WE, Elfenbein GJ (eds): Bone Marrow Transplantation: Foundations for the 21st Century; Annals of the New York Academy of Sciences, Vol. 770. New York: The New York Academy of Sciences, 1995, pp 116–129.
13. Zorsky P, Fang T, Janssen W et al.: The WBC recovery rate after priming with CYVP16+GCSF is a determinant of ANC recovery and survival after BUCY2 and autologous peripheral blood stem cell rescue. *Blood* 86(suppl 1):101a, 1995.
14. Elfenbein GJ, Perkins JB, Janssen WE et al.: Recovery of granulopoiesis after autologous peripheral blood stem cell transplant (PBSCT) varies with the high dose regimen (HDREG). Keystone Symposium, "Blood Cell and Bone Marrow Transplants," p 40, 1996.
15. Partyka JS, Perkins JB, Janssen WE, et al.: Granulocyte and platelet recovery after autologous peripheral blood stem cell transplant (PBSCT) depends on mobilizing regimen (MOBREG). Keystone Symposium, "Blood Cell and Bone Marrow Transplants," p 59, 1996.
16. Perkins JB, Elfenbein GJ, Janssen WE et al.: Lack of difference in rate of hematopoietic recovery after bone marrow and peripheral blood stem cell infusion versus peripheral blood stem cells alone. *Exp Hematol* 23:761, 1995.

17. Bender JG, Unverzagt KL, Walker DE et al.: Identification and comparison of CD34-positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry. *Blood* 77:2591–2596, 1991.
18. Lajtha LG: Bone marrow stem cell kinetics. *Semin Hematol* 4:293–303, 1967.
19. Janssen WE, Elfenbein GJ, Fields KK et al.: Comparison of cell collections and rates of post-transplant granulocyte recovery when G-CSF and GM-CSF are used as mobilizers of peripheral blood stem cells for autotransplantation. In: Dicke KA, Keating A (eds) Autologous Bone Marrow Transplantation, Proceedings of the 7th International Symposium. Arlington, TX, 1995, pp 527–539.
20. Down JD, Mauch PM: The effect of combining cyclophosphamide with total-body irradiation on donor bone marrow engraftment. *Transplantation* 51:1309–1311, 1991.
21. Huang S, Terstappen LW: Formation of haematopoietic microenvironment and haematopoietic stem cells from single human bone marrow stem cells. *Nature* 360:745–749, 1992.
22. Gunther W, Huss R, Schumm M, Kolb HJ: Generation of fibroblast like feederlayer and precursor cells from non mobilized canine peripheral blood. *Blood* 88 (Suppl. 1):1266, 1996.
23. Sackstein R, Janssen WE, Elfenbein GJ (eds) Bone Marrow Transplantation: Foundations for the 21st Century; Annals of the New York Academy of Sciences, Vol. 770. New York, The New York Academy of Sciences, 1995.

IMMUNOCYTOCHEMICAL ANALYSIS OF BONE MARROW TAKEN FROM PATIENTS WITH STAGE IV BREAST CANCER UNDERGOING AUTOLOGOUS TRANSPLANT THERAPY

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ABSTRACT

There is mounting evidence that marrow stem cell transplantation can successfully treat patients with solid malignancies that would otherwise recur with conventional chemotherapy. The marrow, although no longer used frequently as a source of stem cells, is a reservoir for minimal residual cancer in the patient. In an effort to determine if the presence of marrow disease is associated with a higher rate of tumor cell contamination in peripheral blood stem cell (PBSC) collections and decreased disease-free survival (DFS), we performed immunocytochemical (ICC) analysis on paired PBSC and marrow samples taken from 404 patients with stage IV breast cancer. The ICC method used monoclonal antibodies and avidin-biotin alkaline phosphatase staining. Mobilization regimen was available for 127 patients. For patients where the mobilization regimen was known, the incidence of PBSC contamination from patients with immunocytochemistry (ICC) positive marrow was compared with contamination of those with negative marrow. Clinical follow-up was available for 236 patients and Kaplan-Meier survival analysis was performed on all these patients. Patients with ICC positive marrow had the highest incidence of tumor contamination of PBSC products. The lowest incidence of PBSC contamination was found in the ICC negative marrow group mobilized with chemotherapy/cytokine. In addition, patients with ICC positive BM had the lowest DFS ($p<0.04$). We conclude that ICC testing of marrow is a useful test for autologous bone marrow transplant (autoBMT) candidates with stage IV breast cancer.

INTRODUCTION

For breast cancer alone, it is anticipated that over 180,000 individuals will develop this disease in the United States in 1996. Over 45,000 women will die from this disease every year.[1] For high-risk patients, autologous bone marrow transplant (autoBMT) therapy is being used with increasing frequency. However, despite this advancement in the care of these patients, relapse of disease is still the major cause of treatment failure. Consequently the most important questions facing oncologists are 1) when to use high dose therapy (autoBMT therapy), 2) how can we accurately and sensitively determine response to therapy and 3) how can we identify subgroups of patients who would benefit by post-autoBMT or alternative therapy?

We have been using a highly sensitive and specific immunocytochemistry (ICC) breast cancer micrometastatic detection assay since 1990. This assay uses antibreast monoclonal antibodies, alkaline phosphatase staining and can detect down to 1 breast cancer cell among 1,000,000 normal hematopoietic cells.[2,3] Since the marrow may be a measure of minimal residual cancer left in vivo prior to autoBMT therapy, we wished to test the value of this ICC assay. We evaluated paired marrow and peripheral blood stem cell (PBSC) specimens prior to autoBMT therapy in 404 patients with stage IV breast cancer. We then evaluated the association between marrow disease and PBSC contamination in relation to the mobilization regimen. For patients where the clinical follow-up was known, we determined if there was an association between marrow disease and disease-free survival (DFS).

MATERIALS AND METHODS

Patient population and samples tested

From July, 1990, to July, 1996, marrow specimens and PBSC specimens were drawn from 404 patients with stage IV breast carcinoma. The diagnosis of breast cancer and extent of disease was performed using standard histopathology and radiologic testing. All specimens were obtained after the initiation of induction chemotherapy which included a variety of regimens. The conditioning regimens used for autoBMT also was widely varied. Patients were transplanted regardless of response to therapy.

Preparation of marrow specimens and PBSC specimens

Approximately 3 mL of bone marrow was obtained from the posterior iliac crest. PBSC samples were obtained from central venous catheters and ranged from 1–5 mL. Samples from referring hospitals arrived within 24 hours and all specimens were processed within 48 hours of collection. Upon arrival in the laboratory, the marrow was diluted with Liebowitz L-15 medium (L-15) supplemented with 10% fetal bovine serum (FBS; Gibco; Grand Island, NY). Diluted samples were layered over Ficoll-Hypaque (Pharmacia; Uppsala, Sweden) and then

subjected to density gradient centrifugation. The mononuclear cell fraction was tested for viability (Trypan exclusion), cytocentrifuged onto slides and stored at 4°C for immunostaining the next day.

Immunostaining

Patient specimens were removed from 4°C storage and tested for immunoreactivity. Immunostaining was accomplished using the avidin biotin-alkaline phosphatase technique. Slides were fixed in a paraformaldehyde solution at 4°C for 30 minutes and washed thoroughly in phosphate-buffered saline (PBS; GIBCO). Prior to 3/95 immunostaining was performed using the Zymed immunoperoxidase kit. Following fixation, slides were incubated with anti-breast carcinoma antibodies for 45 minutes at room temperature. This was followed with incubation in normal goat serum, followed by Zymed reagents as per protocol. After 3/95 following fixation slides were placed on the Biotek automated immunostainer and incubated with anti-breast carcinoma antibodies for 30 minutes at 37°C. Biotek immunoreagents for alkaline phosphatase staining were performed as per protocol. The specimen was then incubated with Ehrlich's hematoxylin counterstain and cover slipped with cytoseal.

RESULTS

There were 92 patients with ICC positive marrow. For marrow positive patients, tumor contamination of PBSC was 24 of 92 (26%) versus 42 of 312 (13%) for marrow negative patients (p=0.004). The mobilization regimen was known for 127 patients. The PBSC contamination is shown in Table 1. The lowest incidence of PBSC contamination was found in the ICC marrow negative group mobilized with chemotherapy/cytokine. This was significantly better than those who were ICC marrow negative cytokine mobilized (p=0.008) and than those who were ICC marrow positive chemotherapy/cytokine mobilized (p=0.029).

Clinical follow-up was available for 236 patients. The overall DFS was 14% for all patients. This is consistent with most stage IV patient populations despite the heterogeneity of therapy. DFS was significantly reduced for patients with ICC positive marrow (p=0.04) when compared to patients who were ICC negative (Figure 1).

DISCUSSION

There is now clear evidence that autoBMT will result in long-term DFS for patients with high risk stage II, III disease and chemosensitive stage IV disease.[4,5] Little is known regarding why patients with stage IV disease fail autoBMT therapy. The successful outcome of BMT is multifactorial. The tumor burden should be low, the tumor cells must be chemosensitive, and the stem cell graft needs to be tumor-free.[6,7] Recent studies suggest that the clinical outcome of HDC/autoBMT protocols

Table 1. The relationship of ICC marrow disease with PBSC contamination in regards to mobilization regimen

Mobilization regimen	*BM⁻ Group*		*BM⁺ Group*	
	PBSC⁻	*PBSC⁺*	*PBSC⁻*	*PBSC⁺*
cytokine only	13	5 (28%)	17	3 (15%)
chemo/cytokine	68	3 (4%)	14	4 (22%)

may be influenced by the patient's response to intensive induction or re-induction chemotherapy.[5,8] Given that the majority of patients relapse at existing sites of disease, the ability of chemotherapy to clear a patient's disease may be a deciding factor in determining an appropriate HDC/autoBMT candidate.

Only about 15% of patients present with stage IV disease. Currently, the prognosis for this group of patients is poor. Only about 15–25% of patients with chemoresponsive disease will have long-term benefit from autoBMT therapy.[8,9] There is now growing evidence that patients with persistent disease may ultimately fail autoBMT or other high-dose chemotherapy regimens.[8,9] Marrow disease is of

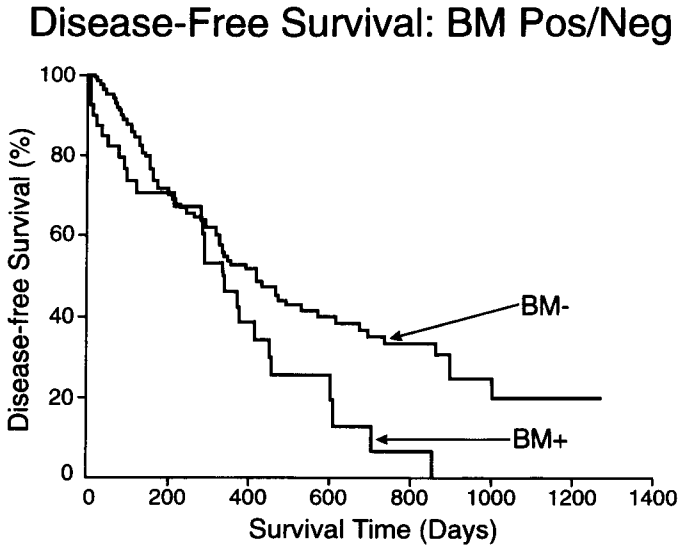

Figure 1. *Comparison of long-term DFS for stage IV breast cancer patients with ICC positive marrow versus those with ICC negative marrow. DFS percentage is on the y-axis and survival time in days in on the x-axis. The number of patients in each group is indicated at the top of the graph.*

particular importance in this group of patients. Approximately 70% of patients with untreated metastatic breast cancer will have marrow disease.[10] This high incidence means that monitoring the marrow is ideal for determining the chemoresponsiveness of the induction therapy and who may be clear of occult disease.

This study presented here adds further evidence that our minimal residual cancer ICC assay of marrow helps to distinguish subgroups of patients. The monitoring of marrow with this assay helps to determine subgroups with: 1) The highest incidence of PBSC contamination; 2) the lowest incidence of PBSC contamination and 3) those with the lowest DFS. With this information, the autoBMT physician can identify high risk subgroups on which to base clinical decisions. Therapeutic options can include: 1) Giving additional induction therapy to ICC marrow positive patients to clear disease before stem cell harvesting; 2) adding post-autoBMT therapy or 3) choosing alternate therapy. Future studies will focus on trying to stratefy these patients into different treatment strategies and to see if DFS can be further increased.

REFERENCES

1. Abeloff M: ABMT in perspective: "Conventional" therapy for metastatic breast Cancer. In: Proceedings of the DATTA Forum on High Dose Chemotherapy and Autologous Hematopoietic Support as a Treatment of Breast Cancer. 1993, pp 3–5.
2. Ross AA, Cooper BW, Lazarus HM et al.: Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques. *Blood* 82:2605–2610, 1993.
3. Moss TJ, Ross A.: The risk of tumor contamination in peripheral stem cell harvests. *J Hematother* 3:225–232, 1992.
4. Peters WP, Ross M, Vredenburgh JJ et al.: High-dose chemotherapy and autologous bone marrow support as consolidation after standard-dose adjuvant therapy for high-risk primary breast cancer. *J Clin Oncol* 11:1132–1143, 1993.
5. Williams et al.: High-dose consolidation therapy with autologous stem-cell rescue in stage IV breast cancer: Follow-up report. *J Clin Oncol* 10:1743, 1992.
6. Brenner MK, Rill DR, Moen RC et al.: Gene marking and autologous bone marrow transplantation. *Ann NY Aca Sci* 716:204–14, 1994.
7. Brenner MK, Rill DR: Gene marking and autologous bone marrow transplantation. *J Hematother* 3:33–6, 1994.
8. Ayash L, Elias A, Wheeler C et al.: Double dose-intensive chemotherapy with autologous marrow and peripheral blood progenitor support for metastatic breast cancer: A feasibility study. *J Clin Oncol* 11:37, 1994.
9. Antman K, Ayash L, Elias A et al.: A phase II study of high-dose cyclophosphamide, thiotepa, and carboplatin with autologous bone marrow support in women with measurable advanced breast cancer responding to standard-dose therapy, *J Clin Oncol* 10:102–110, 1992.
10. Moss TJ: Detection of occult tumor cells in autografts *Immunometh* 5:226–231, 1994.

COMPARISON OF CULTURE VERSUS MOLECULAR DETECTION OF LYMPHOMA IN THE CONTEXT OF A RANDOMIZED PROSPECTIVE TRIAL OF BLOOD VERSUS MARROW FOR RECONSTITUTION AFTER HIGH DOSE THERAPY: AN INTERIM ANALYSIS

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INTRODUCTION

High-dose therapy (HDT) requiring transplantation of a source of hematopoietic stem cells to restore hematopoiesis has become a standard salvage therapy for patients with lymphoma who have failed conventional therapies.[1] Originally, bone marrow (BM) was employed as the source of reconstituting stem cells. Unfortunately, up to 40% of patients eligible for HDT and transplantation had evidence of tumor in their histologically normal BM when this was evaluated using a sensitive culture technique.[2] Retrospective analysis demonstrated that patients with minimal tumor contamination of their transplanted marrow harvest had a poor outcome compared with patients whose harvest had no evidence of tumor.[2,3] This situation, as well as difficulties in obtaining adequate harvests from patients with prior radiation therapy to the pelvis, led investigators at the University of Nebraska Medical Center to evaluate stem cells harvested from blood under steady state conditions (no mobilization), by leukapheresis as an alternative reconstituting source to BM.[4] Evaluation of blood stem cell harvests for tumor contamination employing the same techniques as applied to BM harvests showed that blood stem cell harvests were less frequently contaminated or contaminated with fewer tumor cells than BM harvests.[5] This has since been confirmed by others in a variety of diseases.[6]

Consequently, a retrospective analysis was performed of the outcome of HDT for the treatment of lymphoma with blood stem cells versus BM as the reconstituting stem cell source. The results demonstrated a statistically significant advantage in disease-free survival for patients receiving blood stem cell

transplantation.[7] Although this was potentially due to lower tumor cell contamination of the infused harvests it could not be proven, and other explanations—for example, better immunological reconstitution of blood stem cell recipients—could account for these observations. Also, as this was a retrospective analysis, bias due to inadvertent patient selection cannot be formally excluded as an explanation of the differences.

Consequently, a multicenter prospective randomized study of blood stem cells (PSCT) versus BM (autoBMT) was developed that included molecular and cellular techniques of tumor detection as well as analysis of immunological recovery of the transplanted patients in an attempt to resolve the question regarding whether blood or BM is the best reconstituting source of stem cells. Because of advances in technique, the methods applied differed from the historical studies. Originally, blood stem cells were harvested in steady-state without mobilization. More recently, chemotherapy and/or cytokine mobilized blood stem cell harvests have been shown to provide rapid hematopoietic reconstitution and fewer leukaphereses are needed to obtain an adequate harvest.[4] However, there is evidence that the techniques that mobilize blood stem cells may also mobilize tumor cells,[8] and this is the case in lymphoma.[9] The prospective randomized study employs a cytokine to mobilize blood stem cells. Cultures established from cytokine-mobilized blood stem cell harvests generate many more cells, especially myeloid cells, than nonmobilized harvests. Originally, molecular confirmation of the presence of tumor in cultured harvest employed Southern blot analysis, which was required to match the original tumor.[2] The prospective randomized study relies primarily on the more sensitive technique of PCR to detect *bcl-2* rearrangements or the CDRIII of the immunoglobin genes or the T cell receptor.[10]

At this time, a complete analysis of the available data including the outcome of BM versus blood stem cells as the reconstituting source has not yet been attempted. A comparison has been made between the outcome of culture versus molecular detection of lymphoma in the infused harvest, and potentially interesting differences have been noted that are described in this report.

MATERIALS AND METHODS

Patients

Seventy patients (35 males and 35 females) have been entered in the trial since the initiation in October, 1993. The age range of patients is 18–68 years with a median of 46 years. Thirty-four patients have been randomized to PSCT and 36 to autologous (auto)BMT. The median number of prior chemotherapies is two in each arm with 96% of the patients in the autoBMT arm having chemotherapy sensitive disease and 88% in the PSCT arm.

Culture studies

Samples of peripheral blood and BM were received before harvest, followed by either a sample from the BM harvest or apheresis harvest depending on treatment randomization. Samples were diluted with an equal volume of Hanks Balanced Salt Solution (HBSS), then layered over a cell separation medium (Accu-Prep Lymphocyte, Accurate Chemical and Scientific Corporation, Westbury, NY) and centrifuged at 400*g* for 20 minutes. The cells collected from the interface were washed once by centrifugation (400*g* for 7 minutes), then resuspended in Tris-buffered ammonium chloride (pH 7.2) for five minutes to lyse mature red cells. An equal volume of complete medium (RPMI 1640 medium supplemented with 20% FCS, 1% penicillin/streptomycin, 1% L-glutamine) was added to each tube and the suspensions were washed again. Cultures were established in 25 cm^2 T-flasks using a modification of a technique described by Philip et al.[11] using 1% phytohemagglutinin (PHA; Murex Diagnostics, Inc., Norcross, Georgia) stimulated human spleen cell conditioned medium. Flasks containing 2.0×10^7 cells in 10 mL of medium were cultured at 37°C in 5% CO_2 in air. At the weekly change of half the medium, the supernatant cellularity of each culture was determined using a hemacytometer. Each culture flask was photographed weekly using a Nikon inverted phase contrast microscope. A cytospin of the nonadherent cells was made for morphological analysis and the harvested cells were then centrifuged at 400*g* for five minutes, the supernatant was removed and the pellet frozen for future DNA analysis. These parameters were employed to determine if there was any evidence of lymphoma in the infused harvest. The results were reported to the statistical core for analysis.

Molecular studies

DNA extraction of tissue sections from paraffin-embedded material was performed as described by Wright and Manos,[12] while DNA extraction from fresh cell preparations was performed by the cell lysate method described by Gribben and coworkers.[13] A semi-nested PCR procedure was used to amplify IgH CDRIII using consensus primers as described by Wan et al.[14] The PCR reactions were performed in an OmniGene Thermocycler (Hybaid Ltd., England) with the following cycling parameters: annealing for 40 seconds at 55°C, extension for 40 seconds at 72°C and denaturation for 40 seconds at 95°C. Both the initial and the nested rounds of amplification were performed for 30 cycles. Detection of *bcl*-2 translocation involving the major breakpoint region was performed using primers and reagents described by Gribben et al.[13] The cycling conditions were similar to those used for amplifying IgH CDRIII. Sequencing of the tumor-specific IgH CDRIII was performed directly on the PCR product without prior cloning. If there was substantial contamination by IgH CDRIII products from normal B cells present in the tumor, pure clonal products could be obtained by either denaturing gradient gel electrophoresis of the PCR product or micromanipulation of the tumor sections before the PCR, as described previously.[15]

Table 1. Comparison of culture and molecular positive samples in current study (observed) versus historical data (expected) [Marrow and apheresis data combined]

	Observed[a,b]	*Expected*[c]
culture positive	8/55 (14%)	18–27%
molecular positive	13/27 (48%)	18–27%

[a]*Molecular detection done by PCR versus Southern originally.*
[b]*Cytokine used to mobilize stem cells into apheresis harvest versus nonmobilized originally.*
[c]*Original studies published in references 2 and 3.*

The clonal IgH CDRIII product was recovered after gel purification and sequenced using the Sangers chain termination method with a cycle sequencing kit containing fluorescent dideoxynucleotide terminators according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The sequencing gel was read using the Applied Biosystems 373A Sequenator (Applied Biosystems, Foster City, CA). The V, D and J regions in the CDRIII product were determined with the help of the GCG program and the sequence database (SAW) kindly supplied by Dr. Schroeder, University of Alabama. The tumor-specific oligonucleotide primers and probes were then designed with the help of Olig 4.1 Software (National Biosciences, Plymouth, MN). In general, only one primer could be obtained due to the short length of the CDRIII. In the detection of minimal residual disease, DNA was amplified using first round consensus primers to the CDRIII region as described above. Two microliters of a 1:400 dilution of the PCR product from the first round of amplification was re-amplified using either the consensus V- or J-nested primer coupled with the tumor-specific primer for that particular case. After the nested or semi-nested amplification, the PCR product was electrophoresed in agarose gel and Southern transferred to a nylon membrane. The membrane was then hybridized with the ^{32}P-labeled tumor-specific probe at a temperature 10°C below the Tm calculated according to the formula Tm=4(G+C) +2(A+T) and washed at the appropriate stringency before autoradiography.[17] The results were reported to the statistical core for analysis.

Data collection and analysis

Data were collected on paper forms by the participating institutions and provided to the Statistical and Data Center where it was verified by the study coordinator and then entered into the study database. All data management and analysis was performed using the SAS system. Comparisons of survival were made using the Kaplan-Meier method and comparisons of survival distributions were made using the logrank test.

RESULTS

The outcome of culture versus molecular detection of lymphoma in the harvest in the current study versus the historical studies is presented in Table 1.

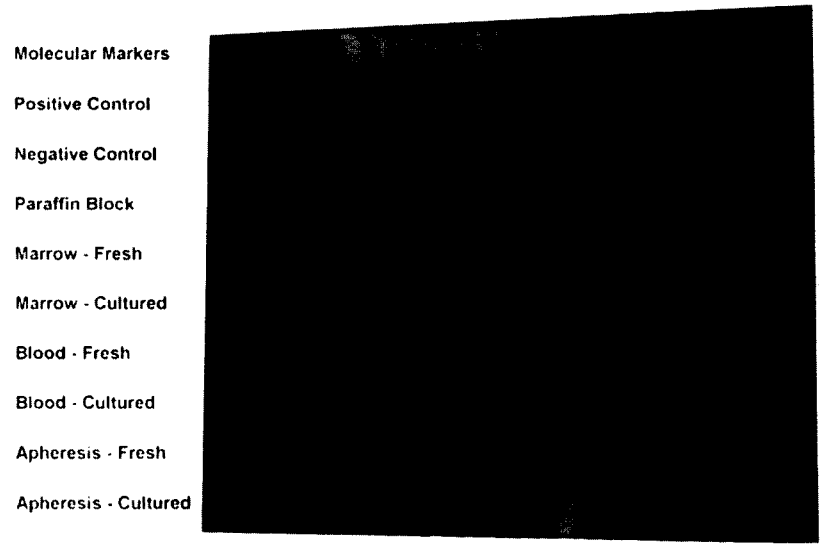

Figure 1. *Molecular analysis for a rearranged bcl2 gene in the original paraffin block of tumor, fresh and cultured marrow aspirates (all positive), fresh and cultured blood sample (negative) and cytokine mobilized fresh and cultured leukapheresis harvest (positive) of a patient with intermediate grade non-Hodgkin's lymphoma.*

The data for the historical studies[2,3] were computed based on the relative proportions of patient BM versus leukapheresis harvests in the current study and combining the range of values from historical studies. It is evident that the proportion of culture-positive harvests in the current study is lower than in the historical studies (18–27%). Potentially, this reflects the likelihood that patients are being offered HDT requiring transplantation at an earlier stage of disease progression when overall burden of disease is lower and when there is less exposure to therapies that have the potential to select resistant clones. At the same time, the proportion of molecularly positive harvests (48%) was higher than in historical studies (18–27%) and it was evident that there was a major discrepancy between the proportion of molecularly positive harvests and culture-positive harvests, which had only been observed infrequently in previous studies. Potentially, the proportion of molecularly positive harvests is higher because of the increased sensitivity of the molecular methods employed in the current study. However, an additional, significant component likely arises because the use of a cytokine to mobilize blood stem cells for collection also mobilizes tumor cells (Figure 1) and this almost certainly increases the frequency of molecularly positive leukapheresis harvests. Note that this occurs both in patients whose BM is positive and in those whose BM is negative. In the latter instance, this observation suggests

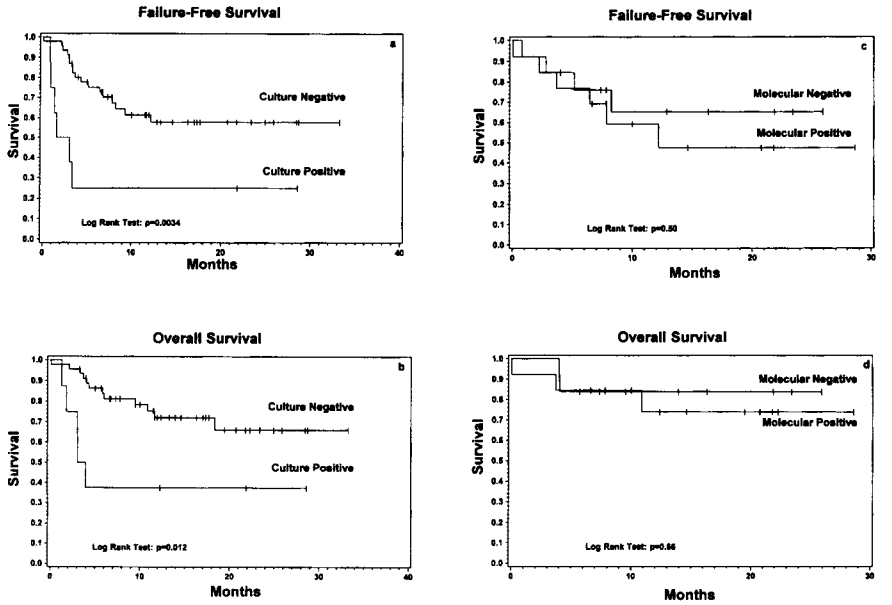

Figure 2. *Failure-free and overall survival curves for patients with intermediate grade non-Hodgkin's lymphoma undergoing HDT and transplantation with a harvest retrospectively identified to be positive or negative for lymphoma by either culture or molecular techniques.*

that the tumor cells are mobilized from sites other than BM (see patient U11 in Table 3), although this could also arise because of sampling variance in the BM aspirates and/or core biopsies. Clearer evidence of the lack of growth in culture of molecularly positive harvests is presented in Table 2, and shows a matched-pairs analysis of culture versus molecular detection of tumor for a number of patients.

It may be noted that 10 molecularly positive harvests did not grow in culture. Also, five culture-positive harvests were not, initially, molecularly positive in studies on the fresh sample. In at least three cases, the cultured cells were molecularly positive, suggesting that in some instances, the combination of culture plus molecular detection is more sensitive than either technique alone and that the combination is likely detecting exceedingly infrequent tumor cells at a frequency estimated to be less than one tumor cell in 10^5 normal cells. The significance of such infrequent tumor cells is hard to assess. One other possibility, noted previously,[2] is the situation in which the culture contains expanding virally infected lymphocytes. The conclusion from the foregoing analysis of the data is that not all harvests molecularly positive for lymphoma cells contain tumor cells that are capable of growth in culture.

Table 2. Relationship between culture positive and molecular positive results for samples with complete data sets

	molecular positive (n=13)	*molecular negative (n=14)*
culture positive	3	5
culture negative	10	9

An important question is whether minimal lymphoma detected by either culture techniques or molecular techniques or both, in histologically negative harvests is clinically significant. This issue is addressed by the failure-free and overall survival curves presented in Figure 2. The failure-free and overall survival of patients, who in retrospect, unknowingly received a culture-positive harvest, was significantly poorer than that of recipients of a culture-negative harvest (Figures 2A and B). In contrast, the failure-free survival of recipients of molecularly positive harvests,

Table 3. Patterns of positivity for lymphoma, by tissue, with and without culture

			Weeks of culture					
Pt.	*Sample*	*Fresh*	*1*	*2*	*3*	*4*	*5*	*6*
	blood	+	+	–	–	–	ND	ND
H3	marrow	+	+	–	–	–	ND	ND
	apheresis	+	–	–	–	–	ND	ND
	blood	+	+	+	–	+	+	+
U7	marrow	+	+	+	+	+	–	+
	apheresis	+	+	+	+	+	+	+
	blood	–	–	–	+	–	ND	ND
U11	marrow	–	–	–	–	–	ND	ND
	apheresis	–	–	+	+	–	ND	ND
	blood	+	+	–	–	–	ND	ND
U13	marrow	+	+	–	–	–	ND	ND
	apheresis	–	+	–	–	–	ND	ND
	blood	–	–	–	–	–	ND	ND
U14	marrow	+	–	–	–	–	ND	ND
	apheresis	+	+	–	–	–	ND	ND
	blood	+	+	+	+	+	–	ND
U17	marrow	+	+	+	+	+	+	ND
	apheresis	+	+	+	+	+	–	ND

ND=no data.

although numerically poorer than that of recipients of molecularly negative harvests, was not significantly different (Figure 2C) and no differences at all were noted in overall survival (Figure 2D). When the patients were combined on the basis of either a culture-positive or molecularly positive harvest their survival was statistically significantly worse than that of recipients of tumor-negative harvests (data not shown). Therefore, evidence of minimal lymphoma contamination detected by outgrowth of lymphoma cells in culture is a very significant predictor of a clinically poor outcome. Detection of lymphoma contamination by molecular techniques is less clinically predictive, implying that while some molecularly detected lymphoma cells may be clinically important, not all molecularly detected lymphoma cells are clinically significant.

Some potential reasons why molecularly detected lymphoma cells may not be clinically important is evident from Table 3, which tracks the weekly outcome of cultures established from different sources (blood, BM, leukapheresis harvest) from a sample of patients whose culture-negative fresh tissue sample was molecularly positive or negative. In general, the patterns of growth are much more reflective of the patient than they are of the source of the cells. This implies that the characteristics of the patient's tumor are more important to outcome than the source of the tumor. Based on hints that growth of lymphoma cells in culture might, in part, be related to the status of their *p53* gene expression,[18] in a very preliminary study, the p53 mRNA status of lymphoma cells growing out in culture from patient harvests has been examined. There is evidence that such lymphoma cells had decreased or absent p53 mRNA expression, perhaps related to *p53* mutations, but this needs to be confirmed.

Potentially, therefore, the culture technique is a surrogate predictor of additional molecular abnormalities of lymphoma cells. These additional abnormalities, such as decreased p53 mRNA levels, confer an aggressive phenotype on the tumor including growth in culture and, potentially, resistance to therapy and a poor outcome.

DISCUSSION

These studies have established that not all molecularly detected lymphoma cells in hematopoietic harvests have the ability to grow in culture. Furthermore, lymphoma outgrowth in culture is a very significant predictor of a poor clinical outcome for patients inadvertently reinfused with harvests containing such cells. In contrast, the presence of molecularly detected lymphoma cells in reinfused harvests is not a significant predictor of clinical outcome. There was a hint from preliminary studies that the outgrowth of lymphoma cells in culture may be a surrogate, predicting the existence of additional molecular abnormalities in some lymphoma cells. These additional molecular abnormalities confer not only the ability of the tumor cells to grow in culture but also an aggressive phenotype and,

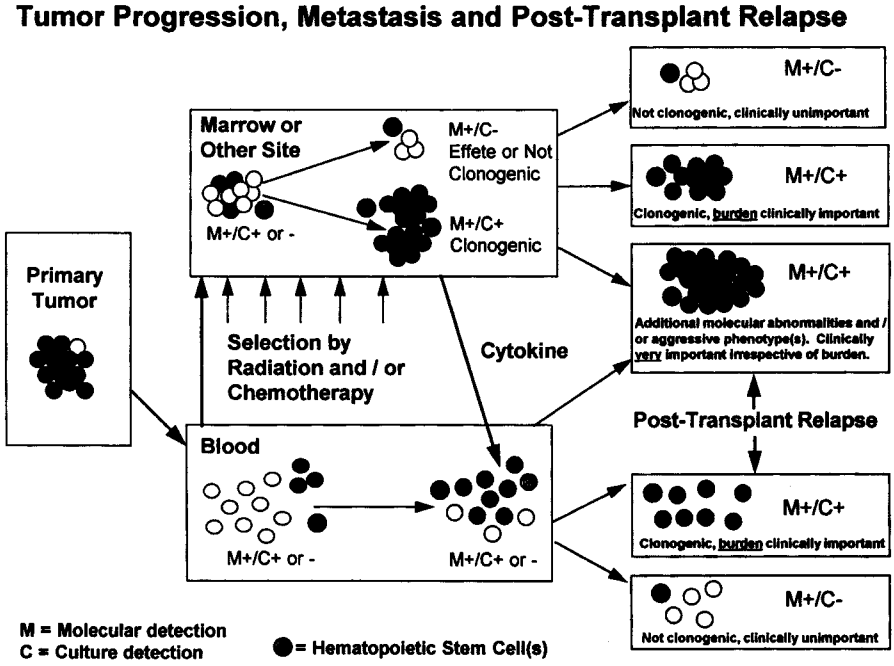

Figure 3. *Illustration of a conceptual model of tumor progression metastasis and post-transplant relapse that would explain current observations of the relationship of the properties of minimal lymphoma in hematopoietic tissues to the clinical outcome of HDT and transplantation.*

potentially, resistance to therapy. These preliminary observations obviously require an additional evaluation. Incidentally, these studies demonstrate also that lymphoma can be mobilized by cytokines into leukapheresis harvests as noted previously[9] and, potentially, sites other than BM may be a source of such cells. A possible conclusion from these observations is that for intermediate grade lymphomas, molecular detection of minimal tumor cells in harvests of marrow or leukapheresis may not be completely reliable in predicting clinical outcome. Currently, this has not been analyzed separately for blood stem cell versus BM harvests as described by Gribben et al.[16] for low grade lymphomas. Perhaps combining molecular detection of lymphoma using a clonal marker (CDRIII and/or *bcl-2* rearrangements) with an assessment of mRNA status of pro-apoptotic (e.g., *p53)* or anti-apoptotic (e.g., *bcl-2*) genes might provide equivalent information to culture detection of lymphoma. This would be desirable because the culture technique is time consuming, technically complex and expensive and not readily adaptable for use at multiple centers.

An attempt has been made to fit these observations into a conceptual model (Figure 3) in order to plan future studies to clarify these issues. There is heterogeneity in the biological behavior of lymphoma cells, such as growth in culture and clinical aggressiveness, not reflected by clonal molecular assays which detect CDRIII sequences or *bcl-2* rearrangements. It is likely that this heterogeneity is in part a consequence of selection of tumor cells during the natural evolution of the tumor and potentially is also promoted by therapeutic agents. As noted in Figure 3, metastatic lymphoma that has established itself in BM has acquired characteristics of survival (at least) and possibly growth at an inappropriate site. In contrast, lymphoma in blood, while it could include such cells, is also more likely to contain a higher proportion of effete or nonclonogenic cells. This might account for the poorer clinical predictive ability of the detection of lymphoma in blood than BM in low grade lymphomas.[16] Also, it may predict that nonmobilized blood is a less likely source of established metastatic cells. However, if a cytokine is used for mobilization, a greater proportion of the cells in blood will be established metastatic lymphoma cells mobilized by the cytokine. A greater number of reinfused tumor cells in the harvest may be associated with a greater risk of relapse, but it might take longer for such cells to expand to the extent of detectable relapse. Therefore, mobilized blood stem cell harvests may contain more potentially aggressive lymphoma cells than nonmobilized blood stem cell harvests but, on the other hand, may still be less likely to re-establish clinically significant metastatic disease than BM. These possibilities are likely to be significantly modified by the selection of lymphoma cells during treatment that, no matter what their source, will dominate outcome. The presence of such aggressive cells may presage a high likelihood of rapid relapse, which could arise either from reinfused tumor cells or tumor cells surviving HDT of the patient.

It is important in the future to test these concepts because they may aid in the selection of the most cost-effective therapy. If the contaminating lymphoma cells in a harvest are effete and/or nonclonogenic, harvest manipulation may not be necessary. Removal or reduction of the burden of potentially clonogenic, but not necessarily highly aggressive, lymphoma cells from the harvest might at least delay relapse or otherwise reduce the proportion of late relapses.

In contrast, this study suggests that harvests containing lymphoma cells that grow in culture or otherwise have an aggressive molecular phenotype, no matter what their source, might still be associated with a poor outcome, even if the harvest is manipulated to reduce tumor cell content. Potentially, such patients might be candidates for alternative therapies. These could include immunotherapy, which might avoid the immunosuppression associated with HDT, thus denying the tumor cells this advantage as well potentially bypassing the likely chemoresistance of such aggressive lymphomas.

ACKNOWLEDGMENTS

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REFERENCES

1. Armitage JO: Bone marrow transplantation. *New Engl J Med* 330:726–838, 1994.
2. Sharp JG, Joshi SS, Armitage JO et al.: Significance of detection of occult non-Hodgkin's lymphoma in histologically uninvolved bone marrow by a culture technique. *Blood* 79:1074–1080, 1992.
3. Sharp JG, Kessinger A, Mann S et al.: Outcome of high-dose therapy and autologous transplantation in non-Hodgkin's lymphoma based on the presence of tumor in the marrow or infused hematopoietic harvest. *J Clin Oncol* 14:214–219, 1996.
4. Kessinger A, Armitage J, Bierman P et al.: Clinical outcome of peripheral blood stem cell support. *Med Oncol* 11:43–46, 1994.
5. Sharp JG, Kessinger A: Minimal residual disease and blood stem cells. In: Gale RP, Juttner CA, Henon P (eds) Blood Stem Cell Transplants. Cambridge: Cambridge University Press, 1994, pp 75–86.
6. Sharp JG, Bishop M, Chan WC et al.: Detection of minimal residual disease in hematopoietic tissues. Annals NY Acad Sci 770:242–261, 1995.
7. Vose JM, Anderson JR, Kessinger A et al.: High-dose chemotherapy and autologous hematopoietic stem-cell transplantation for aggressive non-Hodgkin's lymphomas. *J Clin Oncol* 11:1846–1851, 1993.
8. Brugger W, Bross KJ, Glatt M et al.: Mobilization of tumor and hematopoietic progenitor cells into peripheral blood of patients with solid tumors. *Blood* 83:636–640, 1994.
9. Wu G, Wu GQ, Chan J et al.: Detection of mobilization of tumor cells into the circulation by cytokines is complicated by the dynamics of mobilized cell populations. *J Hematother* 4:214, 1995.
10. Chan WC, Wu G, Greiner T et al.: Detection of tumor contamination in peripheral stem cells in patients with lymphoma using cell culture and PCR technology. *J Hematother* 3:175–184, 1994.
11. Philip I, Philip T, Favrot M et al.: Establishment of lymphomatous cell lines from bone marrow samples from patients with Burkitt's lymphoma. *J Natl Cancer Inst* 73:835–840, 1988.
12. Wright DK, Manos MM.: Sample preparation from paraffin-embedded tissues. In: Innis MA, Gelfand DH, Sninsky JJ (eds) PCR Protocols. A Guide to Methods and Amplifications. London: Academic Press, 1990, pp 153–158.
13. Gribben JG, Freedman AS, Neuberg D et al.: Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B-cell lymphoma. *New Engl J Med* 325:1525–1533, 1991.
14. Wan JH, Sykes PJ, Orell SR et al.: Rapid method for detecting monoclonality of B

cell lymphoma in lymph node aspirates using the polymerase chain reaction. *J Clin Pathol* 45:420–423, 1992.

15. Wu G, Greiner TC, Chan WC: Obtaining clone-specific primer and probe for the immunoglobulin heavy chain gene from paraffin-embedded tissue of B-cell lymphoma: Technical considerations. *Diag Molec Pathol* (Submitted).
16. Gribben JG, Neuberg D, Barber M et al.: Detection of residual lymphoma cells by polymerase chain reaction peripheral blood is significantly less predictive for relapse than detection in bone marrow. *Blood* 83:3800–3807, 1994.
17. Wickert RS, Weisenburger DD, Tierens A et al.: Clonal relationship between lymphocytic predominance Hodgkin's disease and concurrent or subsequent large cell lymphoma of B-lineage. *Blood* 86:2312–2320, 1995.
18. Sharp JG, Mann SL, Murphy B et al.: Culture methods for the detection of minimal tumor contamination of hematopoietic harvests: A review. *J Hematother* 141–148, 1995.

THE DETECTION AND SIGNIFICANCE OF TUMOR CELL CONTAMINATION OF THE BONE MARROW

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INTRODUCTION

Micrometastatic involvement of the bone marrow (BM) is common in breast cancer. At the time of initial diagnosis, 4–48% of patients with Stage I or Stage II breast cancer have tumor cells in their BM as detected by immunologic techniques.[1–6] A number of studies have demonstrated that micrometastatic disease in the BM is a prognostic factor for patients with newly diagnosed breast cancer, and is predictive of early relapse, as well as the site of relapse.[2,3] Patients with lymphoma and neuroblastoma receiving high-dose chemotherapy (HDCT) with hematopoietic support may have a higher relapse rate if their BM or peripheral blood progenitor cells (PBSCs) are contaminated with tumor.[7,8] It is unknown if tumor cells in the cellular support contribute to relapse in breast cancer. However, the detection of malignant cells in the hematopoietic support and the application of techniques to eliminate the contaminating cancer cells may improve the number of long-term, disease-free survivors following HDCT.

A number of different techniques and monoclonal antibodies have been used to detect micrometastatic disease in the BM or tumor cell contamination of the PBSCs.[1–6,9] There have been significant variations in the rate of detection of micrometastatic disease, as well as the clinical implications of the presence of malignant cells, depending on the technique and the antibodies employed.

COMPARISON OF THREE TECHNIQUES TO DETECT TUMOR CELLS IN THE BM

We use a model system with spiked BM samples to compare three techniques to detect micrometastatic disease in the BM, immunohistochemistry, two-color immunofluorescence and fluorescence activated cell sorting (FACS) with histologic examination of the sorted cells.

MATERIALS AND METHODS

We serially diluted breast cancer cells from the CAMA-1 or SKBR3 cell lines into normal BM mononuclear cells at final tumor to marrow cells ratios of 1:10,

1:100, 1:1000, 1:10,000, 1:100,000 and 1:1,000,000. Different samples of each dilution were tested with each method to detect tumor cells. The samples were distributed to the different investigators in a blinded fashion.

FACS with cytology

Triplicate samples of 2×10^6 cells were incubated for 30 minutes at 4°C with 50 μL of a solution containing saturating concentrations (20 μg/mL) of FITC conjugated monoclonal antibodies as previously described,[14] plus a phycoerythrin conjugated anti CD45 monoclonal antibody (Coulter). Samples were washed twice in PBS containing 1% fetal calf serum and 0.02% sodium azide. The samples were analyzed with a Becton Dickinson Fluorescence-activated cell sorter (FACStar^{Plus}). Emission of fluorescence at 530 nm (FITC) and 585 nm (PE) was analyzed after pregating on the basis of forward and right-angle light scattering to eliminate residual red blood cells, nonviable cells, and cellular debris. Two parameter histograms of FITC versus PE were acquired. A sample was considered positive by flow cytometry if it was more than two standard deviations above the negative control. Cells that were FITC positive were sorted into 2 groups, one that was CD45 positive and one that was CD45 negative. Each group was then processed for cytologic examination.

Cytologic analysis following fluorescence-activated cell sorting

Cytospins were made by spinning the samples at 1000 rpm for three minutes. Slides were fixed and stored in 95% ethanol. The slides were stained using a modified Papanicolaou method and the slides were examined under high-power light microscopy by a cytopathologist. The cytospins were scored as positive or negative, and the number of tumor cells were quantitated.

Immunohistochemical method

Cytospin slides were prepared with 5×10^5 BM/breast cancer cells per slide by spinning samples at 1000 rpm for 3 minutes. A minimum of 10 slides, 5×10^6 cells, were examined. Slides were air-dried overnight, fixed for 10 minutes in cold acetone, air dried for 2 hours, then stained using a modification of the alkaline phosphatase-antialkaline phosphatase (APAAP) technique. Slides were washed three times in TBS (pH 7.6), blocked with 5% normal goat serum, then incubated for one hour with antitumor cell antibodies followed by rabbit anti-mouse IgG for 30 minutes, and a complex composed of alkaline phosphatase and murine monoclonal anti-alkaline phosphatase for 30 minutes, with three washes of TBS between incubations. A red color was developed for 15–30 minutes using a new fuchsin substrate containing levamisole to suppress endogenous alkaline phosphatase activity. The cells were lightly counterstained with methylene green. Stained slides were examined using a standard binocular light microscope with

Table 1. Reactivity with normal BM

Method	*Normal bone marrow*[1]	*0.1% breast cancer mix*[1]
two-color immunofluorescence	28/28	4/4
immunohistochemical	27/28	4/4
FACS with cytology	28/28	4/4

[1]*Number correctly identified/total.*

10× and 40× objectives. Both immunohistochemical staining and morphology were used to differentiate tumor cells and normal BM cells.

Two-color immunofluorescence method

The normal BM/breast cancer cell mixtures with 1×10^6 total cells were incubated with the 4 FITC-conjugated monoclonal antibodies for 45 minutes at 4°C in the dark. In addition, an anti-CD45 phycoerythrin directly conjugated monoclonal antibody (Coulter) was included. FITC (green) excites at a wavelength of 495 nm and emits at 525 nm. Phycoerythrin (PE) (red) excites at 488 nm and emits at 578 nm. The mixtures were washed three times with phosphate-buffered saline with 0.1% bovine serum albumin and 0.001% sodium azide (PBS/BSA/AZ). The cells were fixed with the same PBS/BSA/AZ solution and a 1% paraformaldehyde solution, and cytospin slides were prepared, with 2×10^5 cells on each cytospin slide. A minimum of 10 slides, 2×10^6 cells, were examined. These cells were centrifuged onto the slide at 750 rpm for five minutes. Mounting media with 50% PBS and 50% glycerol with 0.1% phenylenediamine was added and the slides were coverslipped. These slides were examined using a fluorescent binocular microscope with bright field capacity with the 25× and the 40× objectives. Both immunofluorescent staining and cellular morphology under bright field were used to differentiate tumor cells and normal BM cells.

RESULTS

Specificity for distinguishing normal from contaminated marrow as demonstrated in Table 1, the 3 immunodetection techniques were essentially identical with excellent specificity.

Sensitivity for detecting tumor cells at different ratios in three separate experiments were carried out in which serial dilution of breast cancer cells were mixed with normal BM cells. As demonstrated in Table 2, the two color immunofluorescence technique was the only technique that consistently detected the contaminating breast cancer cells even at a concentration of one tumor cell in 10^6 normal BM cells.

Table 2. Tumor cell detection

Method	*Tumor cell dilution*[1]			
	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$
two-color immunofluorescence	3/3	3/3	3/3	3/3
immunohistochemical	3/3	3/3	3/3	1/3
FACS with cytology	3/3	2/3	2/3	2/3

[1]*Number correctly identified/total.*

DETECTION OF BREAST CANCER CELLS IN THE BM FROM STAGE IV BREAST CANCER PATIENTS

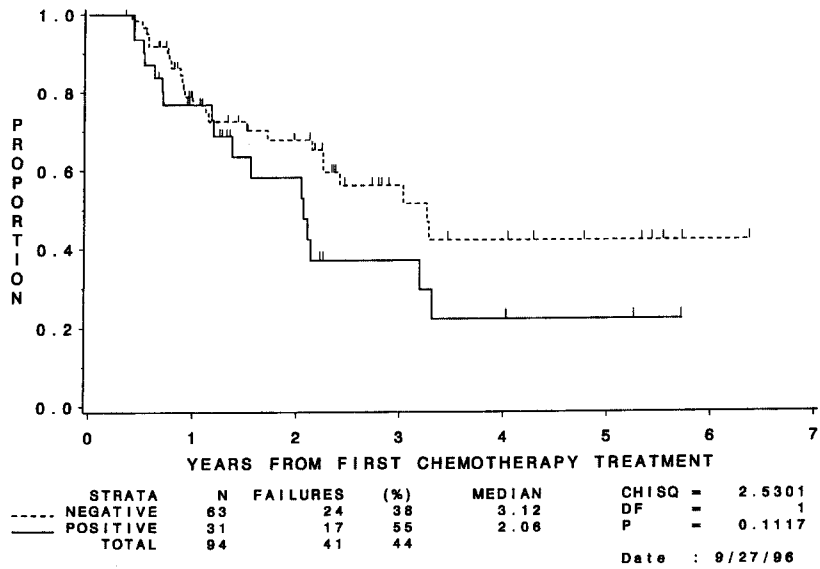
Using our FACS assay with the cytologic examination of the sorted cells, we evaluated the BM from Stage IV breast cancer patients treated on two different protocols. In the first protocol, the patients received no induction chemotherapy and were only treated with one cycle of high dose cyclophosphamide, cisplatin and BCNU with autologous BM support. In the second protocol, the patients received AFM induction chemotherapy for 3–4 cycles followed by the high dose chemotherapy with autologous BM support. As demonstrated in Table 3, the patients treated on the upfront trial had significantly higher incidence of tumor cells in the BM. This was true both for the FAC assay as well as the histologic examination of the sorted cells.

In addition, we have used our two-color immunofluorescence assay to look at the BM from the patients treated on our purge protocol. The patients all had positive BM biopsies, a pelvic bone metastases or more than 3 bone metastases. Before induction chemotherapy, over 80% of the patients treated on the purge protocol had contaminating tumor cells in the BM as detected by the two color immunofluorescence technique. Following induction chemotherapy, one-third of the patients still had tumor cells contaminating the BM. Only 3 of the 31 patients who were positive prior to the immunomagnetic purging remained positive following the purging procedure, and those 3 patients had a marked reduction in the

Table 3. Number of breast cancer patients with antigen positive cells in the BM

Patient group	*Flow cytometry no. (% positive)*	*Cytology of sorted cells no. (% positive)*
Upfront trial	7/15 (47%)	8/15 (53%)
	$p=0.04$[1]	$p<0.0001$[1]
AFM trial	7/49 (14%)	1/45 (2%)
Total	14/64 (22%)	9/60 (15%)

[1]*Comparison of patients treated on the Upfront trial versus the AFM trial.*



number of contaminating tumor cells. The Kaplan-Meier curve above demonstrated that the patients who had no tumor cells contaminating the harvested BM prepurge (negative) have a trend toward a better overall survival compared to the patients who had tumor cells contaminating the BM prepurge (positive), although the differences are not statistically significant (p=0.11).

TUMOR CELL CONTAMINATION OF THE BM IN HIGH-RISK PRIMARY BREAST CANCER

We conducted a pilot study of four cycles of CAF followed by high-dose cyclophosphamide, cisplatin and BCNU with hematopoietic support for patients with high risk primary breast cancers as defined by 10 or more positive lymph nodes. The patients were treated from 1987 through 1990. We had samples of the harvested BM to test for tumor cell contamination using our immunohistochemical technique. Of the 83 patients, 23 have progressed (28%). There was a statistically significant difference for those patients who had tumor cell contamination of the BM compared with those patients who had no tumor cells in the BM, in terms of both disease-free and overall survival. Thirty of the 83 patients (36%) had tumor cells contaminating the harvested BM. Of the 30 positive patients, there have been 15 recurrences (50%), and of the 53 negative patients, there have been 15 progressions (28%) p=0.038. Similarly, the presence of tumor cells in the BM correlated with a worse overall survival, 13 of the 30 positive patients have died (43%) and 10 of the 53 negative (19%), p=0.022.

Using a univariant analysis, the log of the number of tumor cells detected also correlated with a worse disease-free and overall survival, so the higher the number of tumor cells contaminating the BM, the shorter the survival. In a multi-variant analysis, the tumor cell contamination of the BM, as well as the number of antigen positive cells in the BM, predicted for a worse disease-free and overall survival. There were no significant correlations between the tumor cell detection assay and the ER status, PR status, ER or PR status, size of the primary or number of positive axillary lymph nodes. No other two variable model contained significant variables.

TUMOR CELL CONTAMINATION OF PBSC

We have examined the PBSCs for tumor cell contamination using our immunohistochemical and our two color immunofluorescent assays. There is a consistently lower incidence of tumor cell contamination of the PBSCs compared to BM. In the pilot study of patients with 10 or more positive nodes, only 2 of the 65 patients who received PBSCs (4%) had tumor cells detected. In 90 breast cancer patients, stages II through IV, only 4 patients (4%) had tumor cells detected in their PBSC collections. There is no correlation with the presence of tumor cells in the PBSC collections and disease-free or overall survival.

SUMMARY

There are a number of sensitive techniques to detect tumor cells contaminating the BM or PBSCs used as hematopoietic support following high dose chemotherapy. Our two-color immunofluorescent technique consistently detects one tumor cell in 10^6 normal BM or PBSCs. We did find a correlation between the presence of tumor cells contaminating the BM and a worse disease-free and overall survival for patients with 10 or more positive lymph nodes treated with high dose chemotherapy and hematopoietic support. We have not found any correlation between the presence of tumor cells in the BM and the survival for patients with metastatic breast cancer treated with high dose chemotherapy. The incidence of tumor cell contamination of the PBSC collections is significantly lower than BM contamination. Further improvements in the detection techniques by concentrating the tumor cells or eliminating the majority of the normal hematopoietic cells may increase the sensitivity of the detection techniques and allow one tumor cell to be detected in 10^8 or 10^9 cells. In addition, gene marking studies of the hematopoietic support may further define the role of contaminating tumor cells in patients treated with high dose chemotherapy and hematopoietic support. The role of purging the hematopoietic support remains unknown, although there are a variety of effective purging techniques.

REFERENCES

1. Diel IJ, Kaufmann M, Goerner et al.: Detection of tumor cells in bone marrow patients with primary breast cancer: A prognostic factor for distant metastasis. *J Clin Oncol* 10:1534–1539, 1992.
2. Cote RJ, Rosen PP, Lesser ML et al.: Prediction of early relapse in patients with operable breast cancer by detection of occult bone marrow micrometastases. *J Clin Oncol* 9:1749–1756, 1991.
3. Mansi JL, Berger U, Easton D et al.: Micrometastases in bone marrow in patients with primary breast cancer: Evaluation as an early predictor of bone metastases. *BMJ* 295:1093, 1987.
4. Harbeck N, Untch M, Pache L et al.: Tumour cell detection in the bone marrow of breast cancer patients at primary therapy: Results of a 3-year median follow-up. *Brit J Cancer* 69:566, 1994.
5. Giai M, Natoli C, Sismondi P et al.: Bone marrow micrometastases detected by a monoclonal antibody in patients with breast cancer. *Anticancer Res* 10:119–121, 1990.
6. Kirk S, Cooper G, Hoper M et al.: The prognostic significance of marrow micrometastases in women with early breast cancer. *Eur J Surg Oncol* 16:481–485, 1990.
7. Gribben JG, Nadler LM. Detection of minimal residual disease in patients with lymphomas using the polymerase chain reaction. [Review] *Important Adv Oncol* 117–129, 1994.
8. Rill DR, Santana VM, Robert WM et al.: Direct demonstration that autologous bone marrow transplantation for solid tumors can return a multiplicity of tumorigenic cells. *Blood* 84:380–383, 1994.
9. Ross AA, Cooper BW, Lazarus HM et al.: Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques. *Blood* 82:2605–2610, 1993.
10. Vredenburgh JJ, Silva O, Tyer C et al.: A comparison of immunohistochemistry, two-color immunofluorescence, and flow cytometry with cell sorting for the detection of micrometastatic breast cancer in the bone marrow. *J Hematother* 5:57–62, 1996.
11 Leslie DS, Johnston WW, Daly I et al.: Detection of breast carcinoma cells in human bone marrow using fluorescence-activated cell sorting and conventional cytology. *Am J Clin Pathol* 94:8–13, 1990.
12. Vredenburgh JJ, Peters WP, Rosner G et al.: Detection of tumor cells in the bone marrow of stage IV breast cancer patients receiving high-dose chemotherapy: The role of induction chemotherapy. *Bone Marrow Transplant* 16:815–821, 1995.
13. Peters WP, Ross M, Vredenburgh JJ et al.: High dose chemotherapy and autologous marrow support as consolidation after standard-dose adjuvant therapy for high-risk primary breast cancer. *J Clin Oncol* 11:1132–1143, 1993.
14. Vredenburgh JJ, Silva O, DeSombre K et al.: The significance of bone marrow micrometastases for patients with breast cancer and ≥10+ lymph nodes treated with high dose chemotherapy and hematopoietic support. *Proc ASCO* 14, 1995.

IMMUNOCYTOCHEMICAL DETECTION AND IN VITRO GROWTH OF BREAST CANCER CELLS IN MOBILIZED AND NON-MOBILIZED HEMATOPOIETIC GRAFTS

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ABSTRACT

A number of recent studies have documented tumor contamination of autologous hematopoietic grafts in patients with breast cancer. These studies have concluded that: 1) tumor contamination occurs less frequently in peripheral blood stem cell (PBSC) collections than in bone marrow (BM); 2) fewer patients have tumor contamination of PBSC than BM; 3) tumor cells may be present in hematopoietic grafts regardless of clinical status at time of pheresis and 4) hematopoietic-residing tumor cells appear to be capable of clonogenic growth in vitro. Thus, there is justifiable concern that stem cell mobilization procedures may also mobilize tumor cells into PBSC collections.

We used immunocytochemical (ICC) and in vitro tumor cell clonogenic assay (TCA) techniques to analyze premobilized peripheral blood (PB), BM and PBSC collections from breast cancer patients mobilized with cytokine alone (G-CSF) and chemotherapy followed by cytokine (cyclophosphamide [Cy]/GM-CSF). Before mobilization with G-CSF, only 1/37 (3%) PB and 4/36 (11%) BM specimens had ICC-detectable tumor cells. After 5 or 7 days of G-CSF administration, 3/38 (8%) patients had ICC-detectable tumor cells in the large-volume PBSC collection. This difference was not statistically significant. Before mobilization with Cy/GM-CSF, only 1/32 (3%) PB and 4/36 (11%) BM specimens had ICC-detectable tumor cells. After day 15 of Cy/GM-CSF mobilization, 2/37 (5%) patients had ICC-detectable cells in the large-volume PBSC collection. This difference was not statistically significant.

Tumor cells with in vitro clonogenic growth potential in the TCA were observed in the premobilized BM of one patient, and in the PBSC specimens of another two patients in the G-CSF cohort. In the Cy/GM-CSF cohort, one patient showed tumor colony growth in premobilized PB, and one patient showed tumor colony growth in the BM. No patients showed tumor colony growth in PBSC

samples. These data indicate that tumor contamination in chemosensitive, advanced-stage breast cancer patients is not significantly increased in single, large-volume apheresis following mobilization with either G-CSF or Cy/GM-CSF. Additional trials in expanded patient cohorts are needed to verify these initial observations.

INTRODUCTION

High-dose chemotherapy (HDCT) followed by autologous hematopoietic stem cell support is increasingly being used in the treatment of patients with a variety of malignancies. This treatment has shown encouraging results in patients with localized and metastatic breast cancer.[1–4] However, a number of recent studies have documented tumor contamination of bone marrow (BM) and peripheral blood stem cell (PBSC) grafts.[5–9] We developed sensitive immunocytochemical (ICC) and in vitro tumor cell clonogenic assay (TCA) techniques to demonstrate that breast cancer cells are present in both BM and PBSC collections from breast cancer patients.[6,10–12] These studies have concluded that tumor contamination occurs less frequently in PBSC than in BM,[6] tumor cells may be present in PBSC collections regardless of the patient's clinical status at time of apheresis,[12] and that a strong correlation exists between the ICC detection of these tumor cells and their capacity for in vitro clonogenic growth.[6,13]

Additional evidence suggests that breast cancer cells may be released into the peripheral circulation as a result of cytokine mobilization procedures.[14] While the exact role of the infusion of tumor cells in autologous hematopoietic grafts remains to be elucidated, two recent studies have demonstrated that infused tumor cells are present at sites of post-transplant disease relapse.[15,16] Thus, there is justifiable concern that potentially harmful tumor cells may contaminate PBSC infusions and contribute to disease recurrence.

In this study, we examined the possible role of hematopoietic stem cell mobilization procedures in mobilizing tumor cells into PBSC collections. Specifically, we used ICC and TCA assays to monitor the premobilized peripheral blood (PB), BM and single, large-volume mobilized PBSC collections for the presence and in vitro colony growth of breast cancer cells. Our results indicate that no significant mobilization of tumor cells occurred in two cohorts of chemosensitive patients with advanced-stage breast cancer. No increase in tumor cell contamination was observed in PBSC collections following 5 or 7 days mobilization with granulocyte colony-stimulating factor (G-CSF), or in PBSC collections on day 15 following priming with cyclophosphamide followed by granulocyte-macrophage colony-stimulating factor (Cy/GM-CSF).

MATERIALS AND METHODS

Patient population

Between February, 1994, and August, 1995, 38 consecutive patients with advanced-stage breast cancer participated in a study of HDCT with BM and/or PBSC support at the Johns Hopkins Oncology Center. Eligibility criteria included histologically documented Stage III or IV breast cancer, response to conventional-dose chemotherapy, histologically negative BM biopsy, and adequate hematopoietic, renal and hepatic reserves to undergo high-dose treatment. The median age of the patients was 44 years (range 28–61 years). Ten patients had Stage IIIB breast cancer, and 28 patients had Stage IV breast cancer. Of the patients with Stage IV disease, 11 had radiographic evidence of bone metastases. The study was approved by the Joint Committee for Clinical Investigation of the Johns Hopkins Hospital, and written informed consent was obtained from all patients.

Mobilization procedures

All patients underwent premobilization PB draw, BM harvesting and post-mobilization PBSC collection in a single, large-volume apheresis. One cohort of patients was mobilized using human recombinant G-CSF (Amgen, Thousand Oaks, CA) at 5 μg/kg/day. Initially, G-CSF was given for seven days. Once it was established in the first 10 patients that peak PB $CD34^+$ cell concentration occurred on day six, subsequent patients received a five-day course of G-CSF. The single, large-volume apheresis procedure was performed on day eight in 10 patients, and on day six in 28 patients. The historical control patient population was treated on a protocol with similar eligibility requirements. We have reported on this patient population previously.[11] Briefly, PBSC mobilization was accomplished with cyclophosphamide (Cy) at 4 g/m^2 administered over 90 minutes on the day following BM harvest. Human recombinant GM-CSF (Immunex Corp., Seattle, WA) was administered at 5 μg/kg/day for 15 days. A single, large-volume apheresis was performed 15 days after Cy administration.

High-dose chemotherapy

G-CSF mobilized patients received HDCT with Cy and thiotepa (6 g/m^2 and 800 mg/m^2, respectively) by continuous infusion over 96 hours. Hematopoietic support was followed by the induction of autologous graft-versus-host disease with cyclosporine A and γ-interferon as previously described.[17] Cy/GM-CSF mobilized patients received high-dose therapy with Cy and thiotepa (6 g/m^2 and 800 mg/m^2, respectively) by continuous infusion over 96 hours with hematopoietic cell support. Novobiocin, an agent that augments alkylating agent cytotoxicity, was administered during high-dose therapy as part of a Phase II trial at a dose of 4 g/day for seven days as previously described.[18]

For both groups of patients, samples of pre-mobilized PB, harvest BM and post-mobilized PBSC were obtained under aseptic conditions. These samples were analyzed for the presence and in vitro clonogenic growth of tumor cells.

Tumor cell detection by immunocytochemistry (ICC)

A total of 3–5 mL of PB, BM and PBSC specimens containing a minimum of 1×10^7 cells/mL were collected in sterile, sodium heparin tubes. The ICC procedure for the detection of breast cancer micrometastases has been described previously.[6] Briefly, mononuclear cells were separated by Ficoll-Hypaque density gradient separation (Pharmacia, Uppsala, Sweden), washed in Liebovitz L-15 tissue culture medium (GIBCO/BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (GIBCO/BRL), and cytocentrifuged onto glass microscope slides. Following brief storage at 4°C, the slides were fixed in a paraformaldehyde/methanol fixative, washed thoroughly in phosphate-buffered saline (PBS; GIBCO/BRL) and blocked for endogenous peroxidase activity in a phenylhydrazine (Sigma Chemical, St. Louis, MO) solution. A cocktail of murine IgG monoclonal antibodies directed against breast and/or epithelial cell antigens was incubated on the cytospin preparations for 30–60 minutes. Immunoperoxidase staining was accomplished using the Zymed streptavidin immunoperoxidase kit (Zymed, So. San Francisco, CA).

With this ICC assay, the tumor cells stain bright red, and the hematopoietic cells are counterstained blue. In previous series, we have established a tumor-detection sensitivity of one tumor cell in 5.0×10^5 hematopoietic cells.[6,10] Most importantly, this ICC assay preserves cellular morphology for verification of tumor-consistent phenotype, and shows no cross-reactivity with nontumor cells.

For each PB, BM and PBSC ICC-stained specimen, up to 5.0×10^5 cells were directly examined by light microscopy. The number of ICC-positive, morphology-consistent tumor cells was calculated by direct observation, and expressed with a common denominator of 100,000 hematopoietic cells.

Tumor cell clonogenic assay (TCA)

The TCA technique has been described previously.[6,13] Briefly, 5.0×10^5 mononuclear cells/mL were plated in triplicate in 35 mm^2 Petri dishes in a soft agar-based medium supplemented with human recombinant growth factors. Negative control plates consisted of medium without the supplemental growth factors. Following 14 days in culture, the tumor colonies (>40 cells) were enumerated by inverted phase microscopy. The agar cultures were then floated onto large microscope slides, and immunostained in a blinded fashion by Dr. Thomas Moss, at Children's Hospital, Los Angeles, CA. The tumor colonies were stained with FITC-conjugated anti-cytokeratin antibody SB-3 (Accurate Chemical, Westbury, NY) or anti-breast cancer antibodies 520C9, 260F9, and 317G5 (kindly

Table 1. Results of the ICC assay[1]

Peripheral blood[2]		*BM*[2]		*PBSC*[3]	
G-CSF	Cy/GM-CSF	G-CSF	Cy/GM-CSF	G-CSF	Cy/GM-CSF
1/37 (3%)	1/32 (3%)	4/36 (11%)	4/36 (11%)	3/38 (8%)	2/37 (5%)

[1]*Number of patients with tumor cells detected by ICC.*

[2]*Specimens collected on day 0, before mobilization.*

[3]*PBSC specimen collected on day 15 after Cy/Gm-CSF mobilization, and on day 6 or 8 after G-CSF mobilization.*

provided by Baxter Healthcare, Immunotherapy Division, Santa Ana, CA). These antibodies have been used by others for the detection of breast cancer micrometastases.[8,19]

We have tested the TCA assay on over 200 BM and 100 PBSC specimens from patients with breast cancer. These combined studies have shown that the combination of human growth factors and agar-based medium supports the clonogenic growth of breast cancer cells in both BM and PBSC.[6,10,11,13] We have observed no tumor colony growth in normal BM specimens with this assay. Further, we have reported that the in vitro growth of tumor colonies in the TCA assay correlates significantly ($p<0.0001$, chi-square test) with the tumor detection results obtained in the ICC assay.[6]

Statistical analysis

Confidence intervals for the proportion of tumor-positive PB, BM and PBSC collections were calculated using exact binomial confidence intervals. Fisher's Exact Test was used for comparison of categorical data.

RESULTS

In total, 75 consecutive patients with advanced-stage breast cancer participated in two studies of HDCT with hematopoietic support using two different PBSC mobilization regimens. Mobilization with either G-CSF alone or Cy followed by GM-CSF resulted in efficient mobilization of PBSC.

ICC assay results

The ICC results for the detection of tumor cells in PB, BM and PBSC are presented in Table 1. Sixty-six patients had pre-mobilized PB and BM, and postmobilized PBSC specimens analyzed. In the remaining nine patients, ICC analysis was not performed in at least one of the hematopoietic specimens because: 1) the PB sample was not collected prior to BM harvest (six patients); 2) the morphology of the BM sample was inadequate for analysis (two patients) or 3) lack

Table 2. ICC Results: Patients with tumor-positive specimens

Mobilization protocol/patient number	*Peripheral blood*	*BM*	*PBSC*
G-CSF (n = 38)			
1	ND[1]	15[2]	1
2	0	1	0
3	0	0	3
4	0	0	2
5	0	20	0
6	1	1	0
Cy/GM-CSF (n = 37)			
1	0	18	0
2	0	2	0
3	0	8	0
4	0	0	1
5	2	4	1

[1]*Not Done; too few cells for analysis.*

[2]*Number of tumor cells per 10^5 hematopoietic cells.*

of a sufficient number of BM cells to perform the assay (one patient).

By semi-quantitative analysis, tumor cell contamination of specimens varied between 0 and 20 tumor cells per 10^5 hematopoietic cells. Tumor cell counts were similar with both G-CSF and Cy/GM-CSF mobilization regimens. Only 2 of 69 PB specimens (3%) and 8 of 72 BM specimens (11%) had tumor cells detected by the ICC assay. The two patients who had tumor cells detected in the PB sample also had tumor cells detected in the BM sample obtained before mobilization.

After mobilization with G-CSF, 3 of 38 PBSC samples (8%) had tumor cells detected by the ICC assay. In the patients who received Cy/GM-CSF, 2 of 37 PBSC (5%) samples were found to be tumor positive. This difference was not statistically significant (p=1.0). All five patients with ICC-positive PBSC samples had Stage IV disease, only one of whom had radiographic evidence of bone metastases. In total, 5/60 (8%) Stage IV patients had ICC-positive PBSC samples compared with 0/5 Stage IIIB patients (p=0.6).

Five patients with tumor cells detected by ICC in the BM samples obtained before mobilization had tumor-free PBSC samples after administration of G-CSF (2 patients) or after Cy/GM-CSF (3 patients; Table 2). However, three patients had tumor cells detected by the ICC assay in the PBSC sample (two after G-CSF alone and one after Cy/GM-CSF) despite tumor-free premobilization PB and BM samples. At least one hematopoietic specimen was ICC-positive in 10 of 60 Stage

Table 3. TCA results

	Peripheral blood		*BM*		*PBSC*	
Patient number	*ICC*[1]	*TCA*	*ICC*	*TCA*	*ICC*	*TCA*
G-CSF (n=38)						
3	0	ND[2]	0	Neg	3	Pos
4	0	Neg	0	Neg	2	Pos
6	1	Neg	1	Pos	0	Neg
Cy/Gm-CSF (n=37)						
1	0	Neg	18	Incl[3]	0	Neg
2	0	Neg	2	Incl	0	Neg
3	0	ND	8	Incl	0	Neg
5	2	Pos	4	Incl	1	Neg

[1]*ICC results as number of tumor cells per 10^5 hematopoietic cells.*

[2]*Not Done; too few cells for analysis.*

[3]*Inconclusive results.*

IV patients (17%) compared with 1 of 15 (7%) Stage IIIB patients (p=0.4). Of the patients with Stage IV disease, at least one of the hematopoietic samples was ICC positive in 5 of 20 (25%) patients with radiologic evidence of bone metastases compared to 5 of 40 (13%) without documented bone metastases (p=0.3).

TCA assay results

Overall, the TCA assay was positive in only four patients (Table 3). Interestingly, two patients mobilized with G-CSF had tumor cells with in vitro clonogenic potential detected in the PBSC sample despite ICC-negative pre-mobilized PB and BM, and TCA-negative BM. Another patient mobilized with G-CSF, who had ICC-positive PB and BM specimens but ICC-negative PBSC, had a TCA-positive BM sample. One patient, mobilized with Cy/GM-CSF, had ICC and TCA-positive PB and ICC-positive BM, but the tumor cells identified by ICC in the PBSC specimen failed to have in vitro clonogenic potential. In three other patients, all mobilized with Cy/GM-CSF, the TCA results of ICC-positive BM samples were inconclusive. Tumor colonies were detected by inverted-phase microscopic evaluation, but the immunostaining of these TCA specimens was inconclusive. No TCA positivity was observed in ICC-negative specimens.

DISCUSSION

Although the clinical relevance of occult tumor contamination of autologous hematopoietic grafts is unclear, an association between occult BM tumor involvement detected by tissue culture, ICC and polymerase chain reaction (PCR)

and poor post-transplant prognosis has been reported.[5,8,9] However, as we previously reported, occult breast cancer contamination occurs less frequently in PBSC collections than in BM. Analysis of paired PBSC collections and BM harvest samples from patients with advanced-stage disease has documented that PBSC collections are less frequently contaminated with tumor cells than BM. Also, the concentration of tumor cells in tumor-involved specimens is about 30-fold lower in PBSC collections than in BM.[6]

Recently, Brugger et al. analyzed the impact of VIP and G-CSF mobilization on occult tumor contamination of serially collected PB samples using an ICC assay with monoclonal antibodies directed against cytokeratin and epithelial-cell antigens.[14] In their series of previously untreated breast cancer patients, they observed mobilization of tumor cells into the PB of 7/7 breast cancer patients studied. These data suggest that tumor cell release into venous PB, even in patients without light microscopic evidence of breast cancer involvement of BM biopsies, may lead to increased tumor contamination of mobilized PBSC products. This study also suggests, albeit in a small number of patients, that mobilization of tumor cells into the PB in patients without evidence of BM contamination might occur early during mobilization, i.e., between days one and seven. As both the type of PBSC mobilization regimen and the timing of the leukapheresis could be critical to the extent of tumor contamination of PBSC grafts, we have now gone on to prospectively analyze hematopoietic specimens for occult tumor contamination.

In this study, we compared tumor cell contamination of premobilized PB and BM with post-mobilized PBSC in chemosensitive breast cancer patients mobilized with G-CSF. Similar to the results we reported in a previous series of patients mobilized with cyclophosphamide followed by GM-CSF,[11] we observed no significant increase in tumor cell contamination of PBSC specimens. In patients mobilized with G-CSF or with Cy/GM-CSF, we found a low incidence of occult tumor contamination in pre-mobilization PB (1/37 and 1/32, respectively) and BM (4/36 and 4/36, respectively). These data suggest that the patients mobilized with Cy/GM-CSF and those mobilized with G-CSF alone had similar tumor burdens before beginning therapy, as anticipated from the similar eligibility criteria for both high-dose studies. We also found a low incidence of tumor contamination by ICC of PBSC samples obtained six or eight days after initiation of G-CSF-only PBSC mobilization (3/38, 8%), and 15 days after cyclophosphamide administration (2/37, 5%). It is interesting to note that two patients mobilized with G-CSF alone had tumor cells with in vitro clonogenic potential detected in the PBPC collection product, despite ICC and TCA-negative patient PB and BM samples. The one Cy/GM-CSF mobilized patient with an ICC-positive PBSC did not show any in vitro tumor colony growth in the TCA.

In summary, we used sensitive and specific ICC and TCA assays to analyze hematopoietic specimens for occult breast cancer cell involvement and in vitro

growth potential before and after PBPC mobilization. Our sequential series of patients consisted of 38 patients with chemosensitive, advanced-stage breast cancer and histologically negative BM biopsies. Patients were mobilized for PBSC collection with either G-CSF or with cyclophosphamide followed by GM-CSF. We observed a low incidence and a low concentration of occult tumor cell contamination of PB and BM harvest products before PBSC mobilization. Despite efficient mobilization of $CD34^+$ cells into a single, large-volume collection, we observed a similar low incidence of tumor contamination of PBSC collections obtained on day six or day eight after a course of G-CSF, or on day 15 after administration of Cy/GM-CSF. The results of these studies do not support the notion that currently used mobilization regimens significantly increase the number of occult tumor cells in single-collection, large-volume apheresis products.

REFERENCES

1. Peters WP, Ross M, Vredenburgh JJ et al.: High-dose chemotherapy and autologous bone marrow support as consolidation after standard-dose adjuvant therapy for high-risk primary breast cancer. *J Clin Oncol* 11:1132–1143, 1993.
2. Peters WP: Autologous bone marrow transplantation for breast cancer. In: Forman SJ, Blume KG, Thomas ED (eds) Bone Marrow Transplantation (1st. ed.). Boston: Blackwell Scientific Publications, 1994, pp 789–801.
3. Bezwoda WR, Seymour I, Dansey RD: High-dose chemotherapy with hematopoietic rescue as primary treatment for metastatic breast cancer: A randomized trial. *J Clin Oncol* 13:2483–2489, 1995.
4. Vahdat I, Antman KH: Dose-intensive therapy in breast cancer. In: Armitage JO, Antman KH (eds) High-dose Cancer Therapy (2nd ed). Baltimore: Williams & Wilkins, 1995, pp 802–823.
5. Sharp JG, Kessinger A, Vaughan WP et al.: Detection and clinical significance of minimal tumor contamination of peripheral blood stem cell harvests. *Int J Cell Cloning* 10(suppl 1):92–94, 1992.
6. Ross AA, Cooper BW, Lazarus HM et al.: Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques. *Blood* 82:2605–2610, 1993.
7. Douer D, Chaiwun B, Glaspy J et al.: Analysis of peripheral blood progenitor cell harvests for tumor contamination using a sensitive immunohistochemical method. *Proc Am Soc Clin Oncol* 12:51, 1993 [abstr].
8. Vredenburgh JJ, Silva O, de Sombre K et al.: The significance of bone marrow micrometastases for patients with breast cancer and ≥10+ lymph nodes treated with high-dose chemotherapy and hematopoietic support. *Proc Am Soc Clin Oncol* 14:A317, 1995 [abstr].
9. Fields KK, Elfenbein GJ, Trudeau WL et al.: Clinical significance of bone marrow metastases as detected using the polymerase chain reaction in patients with breast cancer undergoing high-dose chemotherapy and autologous bone marrow transplantation.

J Clin Oncol 14:1868–1876, 1996.

10. Passos-Coelho JL, Ross AA, Davis JA et al.: Bone marrow micrometastases in chemotherapy-responsive advanced breast cancer: Effect of ex-vivo purging with 4hydroperoxycyclophosphamide. *Cancer Res* 54:2366–2371, 1994.
11. Passos-Coelho JL, Ross AA, Moss TJ et al.: Absence of breast cancer cell mobilization into peripheral blood progenitor cell collections by priming with cyclophosphamide and granulocyte-macrophage colony-stimulating factor. *Blood* 85:1138–1143, 1995.
12. Ross AA, Farmer SL, Moss TJ et al.: Tumor contamination of peripheral blood stem cell (PBSC) collections from breast cancer patients: Influence of clinical status at time of pheresis. *Breast Cancer Res Treat* 32(suppl):63 [abstr].
13. Ross AA, Moss TJ, Weintraub C et al.: In-vitro clonogenic growth of bone marrow micrometastases from patients with breast cancer. *Breast Cancer Res Treat* 27:169A, 1993 [abstr].
14. Brugger W, Bross KJ, Cilatt M et al.: Mobilization of tumor cells and hematopoietic progenitor cells into peripheral blood of patients with solid tumors. *Blood* 83:636–640, 1994.
15. Brenner M, Rill D, Moen R et al.: Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. *Lancet* 341:85–86, 1993.
16. Rill DR, Santana VM, Roberts WM et al.: Direct demonstration that autologous bone marrow transplantation for solid tumors can return a multiplicity of tumorigenic cells. *Blood* 84:380–383, 1994.
17. Kennedy MJ, Vogelsang GB, Jones RJ et al.: Phase I trial of interferon-gamma to potentiate cyclosporine A-induced graft-versus-host-disease in women undergoing autologous bone marrow transplantation for breast cancer. *J Clin Oncol* 12:249–257, 1994.
18. Kennedy MJ, Armstrong DK, Huelskamp AM et al.: Phase I and pharmacologic study of the alkylating agent modulator novobiocin in combination with high-dose chemotherapy for the treatment of metastatic breast cancer. *J Clin Oncol* 13:1136–1143, 1995.
19. Shpall EJ, Jones RB, Bearman SI et al.: Transplantation of enriched CD34-positive autologous marrow into breast cancer patients following high-dose chemotherapy: Influence of CD34-positive peripheral blood progenitors and growth factors on engraftment. *J Clin Oncol* 12:28–36, 1994.

GENE MARKING STUDIES IN AUTOLOGOUS TRANSPLANTATION

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ABSTRACT

The use of in vitro progenitor cell assays to predict stem cell behavior or gene transfer efficiency is not supported by mounting data from clinical studies and large animal models. Hence, further theories regarding stem cell kinetics and the optimal conditions for durable hematopoietic gene transfer should be validated in clinical trials. All clinical trials to date have used retroviral vectors and all report low hematopoietic gene transfer efficiency. Virtually all trials have involved myeloablated hosts undergoing high-dose therapy for cancer. The highest and most durable marking has been observed in children undergoing high-dose therapy for leukemia and neuroblastoma transplanted with mononuclear cells exposed to retroviral supernatant for 6 hours without growth factors. This study quantified transduction levels based on Neo-resistance of bone marrow (BM) colony-forming unit (CFU) post-transplant and has reported detection of the transgene in BM progenitors at 3 years. Under identical transduction conditions, $CD34^+$ cells from our adult patients with myeloma and breast cancer undergoing similar myeloablative therapy showed poor marking with no long-term marking; we used a nested PCR strategy to detect the transgene in BM and peripheral blood samples. $CD34^+$ cells from our adult patients transduced on autologous stroma for 72 hours without growth factors were also poorly transduced. Better marking of $CD34^+$ cells from our adult patients has been observed in the presence of IL3, IL6 and stem cell factor for 72 hours with 9/11 patients having detectable marked cells in vivo post-engraftment and 3/9 showing persistence of marking over 18 months. We have not detected the marker gene in myeloma or breast cancer cells in our patients, although most remain in complete or partial remission. Many questions remain unanswered such as the differentiating or damaging effect of various culture conditions on true long-term repopulating cells, the appropriate assays for transgene delivery, the possibility of a growth disadvantage for transduced cells or incitement of an immune response from expression of the transgene, and the possibility of long-term toxicity.

INTRODUCTION

The hematopoietic stem cell is an obvious target for gene transfer as it is defined by its potential to reconstitute the hematopoietic and immune systems.[1,2] Retroviral

gene transfer is the best characterized and most developed vector system and is currently under the widest clinical study.[3] Though efficient and reproducible retroviral gene transfer to a high percentage of long-term repopulating cells has been shown in rodents, only limited success has been achieved in larger animals, including man.[1] Whereas murine BM cultured with retrovirus in the presence of IL-3, IL-6 and SCF has provided efficient gene transfer to a significant percentage of hematopoietic stem cells, previous work from our group has shown only minimal levels of gene transfer in circulating progenitors of human long-term repopulating cells when $CD34^+$ cells were transduced using a similar protocol.[4] Higher levels of gene transfer were reported in children recovering from high-dose chemotherapy when their marrow cells were simply exposed to retroviral vector supernatant for 6 hours without the inclusion of exogenous growth factors.[5] Stromal cells or stromal matrix molecules have been shown to substitute for or enhance the effects of exogenous growth factors during transduction of human hematopoietic progenitors and improve gene transfer into primitive cells in in vitro testing and in animal models.[6–8] We recently compared the 6-hour transduction procedure without growth factors in adults to the use of autologous marrow stromal cells during transduction as a means of improving efficiency of gene transfer into primitive repopulating cells. The results of this trial will be summarized along with a more general discussion of gene marking trials in autologous transplantation.

GENE MARKING OF NORMAL HEMATOPOIETIC CELLS

Our transplant procedure and protocol design has been published previously[4,9] and our most recent study differed only in the transduction conditions used. As in our previous clinical protocol of retroviral transduction in the presence of IL-3, IL-6 and SCF, one-third of the harvested bone marrow (BM) or one of three mobilized peripheral blood (PB) collections were CD34-selected and used for transduction. We and others have previously shown CD34-enriched BM and PB cells are equal or better targets for gene transfer as compared with whole mononuclear cells[10,11] and the use of CD34-enriched target cells allowed us to preserve an optimal multiplicity of infection, i.e., at least 10 infectious viral particles/cell. Two retroviral vectors, GlNa.40 and LNL6, carrying an identical bacterial phosphotransferase gene conveying G418 neomycin resistance (Neo) were used to distinguish the PB from the BM grafts. BM and PB from 5 patients were transduced for 6 hours without the addition of other growth factors, herein referred to as "6H" conditions or patients, and BM and PB from 4 patients was transduced for 72 hours in the presence of autologous stroma, herein referred to as "ST" conditions or patients. CFU plating and analysis and cell separations on post-transplant sample and DNA extractions were done and a nested PCR strategy was used to analyze patient blood and BM samples for the Neo gene as outlined previously.[4]

Whether our use of a CD34-enriched cell population might have decreased gene transfer efficiency when compared with the total mononuclear population used at St. Jude is unclear. Under both the 6H and ST conditions, the total and $CD34^+$ cell count and $CD34^+$ percentage fell over the period of transduction for both the BM and PB fractions. This contrasts with our previous cohort of patients transduced for 72 hours with IL-3, IL-6 and SCF where the total cell number, the $CD34^+$ cell number and the $CD34^+$ percentage all increased. Interactions between accessory cells might be important in promoting stem cell survival and proliferative potential without terminal differentiation. Hence, either primitive cell cycling or accessory cell functions could have been compromised under the 6H or ST conditions. Long-term culture conditions, perhaps favoring primitive hematopoietic cell cycling while preserving cell numbers and hopefully differentiation capacity, may offer an advantage over the short in vitro culture conditions used here.[7,12]

As evaluated by plating cells in methylcellulose-containing media, with and without G418, the overall marking efficiency was poor, with great variability between patients and between PB and BM fractions for each patient. There was a significantly greater mean transduction efficiency in the CD34-enriched cells transduced on stroma compared with the 6H group. DNA samples prepared from PB, granulocyte and total mononuclear PB fractions, and BM were analyzed by semi-quantitative PCR for the Neo gene at the time of engraftment and then every 3 months post-transplantation for up to 24 months. Only one ST patient, who received the highest number of CD34-enriched cells, had gene marked cells post-transplantation, detected at 186 days in 0.1% of the mononuclear PB fraction derived from the transduced BM graft. The inclusion of an autologous stromal layer during transduction was prompted by previous evidence that stromal cells or stromal matrix molecules can substitute for or enhance the effects of exogenous growth factors and improve gene transfer into primitive hematopoietic cells in animal models and human cells assayed in vitro.[6,8,13]

Four of five 6H patients had marked cells, and the longest period of marking was observed in a multiple myeloma patient who had marked granulocytes detected 315 days after transplant at a level of 0.1%. The short circulation and survival time of granulocytes means that the positive PCR signal was not simply caused by the prolonged survival of transduced terminally differentiated cells, but rather by the production of daughter cells from previously quiescent progenitors. The fact that low-level marking was observed in 4 of 5 6H patients versus 1 of 4 ST patients suggests that the 6H condition was more favorable for gene transfer. In contrast to our previous study, none of the 6H or ST patients showed consistent marking over time or had any marked cells detected greater than one year post-transplantation. The overall efficiency of retroviral gene transfer in this clinical trial was not better than that seen in our previous cohort of patients whose cells were transduced in the presence of growth factors and is thus unlikely to be useful for therapeutic applications.

GENE MARKING TO DEFINE THE SOURCE OF RELAPSE

Many important issues surrounding tumor biology in autologous transplantation can be addressed using the tools of gene marking. It is now well accepted that tumor cells contaminating the autologous graft can contribute to relapse in several tumors. Several patients with CML have also been shown to have the marker gene in *BCR/ABL*$^+$ colonies post-transplantation.[14] Marking of tumor cells in the children with neuroblastoma and AML at relapse has also been detected.[15] Only one patient with relapsing BC in our current trial was evaluated for gene marking of tumor and nested Neo PCR of tumor cell DNA extracted from malignant pleural effusion cells on day 123 was negative. This patient's cells were transduced on autologous stroma where the cytokines and matrix molecules elaborated by stromal cells would be predicted to have their greatest effects on the primitive hematopoietic cells during transduction. Such a growth environment is unlikely to enhance the growth of contaminating breast cancer cells.[16] Stromal cell coculture, however, may be more beneficial to the growth of contaminating myeloma.[17] Previous attempts to perform gene transfer into myeloma cells have revealed that myeloma cells are fragile and poorly tolerate in vitro handling and incubation.[18]

Ex vivo BM or PB purging techniques often have potentially damaging effects on normal marrow progenitors which makes a realistic assessment of their clinical utility vital. Since neither breast cancer nor multiple myeloma cells express CD34 antigen and the CD34-enrichment process has been shown to serve as a two to four log purge of myeloma and breast cancer tumor cells, very few tumor cells would have been exposed to retroviral vector during transduction in our recent trial.[19,20] No other studies have shown marked relapses in myeloma or breast cancer patients to date, but even if these cells were present in the graft and contributed to relapse, they may not be susceptible to gene transfer under conditions optimized for transfer to more slowly cycling tumor cells (C. Dunbar, unpublished data). Chemical and immunologic purging techniques in other tumors are currently under evaluation at a number of institutions using the tools of gene marking.

ASSESSMENT OF TRANSGENE DELIVERY

An area of technical importance concerns the methods employed to evaluate transgene delivery. The gene transfer efficiency observed in our 6H patients was less than that observed for children treated under similar conditions with identical vectors at St. Jude Children's Research Hospital.[5] Levels of marked BM CFU-C there averaged 5% at 12 to 18 months post-transplantation and have continued to be detected and expressed for up to three years in BM.[21] There are a number of possible reasons for the less successful marking in our patients. The St. Jude patient population consisted of children (range 2–19 years) who underwent prompt collection of autologous marrow just after recovery from high-dose induction chemotherapy. They may have had a relatively high number of

primitive stem cells in cycle, and been susceptible to retroviral gene transfer.[22] Our patients were instead heavily pre-treated older adults with probable stem cell damage or deficiency due to prior alkylating agent chemotherapy. The different methods used for determining marking efficiency post-transplantation may have skewed the comparison. We chose a strategy of nested PCR on total DNA from BM and PB fractions, while the marking efficiency of the St. Jude patients was evaluated by DNA PCR on colonies of Neo-resistant progenitor colonies grown out at various time points post-transplant. Discrepancies between BM Neo-resistant colony and total DNA PCR on mature circulating cells have been noted in other reports.[23,24]

RT-PCR has also been promoted as a sensitive method of transgene detection.[14] While it could improve the detection of rare events, it will only detect functional transgenes whose expression is certain to be highly variable. Reliable estimates for actual transgene integration are therefore difficult. The overall expression of the transgene is an important but separate question. The engraftment of hematopoietic progenitors into immunodeficient mice may also aid in our understanding of gene transfer to the most primitive human hematopoietic cells but is plagued by low levels of human cell engraftment. Approaches to improve engraftment include the co-transplantation of stromal cells secreting human IL-3 and the creation of transgenic mice secreting human cytokines.[25,26]

APPROPRIATE MARKER GENES

Aside from the few studies in which a therapeutic gene of interest has been transferred to autologous marrow or peripheral blood cells, most studies have used vectors to transfer genes not thought to have any physiologic or immunologic consequences. Chief among these is the neomycin phosphotransferase gene (Neo). It has been known since at least 1987 that Neo has the potential to alter growth of HL-60 cells,[27] which might imply that a subtle effect on growth could be induced in other cells including hematopoietic stem cells. Furthermore, a more general concern for all transferred genes, including Neo, is their potential immunogenicity.

A recent retroviral gene transfer study in HIV patients clearly demonstrated that an anti-transgene immune response could be problematic.[28] Autologous $CD8^+$ HIV-specific cytotoxic T lymphocyte clones from six patients were transduced ex vivo with a retroviral vector expressing the bacterial hygromycin resistance gene and the herpes thymidine kinase gene. After reinfusion of large doses of hygromycin-resistant autologous anti-HIV T-cell clones every 14 days, 5/6 patients lost all positive cells or had very low levels by the fourth infusion of cells compared with earlier in the treatment course. These patients were all shown to have developed specific cell-mediated immunity against the hygromycin resistance-tk gene product. This immunity was class I MHC-restricted and very vigorous, with high CTL precursor frequencies despite the patients having active HIV disease. In

contrast, there is some evidence that introducing transgenes via hematopoietic stem cells induces tolerance.[29] A more thorough overview of the problem of immunity and gene transfer can be found elsewhere.[30]

An ideal marker transgene should be easily detected without requiring prolonged ex vivo culture to facilitate selection of transduced cells, both prior to transplantation to avoid transplantation of untransduced cells, and after hematopoietic reconstitution to measure transfer efficiency. Some candidate genes to fit these constraints include CD24 and a truncated non-functional version of the nerve growth factor receptor.[31,32] The use of CD24, a GPI-linked protein, may be problematic in that cell-cell transfer does occur and could abrogate specificity.[33] Another gene of recent interest is the green fluorescent protein (GFP) isolated from *Aequorea victoria.* This gene and its mutants produce a protein that emits green fluorescence when excited by blue light. Our lab has found this gene and its humanized form to be of great utility for transient assays but to confer a selective disadvantage and probable toxicity when expressed over longer periods, thus eliminating it as a potentially useful marking gene (Emmons, unpublished data).

An intriguing way to tip the balance of toxicity in favor of the gene-marked cell is the use of the drug resistance genes in vector constructs. This offers the hope of increasing the number of chemoprotected gene modified cells in vivo using chemotherapy, as has been shown in the mouse model using MDR-I.[34–36] Several clinical protocols are now underway with MDR-1, and other drug resistance genes under intense study include mutant DHFR conferring resistance to trimetrexate,[37] and 06-alkylguanine-DNA-alkyltransferase protecting against alkylating agents such as CCNU.[38]

THE FUTURE

Low gene transfer efficiency is the main obstacle to wider clinical application of genetic manipulation in autologous transplantation. We would like to again stress the importance of marking studies as indices for new gene transfer vectors and transduction conditions. No in vitro assays have yet proven predictive of gene transfer efficiency to human long-term repopulating cells. Additionally, as our assumptions about gene transfer efficiency largely rest on in vitro studies of progenitor populations which may not reflect the dynamics of the most primitive cells, we may make false assumptions about the best course for future gene marking studies. Problems with low efficiency transfer to the most primitive hematopoietic cells may be cell specific because of low cell cycling, insufficient viral receptor density or defenses against integration or vector specific because of potential toxicity or short life span as is true for the current generation of retroviruses. Better understanding and modeling of these most primitive interactions will be necessary before rational proposals for further marking trials are made.

REFERENCES

1. Dunbar CE, Emmons RVB: Gene transfer into hematopoietic progenitor and stem cells: Progress and problems. *Stem Cells* 12:563, 1994.
2. Karlsson S: Treatment of genetic defects in hematopoietic cell function by gene transfer. *Blood* 78:2481, 1991.
3. Mulligan RC: The basic science of gene therapy. *Science* 260:926, 1993.
4. Dunbar CE, Cottler-Fox M, O'Shaughnessy JA et al.: Retrovirally-marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. *Blood* 85:3048, 1995.
5. Brenner MK, Rill DR, Holladay MS et al.: Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. *Lancet* 342:1134, 1993.
6. Moore KA, Deisseroth AB, Reading CL et al.: Stromal support enhances cell-free retroviral vector transduction of human bone marrow long-term culture initiating cells. *Blood* 79: 1393, 1992.
7. Carter RF, Abrams-Ogg ACG, Dick JE et al.: Autologous transplantation of canine long-term marrow culture cells genetically marked by retroviral vectors. *Blood* 79:356, 1992.
8. Moritz T, Patel VP, Williams DA: Bone marrow extracellular matrix molecules improve gene transfer into human hematopoietic cells via retroviral vectors. *J Clin Invest* 93:1451, 1994.
9. Dunbar CE, Nienhuis AW, Stewart FM et al.: Amendment to clinical research projects. Genetic marking with retroviral vectors to study the feasibility of stem cell gene transfer and the biology of hematopoietic reconstitution after autologous transplantation in multiple myeloma, chronic myelogenous leukemia, or metastatic breast cancer. *Hum Gene Ther* 4:205, 1993.
10. Cassel A, Cottler-Fox M, Doren S, Dunbar CE: Retroviral-mediated gene transfer into CD34-enriched human peripheral blood stem cells. *Exp Hematol* 21:585, 1993.
11. von Kalle C, Kiem HP, Goehle S et al.: Increased gene transfer into human hematopoietic progenitor cells by extended in vitro exposure to a pseudotyped retroviral vector. *Blood* 84:2890, 1994.
12. Stewart AK, Dube ID, Kamel-Reid S, Keating A: A phase I study of autologous bone marrow transplantation with stem cell gene marking in multiple myeloma. *Hum Gene Ther* 6: 107, 1995.
13. Bodine DM, Moritz T, Donahue RE et al.: Long term expression of a murine adenosine deaminase (ADA) gene in rhesus hematopoietic cells of multiple lineages following retroviral mediated gene transfer into $CD34^+$ bone marrow cells. *Blood* 82:1975, 1993.
14. Deisseroth AB, Zu Z, Claxton D et al.: Genetic marking shows that Ph^+ cell present in autologous transplants of chronic myelogenous leukemia (CML) contribute to relapse after autologous bone marrow transplantation in CML. *Blood* 83:3068, 1994.
15. Brenner MK, Rill DR, Moen RC et al.: Gene-marking to trace origin of relapse after autologous bone marrow transplantation. *Lancet* 341:85, 1993.
16. O'Shaughnessy JA, Cowan KH, Wilson W et al.: Pilot study of high dose ICE (ifosfamide, carboplatin, etoposide) chemotherapy and autologous bone marrow transplant (ABMT) with neoR-transduced bone marrow and peripheral blood stem cells in patients with

metastatic breast cancer. *Hum Gene Ther* 4:331, 1993.
17. Caligaris-Cappio F, Bergui L, Gregoretti MG et al.: Role of bone marrow stromal cells in the growth of human multiple myeloma. *Blood* 77:2688, 1991.
18. Bjorkstrand B, Dilber MS, Smith CIE et al.: Retroviral-mediated gene transfer into human myeloma cells. *Br J Haematol* 88:325, 1994.
19. Shpall EJ, Jones RB, Bearman SI et al.: Transplantation of enriched CD34-positive autologous marrow into breast cancer patients following high-dose chemotherapy: Influence of CD34-positive peripheral-blood progenitors and growth factors on engraftment. *J Clin Oncol* 12:28, 1994.
20. Vescio RA, Hong CH, Cao J et al.: The hematopoietic stem cell antigen, CD34, is not expressed on the malignant cells in multiple myeloma. *Blood* 84:3283, 1994.
21. Brenner MK: The contribution of marker gene studies to hemopoietic stem cell therapies. *Stem Cells* 13:453, 1995.
22. Miller DG, Adam MA, Miller AD: Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol Cell Biol* 10:4239, 1990.
23. Bordignon C, Notarangelo LD, Nobili N et al.: Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. *Science* 270:470, 1995.
24. Kohn DB, Weinberg KI, Nolta JA et al.: Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. *Nature Medicine* 1:1017, 1995.
25. Nolta JA, Handley MB, Kohn DB: Sustained human hematopoiesis in immunodeficient mice by co-transplantation of marrow stroma expressing human interleukin-3: Analysis of gene transduction of long-lived progenitors. *Blood* 83:3041, 1994.
26. Bock TA, Orlic D, Dunbar CE et al.: Improved engraftment of human hematopoietic cells in severe combined immunodeficient (SCID) mice carrying human cytokine transgenes. *J Exp Med* 182:2037, 1995.
27. von Melchner H, Housman DE: The expression of neomycin phosphotransferase in human promyelocytic leukemia cells (HL60) delays their differentiation. *Oncogene* 2:137, 1988.
28. Riddell SR, Elliott M, Lewinsohn DA et al.: T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients. *Nature Medicine* 2:216, 1996.
29. Sykes M, Sachs DH, Nienhuis AW, Pearson DA, Moulton AD, Bodine DM: Specific prolongation of skin graft survival following retroviral transduction of bone marrow with an allogeneic major histocompatibility complex gene. *Transplantation* 55:197, 1993.
30. Dunbar CE, Young NS: Gene marking and gene therapy directed at primary hematopoietic cells. *Curr Opin Hematol* (In Press) 1996.
31. Pawliuk R, Kay R, Lansdorp P et al.: Use of a cell surface antigen, CD24, as a retroviral marker for the analysis and selection of gene transfer to long-term repopulating cells. *Blood* 82:314a, 1994.
32. Mavilio F, Ferrari G, Rossini S et al.: Peripheral blood lymphocytes as target cells of retroviral vector-mediated gene transfer. *Blood* 83:1988, 1994.
33. Anderson SM, Yu G, Giattina M et al.: Intercellular transfer of a glycosylphosphatidylinositol (GPI)-linked protein: Release and uptake of CD4-GPI from recombinant adeno-associated virus-transduced HeLa cells. *Proc Natl Acad Sci USA* 93:5894, 1996.
34. Sorrentino BP, Brandt SJ, Beline D et al.: Selection of drug resistant bone marrow cells in vivo after retroviral transfer of human MDR1. *Science* 257:99, 1992.

35. Podda S, Ward M, Himelstein A et al.: Transfer and expression of the human multiple drug resistance gene into live mice. *Proc Natl Acad Sci USA* 89:9676, 1992.
36. Hanania KG, Deisseroth AB: Serial transplantation shows that early hematopoietic precursor cells are transduced by MDR-1 retroviral vector in a mouse gene therapy model. *Cancer Gene Therapy* 1:21, 1994.
37. Spencer HT, Sleep SEH, Rehg JE et al.: A gene transfer strategy for making bone marrow cells resistant to trimetrexate. *Blood* 87:2579, 1996.
38. Jelinek J, Fairbairn LJ, Dexter TM et al.: Long-term protection of hematopoiesis against the cytotoxic effects of multiple doses of nitrosourea by retrovirus-mediated expression of human 06-alkylguanine-DNA-alkyltransferase. *Blood* 87:1957, 1996.

HEMATOPOIETIC STEM CELL "CANDIDATES" DERIVED FROM FETAL LIVER, FETAL BONE MARROW, UMBILICAL CORD BLOOD, ADULT BONE MARROW AND MOBILIZED PERIPHERAL BLOOD

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ABSTRACT

As part of our ongoing attempt to identify an optimal source of pluripotent hematopoietic progenitor cells for transplantation and for gene therapy, we have compared the proliferative potentials of $CD34^+$ subsets derived from fetal liver (FLV), fetal bone marrow (FBM), umbilical cord blood (UCB), adult bone marrow (ABM), and mobilized peripheral blood (MPB). We have applied three-color multi-parameter analysis of $CD34^+$ subsets by flow cytometry and single cell culture technique. The highest concentration of $CD34^+$ cells was found in FLV, (11.35+7.45%, mean + SD) followed by FBM, ABM, UCB, and MPB. Further analysis showed that FLV contained the highest proportion of $CD34^+/CD38^-/HLA\text{-}DR^+$ subset among the total $CD34^+$ cells (34.73+8.20% versus <2% in ABM). After 14 days of culture in medium containing stem cell factor (SCF), IL-3, IL-6, GM-CSF, erythropoietin (Epo), insulin-like growth factor-I (IGF-1) and basic fibroblast growth factor (b-FGF), the cloning efficiencies of singly sorted $CD34^+CD38^-$ cells were: FLV (63 ± 26%) > FBM (30 ± 21%) > UCB (15 ± 11%) > ABM (3 ± 2%) > MPB (1.5 ± 2%). Moreover, based on the growth pattern of the colonies, $CD34^+CD38^-/HLA\text{-}DR^+$ cells from FLV yield the highest percentages of colonies with dispersed growth pattern, indicative of a high proportion of primitive stem cells. Cells showing dispersed growth pattern were replated for up to four generations and $CD34^+/CD38^-/HLA\text{-}DR^+$ cells from FLV demonstrated the highest replating potential and ABM the least. Significantly higher expansion was found from $CD34^+CD38^-$ cells cultured in the presence of stromal layer. Each $CD34^+CD38^-$ cell from FLV produced 11,500 progeny cells, 2-fold higher than the same cells cultured without stroma. Our results suggested that fetal liver contains a significantly higher number of very primitive stem cells, which have a markedly greater replating potential and are ideal targets for delivery of gene therapy compared with ABM, or MPB.

INTRODUCTION

The increasing demand for hematopoietic stem cells for clinical transplantation and for gene therapy has spawned numerous studies to identify new sources of stem cells in vivo and developments of various culture systems for expansion in vitro. Stem or progenitor cell products for clinical use should provide both short and long-term engraftment. Short-term engraftment is characterized by recovery of clinically relevant levels of circulating neutrophils and platelets and is probably induced by lineage-committed progenitors, whereas long-term engraftment requires that a pool of pluripotent stem cells is established, providing mature blood cells of all lineages throughout lifetime.[1] Pluripotent hematopoietic stem cells are therefore characterized by both self-renewal capacity and differentiating potential to give rise to myeloid and lymphoid lineages.

The conventional source of hematopoietic progenitor cells for clinical use has been the bone marrow (BM). In the past decade, mobilized peripheral blood progenitor cells have been increasingly used in lieu of BM for autologous transplantation,[2–7] and in the past two years, also in allogeneic transplantation.[8–12] A major advantage is the accelerated hematopoietic recovery achieved by blood-derived versus marrow-derived progenitor cells. Other sources of hematopoietic stem cells for clinical use, such as umbilical cord blood (UCB) and fetal tissue, are being studied and their roles in transplantation and human gene therapy explored.[13–16] Present evidences indicate that there are not only significant phenotypic differences in the proportion of pluripotent versus lineage-committed progenitors but also functional differences among fetal tissue derived progenitors, UCB, adult bone marrow (ABM) and mobilized peripheral blood. The goal of the present study is to explore the potentials of $CD34^+$ cells derived from fetal tissues as a source for expansion of pluripotent stem cells with self-renewal capacity.

Hematopoietic progenitor cells have been demonstrated to be associated with the surface antigenic marker CD34.[17] At steady state, about 1 to 2% of the cells from the BM and <0.1% (nonmeasurable) of mononuclear cells (MNC) from the peripheral blood are positive for CD34.[7,8] By means of multicolor, multidimensional flow cytometry, it has been shown that the $CD34^+$ cells can be subcategorized into phenotypic subsets, some of which possess characteristics of stem cell "candidates" and others of lineage-committed progenitors. The pluripotent stem cell "candidates" are characterized by $CD34^+/CD38^-$ and can be further subclassified according to the presence or absence of HLA-DR, Thy-1, or staining with rhodamine, etc.[18–24] The lineage-committed subsets are characterized by $CD34^+/CD38^+$, and the co-expression of one of the following antigens: CD7, CD10, CD16, CD33, CD61, or CD71, depending on the lineage commitment.[18] It is not definitively known which $CD34^+$ subset represents the population with the highest self-renewal or multilineage potential. Possible candidates include $CD34^+/CD38^-/HLA\text{-}DR^+$, $CD34^+/HLA\text{-}DR^-$,

$CD34^+/Thy1^+/Lin^-$, or $CD34^+/CD45RA^{low}/CD71^{low}$, etc., probably depending on the assay system as well as on the source of the cells.[18–21] In this study, we have compared the functional characteristics of the $CD34^+/CD38^-/HLA\text{-}DR^+$ cells from various sources and have demonstrated that cells of this phenotype from fetal liver possess a 700–fold higher proliferative and replating potential as the cells with the same phenotype from ABM. Fetal liver (FLV) is a possible source for stem cell "candidates" with high self-renewal capacity.

MATERIALS AND METHODS

Cell preparations

Fetal liver or fetal BM cells were obtained from aborted or miscarried fetuses of 12 to 25 weeks of gestational age and used as approved by the Human Subjects Committee of the University of California, San Diego (UCSD). Umbilical cord blood samples were collected as previously described and as also approved by the Human Subjects Committee.[22] BM cells were isolated by flushing intramedullary cavities of the femurs with RPMI 1640 with 10% fetal calf serum (FCS).[18] The fetal liver or BM mononuclear cells were separated by Ficoll-Hypaque (Histopaque 1077; Sigma Chemical Co., St. Louis, MO) and washed twice.

For BM samples, healthy subjects between the ages of 18 to 65 were recruited for BM samples. Approximately 40–50 mL of BM were drawn from multiple sites (10 to 15 mL each time) from the posterior iliac crest. Subjects gave informed consent to have marrow samples collected.

For mobilized peripheral blood, healthy subjects were recruited using inclusion and exclusion criteria similar to those used for blood donors. The projects were reviewed and approved by the UCSD Human Subjects Committee.[8] Subjects gave informed consent to participation after explanation of the project. Baseline values of CD34 cells were determined and colony assays (see below) were performed. They were randomized into 3 groups, receiving a) 10 μg/kg/day G-CSF, b) 10 μg/kg/day GM-CSF or c) 5 μg/kg/day of each G-CSF and GM-CSF (G/GM). Growth factors were administered and leukapheresis was performed as previously described.[8] For the present comparative study, we have made use of the data from G/GM mobilization.

Flow cytometry

The progenitor cell preparations were labeled with CD34 (8G12 FITC), CD38 (Leu17 PE), and HLA-DR biotin followed by an incubation with streptavidin allophycocyanin (APC; Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Flow cytometric analysis and sorting was performed on a FACStar^{Plus} equipped with an Argon ion laser tuned at 488 nm and a Helium Neon laser (633 nm) or an Innova 90 tuned at 647 nm (BDIS). Data acquisition was performed with Lysis 2.0 (BDIS). Forward light scatter, orthogonal light scatter, and 3 fluorescence

signals were determined for each cell and the listmode data files were analyzed with the Paint-A-Gate^{Plus} software (BDIS).[18,19]

Cell sorting was performed on a FACStar^{Plus} (BDIS) using the Automated Cell Deposition Unit (ACDU), which permits single-cell sorting with an accuracy of greater than 99%. An index sorting device was used on the forward light scatter parameter and this new feature allows the linkage of list mode flow cytometry data to the location of the well in the microtiter plate. In addition, cells with large orthogonal and forward light scatter were excluded.

Clonogenic cultures

In the liquid culture system, each well contained a 200 μL mixture of myeloid long-term culture medium (Terry Fox Laboratory, Vancouver, BC, Canada) containing 12.5% horse serum, 12.5% FCS, 10^{-4} mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 0.2 mmol/L i-inositol, 20 μmol/L folic acid, and antibiotics, and supplemented with 2.5 U/mL recombinant human erythropoietin (rhEpo; Amgen, Thousand Oaks, CA), 10 ng/mL recombinant human interleukin-3 (rhIL-3), 500 U/mL rhIL-6, 10 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) with 2.5 ng/mL recombinant human basic fibroblast growth factor (rhbFGF), 10 ng/mL recombinant human insulin-like growth factor-1 (rhIGF-1; Collaborative Research, Bedford, MA), and 50 ng/mL recombinant human stem cell factor (rhSCF; Genzyme, Boston, MA). All cultures were incubated in 5% CO_2 in air at 37°C in a fully humidified incubator. Cell growth was checked on days 3, 5, 7, and 10 and scored for the presence of dispersed cells, cell clusters, or a mixture of both on day 14. Cells were scored as dispersed cells when an expansion was generated from a single cell of a minimum of 40 cells that still appeared as dispersed round translucent cells after 14 days. Replating of the cell progeny was performed by dispersion of the cells into 10 wells of a 96-well flat-bottom plate in identical culture conditions as described above. Plates were scored 14 days after replating of the cells originated from the single cells. Repetitive replating was performed under identical conditions.[18]

Staining for morphology and fluorescent examination

For flow cytometric analysis of the progeny of the single sorted cells, the cells in each well were harvested with a pipette, washed, and stained with a pretitered reagent mixture containing CD34 PE/CY5 (kindly provided by K. Davis, BDIS), CD33 PE, and CD19 FITC, or a mixture of fluorescently labeled isotype control antibodies. The immunostained cells were washed once, resuspended in 0.5 mL of a 0.5% paraformaldehyde solution and analyzed on a FACScan (BDIS).

Statistical analysis

For statistical analysis, a personal computer program, Testimate, supplied by IDV Datenanalyse, Gauting-Munich, Germany, was used. Data reported are mean

Table 1. Percentages of $CD34^+$ and $CD34^+/CD38^-$ subpopulations among MNC preparations

	CD34⁺	*FLV*	*FBM*	*UCB*	*ABM*	*MPB*
	n	8	9	12	9	7
$CD34^+$	mean ± SD	11.35 ± 7.45	10.46 ± 3.20	0.84 ± 0.71	1.71 ± 1.17	0.77 ± 0.53
$CD34^+/CD38^-$	mean ± SD	3.54 ± 3.96	1.62 ± 2.76	0.11 ± 0.17	0.11 ± 0.15	0.11 ± 0.14

± standard deviation, or medians and ranges, wherever applicable. The Kruskal-Wallis Analysis was applied to validate the differences in $CD34^+$ subsets among the MNC samples from different sources. The Mann-Whitney-U Test was then applied to verify the differences in concentrations of $CD34^+$ subsets between two different categories, e.g., FLV compared with fetal BM (FBM), UCB, ABM or mobilized peripheral blood (MPB).

RESULTS

In previous clinical trials, we have compared the immunophenotypes and colony formation capacities of the $CD34^+$ cells mobilized by different growth factors.[23] Using three-color and five-dimensional flow cytometry to compare the pluripotent CD34 positive subsets, we have provided evidences that the combination of G-CSF and GM-CSF stimulates a significantly higher proportion of pluripotent CD34 subsets than G-CSF alone, whereas GM-CSF by itself is not very efficient in mobilizing and adequate number of $CD34^+$ cells.[8,23] The combination G+GM mobilizes a significantly higher number of total $CD34^+/CD38^-$ cells and $CD34^+/CD38^-/DR^+$ subsets in the leukapheresis products than G-CSF alone. Data from this group of normal subjects (G+GM) were used for comparisons in the present study.

The percentages of the $CD34^+$ cells from various sources are summarized in Table 1. The MNC preparations from FLV and FBM contain the highest percentages of $CD34^+$ cells, followed by ABM, UCB and MPB. The highest percentage of $CD34^+/CD38^-$ cells is found in FLV (3.54 ±3 .96%), followed by FBM (1.62 ± 2.76%). UCB, ABM and MPB all contained very low levels of $CD34^+/CD38^-$ subsets. The difference between FLV versus UCB, ABM or MPB is highly significant (p=0.005).

Further characterization by coexpressions of HLA-DR or CDw90 (Thy-1)[4,18,19,23,24] has been shown to identify the primitive and pluripotent stem cell candidates. Figure 1 illustrates the results of three-color multiparameter flow cytometric analysis of the $CD34^+$ subpopulations of MNC samples from various sources. According to the coexpressions of CD38 and HLA-DR, the $CD34^+$ cells were sorted into four different categories; $CD34^+/CD38^-/DR^-$,

$CD34^+/CD38^-/DR^+$, $CD34^+/CD38^+/DR^-$ and $CD34^+/CD38^+/DR^+$ (represented in Figure 1 by 38^-DR^-, 38^-DR^+, 38^+DR^-, 38^+DR^+). The most prominent finding was the high proportion of $CD34^+/CD38^-/DR^+$ cells in FLV, i.e., 34.73 ± 8.20% (Mean ± SD) of total $CD34^+$ cells and to a much less extent in FBM, i.e., 7.27 ± 10.76%. Whereas UCB and MPB also contained a fairly high percentage of $CD34^+/CD38^-/DR^+$ cells (10.50 ± 5.77% and 14.19 ± 15.41%, respectively) among the total $CD34^+$ cells (which is equal to 100%), such phenotypically pluripotent cells are rare in ABM. The differences in percentages of $CD38^-/DR^+$ cells between ABM versus FLV or UCB are significant (p=0.0143, 0.0381, respectively), whereas no differences in percentages of $CD34^+/CD38^-/DR^+$ cells were found among FLV, FBM, UCB or MPB.

To determine the functional differences among the progenitors from various sources with the same phenotype, we have applied index sorting and single cell culture technique to study the growth characteristics of $CD34^+$ cells and their subsets. Cloning efficiency (CE) was determined by the number of wells, each initially containing one single cell sorted into 96-well plates, that grow into colonies after 14 days. Our previous reports have shown that the pluripotency and replating potential of the hematopoietic progenitor cells are indicated by distinct growth patterns in the single cell culture system. Pluripotent stem cells show a dispersed growth pattern and 70% of these dispersed cells gave rise to a second, third and eventually fourth generation of colonies after repetitive replating, characteristic of pluripotent stem cells.[18,19]

The colony efficiencies (CE) of the $CD34^+$ subsets from various sources are summarized in Figures 2A–E, in which the percentage of wells containing colonies are represented by the stacked columns. The pluripotency of the individual cells deposited was reflected by the growth pattern after 14 days of culture. Some demonstrated a dispersed growth pattern (represented by the dark columns), others grow into a mixed pattern with clusters scattered among dispersed blast cells (represented by shaded columns), whereas others grow into typical granulocytic clusters (represented by criss-crossed columns) or erythroid clusters (represented by white columns). Overall, the highest colony efficiencies were found in FLV, followed by FBM, UCB, MPB and ABM. Using the combination CD34, CD38 and HLA-DR as the discriminating panel for stem cell "candidates", $CD34^+/CD38^-$/HLA-DR^+ cells demonstrated the highest CE among samples of FLV, BVM, and UCB, whereas $CD34^+/CD38^+$ subsets among ABM and MPB might show higher CE than the $CD38^-$ subsets. Whereas cells with dispersed growth pattern were found predominantly in the $CD38^-$ subsets, such cells were occasionally encountered among $CD38^+$ subsets among UCB, ABM and MPB.

We have studied the impact of BM stroma on ex vivo culture of $CD34^+/CD38^-$ cells. $CD34^+CD38^-$ and $CD34^+CD38^+$ cells derived from FBM were sorted (20 cells/well) directly onto irradiated marrow stromal layer with and without growth

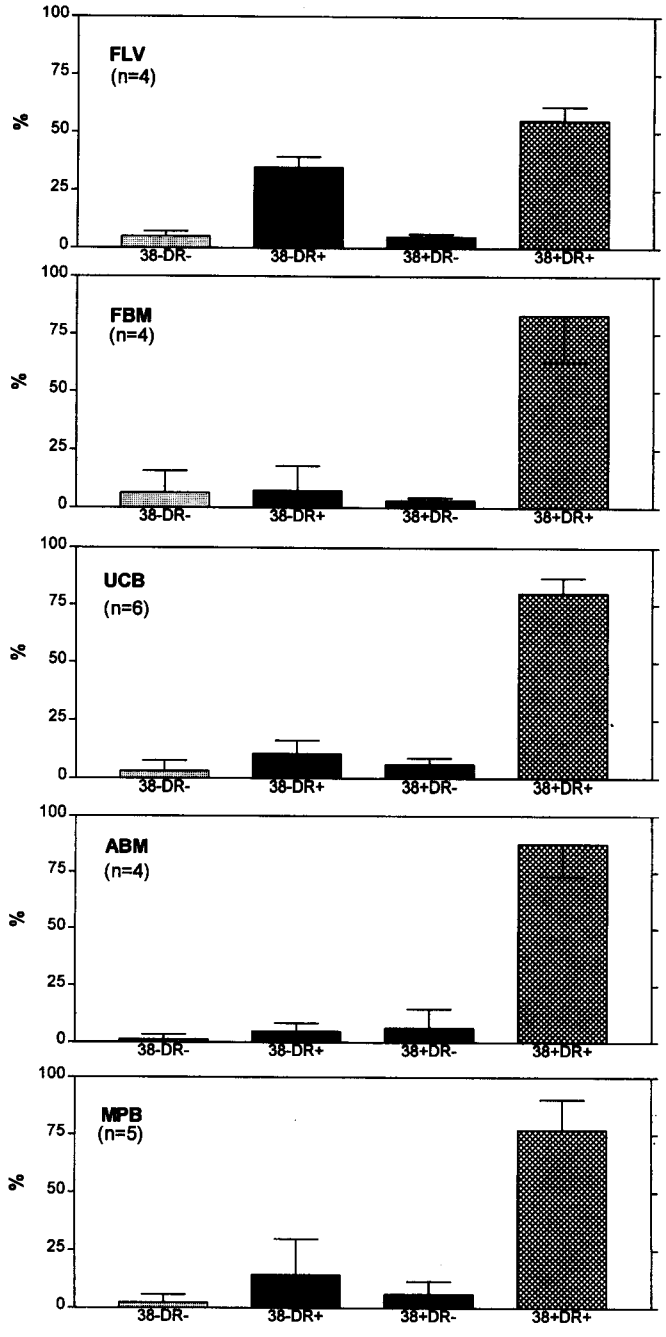

Figure 1. *CD34⁺ subsets from FLV, FBM, UCB, ABM and MPB (mobilized with GM-CSF and G-CSF).*

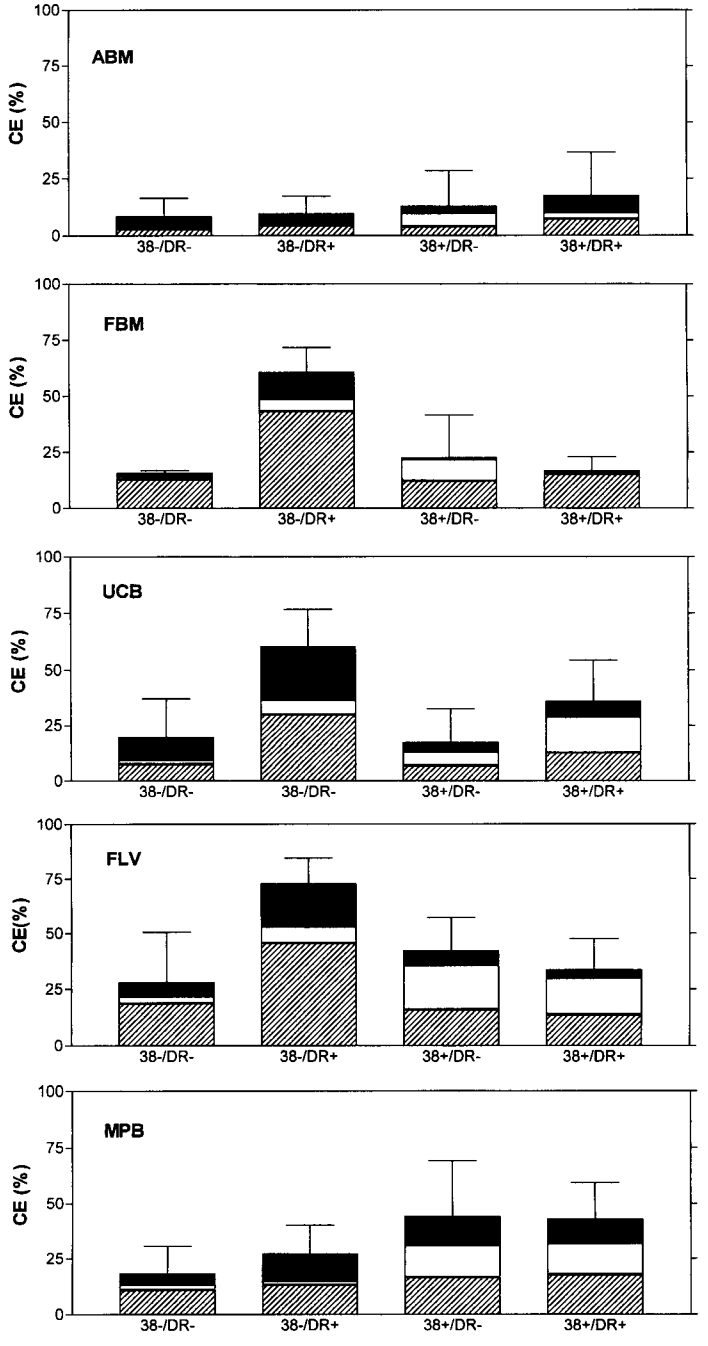

Figure 2. *Colony efficiency and growth pattern of the colonies from CD34⁺ subsets derived from FLV, FBM, UCB, ABM and MPB.*

factors (GF). After 14 days of culture, significantly greater expansion was detected from $CD34^+CD38^-$ cells cultured on the stroma with GF. More cells with dispersed growth pattern appeared in the $CD34^+CD38^-$ subset. Each $CD34^+CD38^-$ cell produced 11,500 progeny cells, 2-fold higher than the same subset cultured in GF without stroma. However, if the $CD34^+CD38^-$ cells were subsequently replated in media containing GFs after the initial exposure to GF alone, stroma alone or to both, the overall increase in cell number was similar in all circumstances. The cells originally cultured in stroma only reached a maximum after 4 additional weeks (total of 6 weeks), whereas cells cultured in GF or GF + stroma reached the highest cell number in 4 weeks. The results indicated that 1) the marrow stroma layer can maintain the primitive stem cells in their quiescent status until exposure to GF; 2) the proliferative potential of the $CD34^+CD38^-$ cells is realized on exposure to GF; and 3) although stroma layer can enhance the expansion of cells by GF in short-term culture, the overall expansion capacity of progenitor cells remains unaffected.

DISCUSSION

Transplantation of hematopoietic stem cells, derived from fetal marrow or fetal liver have been proposed as a novel strategy for acquired and hereditary BM disorders.[25–30] In utero transplantation of fetal stem cells has been performed successfully.[25,26] It seems that the fetuses are suitable for stem cell transplantation both as donors and recipients, and that engrafted cells can respond functionally to hematologic stimuli just as the endogenous cells.[25–28] Completely HLA-mismatched fetal liver stem cells have been used successfully in the treatment of patients with severe combined immunodeficiency and in patients with aplastic anemia.[28–30] Additional advantages include higher self-renewal and proliferative potential of fetal-derived stem cells,[31] which are more susceptible to retroviral mediated gene transfer than quiescent stem cells derived from adult BM or peripheral blood. Stem cells derived from fetal liver may therefore represent another source for expansion as well as for establishing universal stem cell cultures for transplantation.

As part of our ongoing efforts to identify an optimal source of pluripotent stem cells for expansion, for transplantation and for gene therapy, we have compared the proliferative potentials of $CD34^+$ subsets derived from FLV, FBM, UCB, ABM and MPB. Although a series of reports have indicated that fetal tissues contain a significantly higher concentration of stem cells,[25–29] comparison on the quantitative and qualitative differences among the $CD34^+$ cells from various sources is lacking. We have previously shown that the proportion of rare pluripotent stem cells, represented by $CD34^+/CD38^-$ or $CD34^+/CD38^-/HLA\text{-}DR^-$ cells is significantly higher in leukapheresis products mobilized by the combination of G-CSF and GM-CSF versus G-CSF alone, despite similar numbers of total $CD34^+$ cells in the

product.[8,23] As an adequate proportion of primitive pluripotent stem cells will be essential for a sustained, life-long reconstitution after allogeneic transplantation, or for long-term effect after gene therapy, analysis of the $CD34^+$ subsets and their functional integrity are of significance for choice of starting material for expansion or for gene manipulation.

In this study, we have demonstrated that mononuclear preparations from FLV contain not only the highest concentrations of $CD34^+$ cells, but also the highest proportion of pluripotent subsets $CD34^+/CD38^-$, and $CD34^+/CD38^-$ /HLA-DR^+ cells among the total $CD34^+$ cells. In addition, the cloning efficiencies of singly sorted $CD34^+/CD38^-$/HLA-DR^+ cells from FLV were at least one log higher than those from ABM or MP. Moreover, based on the growth pattern of the colonies, $CD34^+/CD38^-$/HLA-DR^+ cells from FLV yield the highest percentages of colonies with dispersed growth pattern, indicative of a high proportion of primitive stem cells. Upon replating, these cells were able to yield further blast colonies and colonies with mixed growth pattern for up to four generations. Cells with the same immunophenotype from FBM, UCB, or MPB have substantially less and those from ABM have hardly any replating potential. We estimate that the proliferative potential of each $CD34^+/CD38^-$/HLA-DR^+ cell from FLV is approximately 700-fold higher than a corresponding cell from ABM. When cultured in the presence of stromal layer, each $CD34^+CD38^-$/HLA-DR^+ cell from FLV produced respectively 11,500 progeny cells, 2-fold higher than the same cells cultured without stroma.

In vitro expansion of pluripotent stem cells have been the focus of many studies.[32–40] Expansion of primitive, pluripotent progenitors is likely the result of a balance between self-renewal with maintenance of pluripotency, and differentiation with loss of long-term culture-initiating cells (LTC-IC). Although many culture systems have been developed that allow expansion of candidate stem cells, no system has yet been described that allows long-term expansion of primitive progenitors. Stroma layers derived from BM (autologous, allogeneic or xenogeneic) have been shown to support early progenitor cell proliferation and differentiation for 5 or more weeks but only 20% of LTC-IC are maintained.[32–35] Primitive progenitors can also be induced to proliferate and differentiate in stroma-free cytokine-supplemented long-term cultures but LTC-IC are rarely expanded or maintained beyond 2 to 4 weeks.[19,36–37] When cocultured with stroma but separated from the stroma layer by a microporous membrane (stroma-noncontact cultures), a fraction of primitive LTC-IC seems to be maintained for 5 to 8 weeks of culture.[38] The addition of IL-3 and macrophage inflammatory protein-1α *(MIP-1α)* resulted in complete maintenance or expansion for up to 8 weeks.[39] Using a three dimensional culture system (bioreactor), Koller et al was able to expand LTC-IC numbers.[33] It remains unresolved whether the primitive cells are permanently lost from the stem cell compartment during ex vivo expansion, resulting in reduction of long-term engraftment potential. Some authors reported that cells with the highest

proliferative capacity remained quiescent during in vitro culture[40,41] and conditions for maximizing self-renewal capacity may be very different from those for conventional "expansion" of hematopoietic progenitors.

Our results suggest that fetal liver contains a significant number of very primitive stem cells that are not found in similar magnitude in FBM or UCB. The frequency of such phenotypically and functionally primitive stem cells in adult material such as MPB or ABM is extremely rare. The aim of ex vivo expansion of human progenitor cells for allogeneic transplantation and for gene therapy is to multiply both cells with the highest self-renewal capacity for life-long reconstitution, as well as lineage-committed progenitors for rapid recovery. Fetal liver derived stem cells probably represent an excellent cell source but optimal conditions for expansion of these primitive stem cells has yet to be established.

In summary, although many studies have demonstrated that candidate stem cells from adult BM or peripheral blood can be expanded exponentially in number, such ex vivo cultures are always associated with depletion of primitive LTC-IC and no expansion of pluripotent stem cells has yet been shown. The use of stroma combined with new cytokines or three-dimensional culture system allows a limited multiplication of pluripotent stem cells, probably only for a limited period of time.[23,24] Hematopoietic cells derived from fetal tissues contain, however, significantly higher proportion of pluripotent stem cells and few, if any, alloreactive cells, they might represent ideal resources for expansion of primitive stem cells for transplantation and for gene therapy.

REFERENCES

1. Williams DA: Ex vivo expansion of hematopoietic stem and progenitor cells—Robbing Peter to pay Paul. *Blood* 82:3169, 1993.
2. Kessinger A, Armitage JO: The evolving role of autologous peripheral stem cell transplantation following high-dose therapy for malignancies. *Blood* 77:211–213, 1991.
3. Körbling M, Dörken B., Ho AD et al.: Autologous transplantation of blood-derived hemopoietic stem cells after myeloablative therapy in a patient with Burkitt's lymphoma. *Blood* 67:529–532, 1986.
4. Kessinger A, Armitage JO, Landmark JD et al.: Reconstitution of human hematopoietic function with autologous cryopreserved circulating stem cells. *Exp Hematol* 114:192–196, 1986.
5. Sheridan WP, Begley CG, Juttner CA et al.: Effect of peripheral-blood progenitor cells mobilized by filgrastim (G-CSF) on platelet recovery after high-dose chemotherapy. *Lancet* 339:640–644, 1992.
6. Bensinger W, Singer J, Appelbaum F et al.: Autologous transplantation with peripheral blood mononuclear cells collected after administration of recombinant granulocyte stimulating factor. *Blood* 81:3158–3163, 1993.
7. Ho AD, Glück Germond C, Sinoff C, Dietz G, Maruyama M, Corringham RET: Optimal

timing for collections of blood progenitor cells following induction chemotherapy and granulocyte-macrophage colony-stimulating factor for autologous transplantation in advanced breast cancer. *Leukemia* 7:1738–1746, 1993.

8. Lane TA, Law P, Maruyama M, Young D, Burgess J, Mullen M, Mealiffe M, Terstappen LWMM, Hardwick A, Moubayed M, Oldham F, Corringham RET, Ho AD: Harvesting and enrichment of hematopoietic progenitor cells mobilized into the peripheral blood of normal donors by granulocyte macrophage-colony stimulating factor (GM-CSF) or G-CSF: Potential role in allogeneic marrow transplantation. *Blood* 85:275–282, 1995.
9. Zecca M, Perotti C, Marradi P, Montagna D, Giorgiani G, Balter R, Prete L, Locatelli F: Recombinant human g-csf-mobilized peripheral blood stem cells for second allogeneic transplant after bone marrow graft rejection in children. *Br J Haematol* 92:432, 1996.
10. Bensinger WI, Weaver CH, Appelbaum FR, Rowley S, Demirer T, Sanders J, Storb R, Buckner CD: Transplantation of allogeneic peripheral blood stem cells mobilized by recombinant human granulocyte colony-stimulating factor [see comments]. *Blood* 85:1655, 1995.
11. Korbling M, Huh YO, Durett A, Mirza N, Miller P, Engel H, Anderlini P, van B K, Andreeff M, Przepiorka D, et al: Allogeneic blood stem cell transplantation: peripheralization and yield of donor-derived primitive hematopoietic progenitor cells ($CD34^+$ thy-1 dim) and lymphoid subsets, and possible predictors of engraftment and graft-versus-host disease. *Blood* 86:2842, 1995.
12. Corringham RET, Ho AD: Rapid and sustained allogeneic transplantation using immunoselected $CD34^+$-selected peripheral blood progenitor cells mobilized by recombinant granulocyte- and granulocyte-macrophage colony-stimulating factors. *Blood* 86:2052–2054, 1995.
13. Broxmeyer, HE, Hangoc G, Cooper S, Ribeiro RC, Graves V, Yoder M, Wagner J, Vadhan-Raj S, Bennminger L, Rubinstein P, Broun ER: Growth characteristics and expansion of human umbilical cord blood and estimation of its potential for transplantation in adults. *Proc Natl Acad Sci USA* 89:4109, 1992.
14. Gluckman E, Thierry D, Lesage S et al.: Clinical applications of stem cell transfusion from cord blood. *Transfusion Sci* 13:415, 1992.
15. Rubinstein P, Rosenfield RE, Adamson JW, Stevens CE: Stored placental blood for unrelated bone marrow reconstitution. *Blood* 81:1679, 1993.
16. Kurtzberg J, Laughlin M, Graham ML, Smith C, Olson JF, Halperin ED, Ciocci G, Carrier C, Stevens CE, Rubinstein P: Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. *New Engl J of Med* 335:157–166, 1996.
17. Civin I, Banquerigo ML, Strauss LC, Loken MR: Antigenic analysis of hematopoiesis. IV. Characterization of MY10-positive progenitor cells in normal human bone marrow. *Exp Hematol* 15:10–17, 1987.
18. Huang S, Terstappen LW: Lymphoid and myeloid differentiation of single human $CD34^+$, $HLA\text{-}DR^+$, $CD38^-$ hematopoietic stem cells. *Blood* 83:1515–1526, 1994.
19. Terstappen LWMM, Huang S, Safford M, Lansdorp PM, Loken MR: Sequential generations of hematopoietic colonies derived from single nonlineage-committed $CD34^+/CD38^-$ progenitor cells. *Blood* 77:1218–1227, 1991.
20. Bender JG, Unversagt KL, Walker DE, Lee W, Van Epps DE, Smith DH, Stewart CC,

To LB: Identification and comparison of CD34-positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry. *Blood* 77:2591–2596, 1991.

21. Lansdorp PM, Dragowska W: Long-term erythropoiesis from constant numbers of $CD34^+$ cells in serum-free cultures initiated with highly purified progenitor cells from human bone marrow. *J Exp Med* 175:1501–1509, 1992.
22. Yu Mang, Leavitt MC, Maruyama M, Yamada O, Young D, Ho AD, and Wong-Staal, F: Intracellular immunization of human fetal cord blood stem/progenitor cells with a ribozyme against human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* 92:699–703, 1995
23. Ho AD, Young D, Maruyama M et al.: Pluripotent and lineage-committed $CD34^+$ subsets in leukapheresis products mobilized by G-CSF, GM-CSF versus a combination of both. *Exp Hematol* (In Press), 1996.
24. Mayani H, Lansdorp PM: Proliferation of individual hematopoietic progenitors purified from umbilical cord blood. *Exp Hematol* 23:1453–1462, 1995.
25. Zanjani ED, Ascensao JL, Flake AW, et al: The fetus as an optimal donor and recipient of hemopoietic stem cells. *Bone Marrow Transplantation* 10:107–114, 1992.
26. Zanjani ED, Ascensao JL, Tavassoli M: Liver-derived fetal hematopoietic stem cells selectively and preferentially home to the fetal bone marrow. *Blood* 81:399–404, 1993.
27. Rice HE, Emani VR, Skarsgard RA, et al: Human fetal liver hematopoietic cell expansion with a novel bioreactor system. *Transplantation Proceedings* 26:3338-3339, 1994.
28. Roncarlo MG, Bacchetta R, Bigler M, et al.: A SCID patient reconstituted with HLA-incompatible fetal stem cells as a model for studying transplantation tolerance. *Blood Cells* 17:391–402, 1991.
29. Roncarlo MG, Bacchetta R: T cell repertoire and tolerance after fetal stem cell transplantation. *Bone Marrow Transplant* 9(suppl.):127–128, 1992.
30. Han JR, Yuan SW, Ren QF: Clinical and experimental study of treating aplastic anemia with fetal liver cell suspension and fetal liver cell-free suspension. *Chung-Hua Nei Ko Tsa Chih* (Chinese Journal of Internal Medicine) 29:347–349, 1990.
31. Pechle C, Migliaccio AR, Migliaccio G et al.: Identification and characterization of three classes of erythroid progenitors in human fetal liver. *Blood* 58:565–572, 1981.
32. Fraser C, Szilvassy S, Eaves C, Humphries R: Proliferation of totipotent hematopoietic stem cells in vitro with retention of long-term competitive in vivo reconstituting ability. *Proc Natl Acad Sci USA* 89:1968, 1992.
33. Koller MR, Palsson MA, Manchel L, Palsson BO: Long-term culture-initiating cell expansion is dependent on frequent medium exchange combined with stromal and other accessory cell effects. *Blood* 86:1784-1793, 1995.
34. Koller MR, Manchel I, Newsom BS, Palsson MA, Palsson BO: Bioreactor expansion of human bone marrow: Comparison of unprocessed, density-separated, and CD34 enriched cells. *J Hematother* 4:159–169, 1995.
35. Davis TA, Robinson DH, Lee KP, Kessler SW: Porcine brain microvascular endothelial cells support the in vitro expansion of human primitive hematopoietic bone marrow progenitor cells with a high replating potential: Requirement for cell-to-cell interactions and colony-stimulating factors. *Blood* 85:1751–1761, 1995.

36. Brandt J, Srour E, Van Besien K, Bridell R, Hoffman R: Cytokine-dependent long-term culture of highly enriched precursors of hematopoietic progenitor cells from human marrow. *J Clin Invest* 86:932, 1990.
37. Mayani H, Lansdorp PM: Proliferation of individual hematopoietic progenitors purified from umbilical cord blood. *Exp Hematol* 23:1453–1462, 1995.
38. Verfaillie CM, Miller JS: A novel single-cell proliferation assay shows that long-term culture-initiating cell (LTC-IC) maintenance over time results from the extensive proliferation of a small fraction of LTC-IC. *Blood* 86:2137–2145, 1995.
39. Mayani H, Little MT, Dragowska W, Thornbury G, Lansdorp PM: Differential effects of the hematopoietic inhibitors MIP-1α, TGF-β, and TNF-α on cytokine-induced proliferation of subpopulations of $CD34^+$ cells purified from cord blood and fetal liver. *Exp Hematol* 23:422–427, 1995.
40. Traycoff CM, Kosak ST, Grigsby S, Srour EF: Evaluation of ex vivo expansion potential of cord blood and bone marrow hematopoietic progenitor cells using cell tracking and limiting dilution analysis. *Blood* 85:2059–2068, 1995.
41. Young JC, Varma A, DiGiusto D, Backer MP: Retention of quiescent hematopoietic cells with high proliferative potential during ex vivo stem cell culture. *Blood* 87:545–556, 1996.

IN VITRO EXPANSION OF PERIPHERAL BLOOD PROGENITOR CELLS

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Hematopoietic progenitor cells derived from peripheral blood are increasingly used to restore the formation of blood after high-dose chemotherapy (HDCT) for solid tumors or hematologic cancers. Compared to rescue by autologous bone marrow (BM) cells, restoration with peripheral blood progenitor cells (PBPC) shortens the period of pancytopenia after transplantation and reduces the risk of infection and bleeding. Interestingly, recent data suggest that cytokine-stimulated BM cells may also mediate rapid hematopoietic recovery with a time-course of neutrophils comparable with PBPC transplantation (Elfenbein, 8th AMBT 1996; Arlington, TX).

A combination of chemotherapy followed by administration of G-CSF enables $>2\times10^6$ CD34 positive cells per kg of body weight to be harvested in most patients. Transplantation of this population after HDCT routinely leads to rapid hematopoietic recovery, mostly with neutrophil counts greater than 500 per cubic millimeter within 10–15 days and platelet counts greater than 20,000 per cubic millimeter within 11–16 days. However, even when extensive numbers of PBPC are harvested and transplanted, further shortening of the cytopenic period has not been observed.

Also, harvesting and transplanting excessive numbers of autologous PBPC cells may increase the risk of graft-mediated relapse of disease, since—by analogy to conventional BM grafts—these PBPC grafts may be contaminated with tumor cells.[1] Tumor cells are co-mobilized together with hematopoietic PC by chemotherapy or granulocyte colony-stimulating factor (G-CSF), although possibly with a different kinetic pattern. These graft-contaminating tumor cells are biologically active and can mediate relapse of neoplastic disease, as shown in clinical retroviral marking studies.[2]

Therefore, as a first approach to reducing the tumor cell load in PBPC grafts, we positively selected cells carrying the CD34 antigen, using a CellPro immunoaffinity column. Autologous transplantation of these CD34-enriched PC also leads to rapid and complete hematopoietic restoration after HDC, and follows an identical time course and pattern as compared to unseparated PB mononuclear cells.[3]

By CD34-enrichment we have also effectively reduced the tumor cell load of our grafts by 2–3 logs compared with unseparated cells. This is provided, however, that

tumors are not carrying the CD34 antigen and are not themselves enriched. In specific cases, such as breast cancer and other malignancies, this may pose a problem.

In order to further reduce the number of tumor cells reinfused into the patient, we used in vitro culture technology to expand normal progenitor and stem cells and to provide a biologic purging effect. We hypothesized that harvesting only a small number of PBPC, and specifically amplifying these cells ex vivo to numbers sufficient for transplantation, would allow us to harvest and transplant the minimum number of tumor cells.

In order to make an ex vivo expansion strategy successful overall, conditions had to be defined in which PBPC, but not contaminating tumor cells, are expanded in vitro. This would result in several benefits, including a sufficient number of clonogenic progenitors, even for repetitive clinical use, and avoiding leukapheresis because a small volume of 100 mL of PB might be sufficient. Moreover, freezing the cells might not be necessary, since the cells are cultured while the patient is undergoing HDC.

To determine the conditions for this concept, our group has developed an experimental protocol for growing progenitor cells ex vivo from a relatively small volume of blood.[4] $CD34^+$ cells were cultured for up to 28 days using mainly RPMI medium, a cocktail of rhCSFs and autologous plasma. All features of the experimental scale protocol also had to provide the option for clinical scale application. For this reason, a stromal cell layer was not included to feed cultured hematopoietic cells. Numerous conditions and combinations of HGF were tested to obtain optimal yields of colony-forming cells (CFC). A combination of rhIL-1β, IL-3, IL-6, SCF and EPO provided best results in terms of amplification of total mononuclear cells and number of CFC.[4] The total number of CFC was expanded about 100–200-fold, and the maximum expansion was reached after a two week culture period.

To achieve sustained production of post-progenitor and effector cells after transplantation, it is critical to maintain the primitive progenitor and stem cells with extensive proliferative capacity. To test for primitive progenitor cells, we assayed long-term culture-initiating cell (LTC-IC) at different time points and demonstrated that LTC-IC levels are maintained for 2–3 weeks.[5]

Based on these preclinical data, we moved from bench to bedside and tested our protocol in a clinical study. The ability of ex vivo–expanded progenitor cells to mediate hematopoietic reconstitution after high-dose therapy was tested.[6] Ten patients with advanced cancer (NSCL, n=5; nasopharyngeal, n=1; breast, n=1; soft tissue sarcoma, n=1; CUP, n=2) who were eligible for HDCT were included in this phase I-II trial. They were between 25 and 57 years of age.

Patients received two cycles of induction chemotherapy at an interval of three weeks, consisting of etoposide (500 mg/m^2), ifosfamide (4000 mg/m^2), cisplatin (50 mg/m^2) and epirubicin (50 mg/m^2), a regimen previously shown to be active

against a variety of cancers.[7] Twenty-four hours after the second cycle of chemotherapy, the patients received filgrastim (G-CSF; Neupogen, AMGEN) at a dose of 5 micrograms per kg of body weight subcutaneously to treat chemotherapy-associated neutropenia and to mobilize PBPC. PBPC were collected in a single leukapheresis in which 7 liters of blood were processed.[8] CD34-positive cells were selected by immunoadsorption columns (Ceprate SC; CellPro).

For clinical transplantation, the starting population for ex vivo culture consisted of a total of 15×10^6 cells after CD34 selection (about 10% of the leukapheresis yield). The cells were grown in RPMI 1640 medium, 2% autologous plasma, rhSCF, rhIL-1β, rhIL-3, rhIL-6 and rhEpo for 12 days.[4] Nonadherent cells were collected, washed and resuspended for reinfusion. High-dose chemotherapy (etoposide 1500 mg/m^2, ifosfamide 12 g/m^2, carboplatin 750 mg/m^2, epirubicin 150 mg/m^2) was administered three weeks after the most recent induction treatment, and the expanded progenitor cells were reinfused 24 hours after this therapy. No toxic side effects were observed by infusion of the cells generated ex vivo.

The grafts contained a median of 12×10^6 expanded nucleated cells per kg, representing a 62-fold median increase. Cultured cells gave rise to erythroid, granulocyte-macrophage and multi-lineage colonies, with a 50-fold increase in clonogenic cells. A median of 1.2×10^5 CFCs per kg were generated and transplanted.[6]

Four patients simultaneously received uncultured CD34-positive cells in addition to cells generated ex vivo, in order not to challenge hematopoietic recovery while possible toxic effects induced by cultured cells were still being evaluated.

Hematopoietic recovery was rapid in 9 patients, while one patient died on day 14 due to neutropenic septicemia. After transplantation of uncultured and expanded cells, the median duration of a neutrophil count below 100 cells per cubic millimeter was 5 days (range 5–7), and median duration of a platelet count below 20,000 was 12 days (range 11–15). When expanded cells only were transplanted, neutropenia lasted 6 days (range 3–11), and thrombocytopenia 12 days (range 11–15). No secondary cytopenic nadir was observed in any patient.

This study demonstrates for the first time the ability of autologous progenitor cells generated ex vivo to restore hematopoiesis after HDCT in cancer patients.

The degree, time course and pattern of reconstitution were similar to historical control patients treated either with unseparated mononuclear cells or with CD34 selected cells.[3] We had shown earlier that PBPC significantly shorten the duration of neutropenia and thrombocytopenia by 5–7 days in patients receiving VIP, compared with no cellular support.[8] Compared with this study, the cytotoxic dose was higher in the ex vivo expansion and high-dose regimen.

To test whether we also had reduced the number of contaminating tumor cells in expanded grafts compared with uncultured cells, and whether tumor cells are

co-expanded together with hematopoietic PC in vitro, cells derived from tumor cell lines were co-cultured with $CD34^+$ fractions at varying ratios.[9] Whereas total MNC expanded about 100-fold, tumor cells from MCF-7, LXFS or primary RS-85 were maintained in culture but did not increase in number for two weeks. Upon replating residual tumor cells into cultures conditions that favor growth of these tumor cell lines, tumor cells did regrow and thereby continued to express biological activity in vitro.

Thus, we have shown that clinical ex vivo expansion is clinically feasible and safe, and that normal PBPC are specifically amplified as opposed to tumor cells.

To transfer our protocol to complete myeloablative and allogeneic settings, it is critical to clarify whether long-term repopulating stem cells are maintained ex vivo using our conditions. Our clinical expansion study could not address this issue, since endogenous hematopoiesis can substantially contribute to hematopoiesis if HDC is not completely myeloablative, and endogenous hematopoiesis cannot be differentiated or ruled out in an autologous situation.

For a long time, there has been no assay for pluripotent hematopoietic stem cell (PHSC) in the human system, since all cells defined and assayed in vitro have proven to be primitive progenitors rather than true PHSC, including LTC-IC, CAFC and CFU-Blast.[10] As a novel approach, primitive stem cells can be assayed using immunodeficient mice into which human hematopoietic cells can be xenotransplanted and monitored for extended periods.[11]

To develop an improved in vivo system, we have generated transgenic immunodeficient SCID mice that carry and express the genes for three human growth factors: hIL-3, GM-CSF and SCF.[12] These transgenic mice support human hematopoiesis for several months, and one can identify and classify these human hematopoietic cells within the murine background. We are currently using this model as a pre-clinical test to determine whether ex vivo expanded cells contain human PHSC.

We therefore can conclude that transplantation of autologous PBPC generated ex vivo restores hematopoiesis after HDCT in cancer patients. No toxic side effects related to the cellular product were observed. The degree of reconstitution is similar to results in historical control patients treated either with unseparated mononuclear cells or CD34 selected cells.

CD34-selected cells (10×10^8) yield sufficient numbers of progenitor cells to permit rapid and sustained hematopoietic recovery after HDCT in adults. This represents about 1/10 of the number of uncultured cells that are harvested for transplantation. When logistics were optimized, ex vivo culture was performed in parallel to HDC and the graft cells did not require cryopreservation.

Overall, the combined modalities of specific ex vivo expansion and CD34 enrichment of PBPC reduces the number of tumor cells that are reinfused into the patient by 4–5 logs.

Ex vivo expansion appears to be an attractive strategy for reducing tumor cell contamination of PBPC grafts. Its future potential includes the generation of tumor cell-reduced stem cell grafts sufficient even for repetitive clinical use. Moreover, lineage-specific generation of myeloid or megakaryocytic post progenitor cells might provide the option to further reduce the period of pancytopenia after transplantation.

A future prospect is the generation of immune cells, such as dendritic cells from PBPC grafts, that may induce autologous tumor-specific responses.

REFERENCES

1. Brugger W, Bross KJ, Glatt M et al.: Mobilization of tumor cells and hematopoietic progenitor cells into peripheral blood of patients with solid tumors. *Blood* 83:636–640, 1994.
2. Rill DR, Santana VM, Roberts WM et al.: Direct demonstration that autologous bone marrow transplantation for solid tumors return a multiplicity of tumorigenic cells. *Blood* 84:380–383, 1994.
3. Brugger W, Henschler R, Heimfeld S et al.: Positively selected autologous blood CD34+ cells and unseparated peripheral blood progenitor cells mediate identical hematopoietic engraftment after high-dose VP16, ifosfamide, carboplatin, and epirubicin. *Blood* 84:1421–1426, 1994.
4. Brugger W, Möcklin W, Heimfeld S et al.: Ex vivo expansion of enriched peripheral blood $CD34^+$ progenitor cells by stem cell factor, interleukin-1β (IL-1β), IL-6, IL-3, interferon-γ, and erythropoietin. *Blood* 81:2579–2584, 1993.
5. Henschler R, Brugger W, Luft T et al.: Maintenance of transplantation potential in ex vivo expanded $CD34^+$-selected human peripheral blood progenitor cells. *Blood* 84:2898–2903, 1994.
6. Brugger W, Heimfeld S, Berenson RJ et al.: Reconstitution of hematopoiesis after high-dose chemotherapy by autologous progenitor cells generated ex vivo. *N Engl J Med* 333:283–287, 1995.
7. Brugger W, Frisch J, Schulz G et al.: Sequential administration of interleukin-3 and granulocyte-macrophage colony stimulating factor following standard-dose combination chemotherapy with etoposide, ifosfamide, and cisplatin. *J Clin Oncol* 10:1452–1459, 1992.
8. Brugger W, Birken R, Bertz H et al.: Peripheral blood progenitor cells mobilized by chemotherapy plus granulocyte-colony stimulating factor accelerate both neutrophil and platelet recovery after high-dose VP16, ifosfamide and cisplatin. *Br J Haematol* 84:402–407, 1993.
9. Vogel W, Behringer D, Scheding S et al.: Ex vivo expansion of $CD34^+$ peripheral blood progenitor cells: Implications for the expansion of containing epithelial tumor cells. *Blood* 88:2707–2713, 1996.
10. Orlic D and Bodine DM: What defines a pluripotent hematopoietic stem cell (PHSC): Will the real PHSC please stand up? *Blood* 84:3991–3994, 1994.
11. Dick JE, Lapidot T, Pflumio F: Transplantation of normal and leukemic human bone

marrow into immune-deficient mice: Development of animal models for human hematopoiesis. *Immunol Rev* 124:25–43, 1991.

12. Bock TA, Orlic D, Dunbar CE et al.: Improved engraftment of human hematopoietic cells in severe combined immunodeficient (SCID) mice carrying human cytokine transgenes. *J Exp Med* 182:2037–2043, 1995.

IN VIVO PRIMING WITH G-CSF BEFORE HARVEST ENHANCES THE HEMATOPOIETIC RECOVERY POTENTIAL NOT ONLY OF PERIPHERAL BLOOD PROGENITOR CELLS BUT ALSO OF MARROW CELLS

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INTRODUCTION

Hematopoietic recovery after infusion of marrow progenitor cells is obtained within three weeks.[1,2] A similar recovery pattern is achieved with progenitors collected from steady state peripheral blood.[3] In contrast, transplantation of stem cells mobilized to the blood during recovery from chemotherapy, growth factor administration or a combination of both enhance the rate of recovery; within 12 days neutrophils >1000/mm^3 and platelets >20,000/mm^3.

We report here that increased numbers of progenitors in the marrow—cells which are CD34$^+$—can be harvested after a brief exposure to granulocyte colony-stimulating factor (G-CSF). Transplantation of these cells after high-dose chemotherapy in stage IV breast cancer patients hastens recovery, similar to the rate observed when comparable numbers of mobilized peripheral stem cells are infused. It appears in these studies that G-CSF has an activating effect on the engraftment potential of progenitor cells in the marrow prior to mobilizing them into the bloodstream.

METHODS

Patients and pre-harvest evaluation

Fifty-seven consecutive Stage IV breast cancer patients, ranging in age from 32–60 years, consented to have marrow harvested under local anesthesia before and after the administration of varying doses of G-CSF. All were receiving chemotherapy in 21 day cycles according to the CAVE (Cyclophosphamide 500 mg/m^2, Adriamycin 50 mg/m^2 and Etoposide 80 mg/m^2) regimen as approved by the Arlington Cancer Center Institutional Review Board. Patients were screened for Epstein-Barr, HIV, hepatitis A, B and C, cytomegalovirus and herpes simplex I and II. Patients with active infections or positive for HIV or hepatitis B or C were not eligible. A bone marrow (BM) aspirate was performed to assess the progenitor cell content and possible tumor involvement; patients with marrow involvement were excluded from harvesting. On the day of harvest, PT/PTT were normal, WBC >5.0 K/μL, hemoglobin >10 gm/dL and platelets >120 K/μL.

Outpatient BM harvest procedure

The collection of BM under local anesthesia, as approved by the Arlington Cancer Center Institutional Review Board, has been previously described in detail.[8] Briefly, the patient received continuous IV Ringer's lactate prior to and during the procedure. Patients were premedicated with 0.5–1.0 mg lorazepam (Ativan) and 0.5–1.0 mg hydromorphone hydrochloride (Dilaudid). Local anesthesia was administered; 1% lidocaine hydrochloride (Xylocaine) + sodium bicarbonate ($NaHCO_3$) to the skin sites and tissue, and 2% Xylocaine + $NaHCO_3$ directly onto the bone. An 11-gauge harvest needle (Lee Medical, Minneapolis, MN) was used to collect 60 mL per site from 8–10 bone punctures for a total of 500 mL per posterior iliac crest. The procedure was repeated on the other side the next morning.

G-CSF administration for marrow harvest

The informed consent and protocol for studying the effects of G-CSF on BM were approved by the Arlington Cancer Center Institutional Review Board. Immediately after their first harvest procedure, patients received 5 μg/kg G-CSF (Amgen, Thousand Oaks, CA) subcutaneously every 12 hours. Since the optimal number of injections was not known we studied groups of 15–20 patients using 2, 3 and 4 doses. Immediately before the harvest a blood sample was analyzed for $CD34^+$ cells to ensure that significant mobilization ($>1\times10^5$ $CD34^+$ cells/mL blood) had not occurred that would preclude marrow collection in favor of peripheral stem cell apheresis. Patients reported minor bone pain easily managed with over-the-counter analgesics. In heavier patients an additional 1–2 procedures were performed to yield $1–2\times10^6$ activated $CD34^+$ cells/kg of body weight.

Patients undergoing peripheral stem cell collection were mobilized with 10 μg/kg G-CSF every 24 hours for 6 days. Apheresis procedures were performed on days 5, 6 and 7 using a Fenwal CS3000 (Baxter, Deerfield, IL) instrument to process 10–12 liters of whole blood at a rate of 85 mL/minute. This consistently produced sufficient numbers of $CD34^+$ cells for transplant—a median of 2.2×10^6 cells/kg.

Processing and storage of progenitor cells

All procedures were sterile. The marrow was gently mixed, equally divided into 100 mL centrifuge tubes and spun down at 1000*g* for 15 minutes. Buffy layers from each tube were pooled. Apheresis products were spun down at 1000*g* for 15 minutes to remove saline and platelets. Cell counts were performed on the whole marrow, blood and pooled buffy using a Sysmex F800 (Baxter Fenwal, Deerfield, IL). Cells were frozen in 4.5 mL vials at a concentration of 200×10^6 cells/mL using controlled-rate freezing. The final freezing solution contained 10% dimethyl sulfoxide (DMSO), 10% autologous serum and 80% Hank's balanced salt solution (HBSS). Vials were stored in the vapor phase of liquid nitrogen.

Determination of CD34+ progenitor cells

Whole marrow, whole blood or processed cells (1×10^6 total white count) were incubated with 20 μL of an IgG_1-PE/FITC isotype or, HPCA-2 anti-CD34-PE and anti HLA-DR-FITC antibodies (Becton Dickinson, Mountain View, CA) at room temperature in the dark for 20 minutes. Red cells were lysed by adding 1 mL of FACSlyse (Becton Dickinson) to each tube and vortexing briefly. Labeled cells were washed 2× with phosphate buffered saline (PBS) and resuspended in 1 mL of PBS for analysis using a Facsort (Becton Dickinson) flow cytometer. Markers and quad stats were set for each specimen based on their isotypic control. Background staining was kept to <0.05%. At least 50×10^3 cells in each specimen were analyzed using a live gate. Results are reported as percentages of all cells stored for the patient (%$CD34^+$, %HLA-DR^+, %$CD34^+$ HLA-DR^-).

MTVB program

After completion of induction chemotherapy, patients were treated with a combination of Mitoxantrone 20 mg/M^2, Thiotepa 200 mg/M^2, VP-16 600 mg/M^2 and BCNU 300 mg/M^2 according to the MTVB protocol as approved by the Arlington Cancer Center Institutional Review Board. This regimen was administered over 4 days with marrow infusion on day 7. The median days to neutrophil and platelet recovery are determined from the day of marrow infusion. The major toxicity of this regimen is myelosuppression. In the first five patients studied, without stem cell support, granulocytes stayed <100/mm^3 for at least 30 days and platelets <20,000/mm^3 for 40 days even with G-CSF support (data not shown).

Reinfusion of progenitor cells

Reinfusion also took place as an outpatient. The patient was premedicated with 50 mg diphenhydramine hydrochloride (Benadryl) and 250 mg hydrocortisone sodium succinate (Solu-Cortef) and received a continuous infusion of Ringer's lactate. Blood pressure, O_2 saturation and cardiac function were monitored throughout the procedure. Vials of marrow or bags of peripheral stem cells were rapidly thawed and, under sterile conditions, drawn up through an 18-gauge spinal needle into a 30 cc syringe. A 400 μL sample was removed for counts, microbiology and CD34 analysis. A 25-gauge × $1^1/_2$ inch needle was placed on the syringe and the cells were pushed over 15–20 minutes into the Y port of a free-flowing IV of Ringer's lactate.

Statistical analysis

Hematopoietic recovery is expressed as median values with ranges. Blood and marrow cell counts and progenitor cell analyses are expressed as mean values and the standard error of the mean. Comparisons of marrow cell counts, overall

Table 1. Analysis of harvested marrow before and after G-CSF administration[a]

	Cell #/mL×10⁶	*CD34⁺/mL×10⁴*	*%CD34*
steady state	10.7 (±0.9)	06.8 (±1.2)	0.65 (±0.08)
2 dose (n=21)	25.7 (±2.8)	12.1 (±1.3)	0.50 (±0.05)
	p=0.001	p=0.001	p=0.06
steady state	09.3 (±0.7)	06.3 (±0.79)	0.70 (±0.08)
3 dose (n=20)	29.0 (±2.5)	14.9 (±2.1)	0.49 (±0.06)
	p=0.001	p=0.004	p=0.05
steady state	09.6 (±0.7)	07.4 (±1.3)	0.81 (±0.16)
	28.4 (±2.5)	17.1 (±4.0)	0.66 (±0.15)
	p=0.001	p=0.005	p=0.43

[a]*Mean ± SEM.*
p values were determined using a 1-sample t-test for paired data.

progenitor cell content and subpopulations were made using a paired analysis. Comparisons of blood counts and progenitor cell content were made using a 2 sample *t*-test; p values <0.05 are considered statistically significant.

RESULTS

The overall cellularity and $CD34^+$ progenitor cell content per mL of steady state marrow was extremely consistent across all three groups and significantly increased after 2, 3 or 4 injections of G-CSF (Table 1). Surprisingly, the cell number quickly reached 25.7×10^6/mL after 2 doses but demonstrated relatively moderate increases with additional G-CSF, 29.0×10^6/mL and 28.4×10^6, after 3 and 4 doses, respectively. Although the $\%CD34^+$ cells initially decreased from 0.65% to 0.50% with 2 doses and from 0.70% to 0.49% with 3 doses, it never reached statistical significance, and showed signs of recovery to 0.66% with continued G-CSF. As a result of the increases in cell number and little change in %CD34, the number of $CD34^+$ cells per mL of marrow significantly increased with all three dosing schedules, ranging from a 1.8-fold increase with 2 injections to a 2.3-fold increase after 4 injections. The total number of $CD34^+$ cells that can be harvested in 450–500 mL of marrow increased from 34×10^6 (±6.3) to 52×10^6 (±6.6) after 2 doses, from 28×10^6 (±3.6) to 65×10^6 (±8.5) after 3 doses and from 28×10^6 (±5.4) to 75×10^6 (±18) after 4 doses (Table 2). Subpopulations of $CD34^+$ cells were analyzed using HLA-DR since the absence or dim expression of this marker defines a population of cells believed to contain more immature

Table 2. Analysis of subpopulations of $CD34^+$ cells[a]

	Total $CD34^+ \times 10^6$	*HLA-DR^+*	*HLA-DR^-*
steady state	34 (±6.3)	20 (±2.9)	14 (±3.4)
2 dose	52 (±6.6)	34 (±4.2)	18 (±3.2)
	p=0.02	p=0.46	p=0.004
steady state	28 (±3.6)	18 (±2.1)	10 (±2.3)
3 dose	65 (±8.5)	42 (±7.2)	23 (±4.6)
	p=0.003	p=0.022	p=0.012
steady state	28 (±5.4)	22 (±5.3)	6 (±2.0)
4 dose	75 (±18)	59 (±13)	16 (±2.0)
	p=0.03	p=0.014	p=0.20

[a] *Mean ± SEM.*
p values were determined using a 1-sample t-test for paired data.

progenitors.[8–10] Compared with steady state marrow harvests, all 3 schedules of G-CSF administration resulted in significant increases in mature progenitors expressing both CD34 and HLA-DR. After 2 doses of G-CSF there was no change in the $CD34^+HLA\text{-}DR^-$ component, 14 (±3.4) versus 18 (±3.2)$\times 10^6$ (p=0.46). However, there appeared to be a transient increase in the number of immature progenitor cells after 3 doses, 10 (±2.3) versus 23 (±4.6)$\times 10^6$ (p=0.012) which reverted to a steady state value of 16 (±2.0)$\times 10^6$ (p=0.20) after 4 doses. Apparently not only can the quantity of available progenitor cells be increased, but the qualitative make-up of $CD34^+$ cells changes with G-CSF exposure (Table 2). Blood samples from 41 patients (Table 3) were analyzed for $CD34^+$ cells prior to each marrow harvest procedure to detect possible spillover into the periphery. Statistically significant increases in the circulating white blood cell count were noted for all dosing schedules compared with steady state blood. The progenitor cell content in the blood increased from a steady state mean of 0.86×10^4 to 4.37 after 2 doses, 7.43 after 3 doses and 8.62 after 4 doses; levels comparable to those reported in Table 1 for steady state marrow. Also, there was a trend toward increasing percentages of $CD34^+$ cells in the bloodstream as the number of G-CSF injections increased, suggesting the onset of mobilization, although the comparison never quite reached statistical significance, p=0.06 after 4 doses. Fifteen patients have been reinfused with activated marrow after MTVB chemotherapy, receiving a median of 1.6×10^6 $CD34^+$ cells/kg (Table 4). This number is comparable to a median of 1.2×10^6 for 10 historical steady state marrow reinfusions and a median of 2.2×10^6 for 11 reinfusions of peripheral stem cells, all in solid tumor patients

Table 3. Effects of G-CSF on $CD34^+$ cell content in the bloodstream[a]

	Cell#/mL $\times 10^6$	*CD34⁺/mL* $\times 10^4$	*%CD34*
steady state (n=41)	5.1 (±0.54)	0.86 (±0.2)	0.17 (±0.03)
2 dose (n=13)	26.6 (±3.8) p=0.001	4.37 (±1.3) p=0.021	0.19 (±0.08) p=0.79
3 dose (n=17)	28.7 (±2.7) p=0.001	7.43 (±1.5) p=0.001	0.24 (±0.05) p=0.16
4 dose (n=11)	31.7 (±4.5) p=0.001	8.62 (±1.7) p=0.001	0.27 (±0.04) p=0.06

[a] *Mean ± SEM.*
p values were determined using a 2-sample t-test.

following the same induction therapy and MTVB regimen. Hematopoietic recovery (Table 4) was hastened when activated marrow was reinfused, compared with steady state marrow. Comparable recoveries were seen when activated marrow or peripheral stem cells were used with an ANC >100/mm^3 by days 9 and 10, and platelet counts >20,000 by days 19 and 18, respectively.

DISCUSSION

Brief exposure to subcutaneously administered G-CSF, 2–4 injections every 12 hours, results in a 2–3-fold increase in the total $CD34^+$ cell content of BM, thereby allowing the collection of larger numbers of progenitor cells per harvest procedure. Circulating levels of $CD34^+$ cells increased in the bloodstream with additional injections of G-CSF, suggesting the onset of mobilization. Analysis of subpopulations of marrow progenitors, $CD34^+HLA\text{-}DR^+$ versus $CD34^+HLA\text{-}DR^-$, indicated significant increases in the more committed, dual-positive component regardless of the number of G-CSF injections. The more immature cells that are $HLA\text{-}DR^-$ did not change after 2 doses, significantly increased after 3 doses, and reverted to baseline levels after four doses. This could be the result of a direct proliferative effect of G-CSF on progenitor populations in the $HLA\text{-}DR^-$ compartment. Although there are no definitive studies demonstrating the presence of high affinity G-CSF receptors on early progenitors,[11,12] other receptors with low affinity could become biologically relevant in the presence of pharmacologic doses of G-CSF. A more plausible explanation would be the immediate effect on $HLA\text{-}DR^+$ progenitor cells,

Table 4. Transplantation and hematopoietic recovery[a]

	Reinfusion procedures		
	Steady state marrow (n=10)	*Activated marrow (n=15)*	*Mobilized PSC (n=11)*
#MNC × 10^6/kg	64 (35–267)	131 (28–228)	107 (30–262)
#CD34$^+$ × 10^6/kg	1.2 (0.2–3.3)	1.6 (1.1–5.5)	2.2 (0.3–11.6)
	Hematopoietic recovery		
	Steady state marrow (n=10)	*Activated marrow (n=15)*	*Mobilized PSC (n=11)*
days to:			
ANC >100/mm^3	14 (6–32)	9 (5–19)	10 (9–11)
ANC >1000/mm^3	19 (9–42)	15 (10–34)	13 (10–19)
Plts >20,000	21 (14–45)	19 (12–41)	18 (12–56)
days ANC <100/mm^3	10 (8–12)	6 (3–12)	6 (4–11)

[a]*Median values with ranges.*

such as CFU-GM, to differentiate along myeloid lineages in response to G-CSF. As this compartment is depleted, earlier progenitor cells would subsequently be recruited, requiring the initiation of self-renewal mechanisms in the marrow to maintain the immature progenitor pool. After 3 doses, self-renewal appeared to be accelerated as demonstrated by the significant expansion in HLA-DR$^-$ cells despite the continued increase in the number of HLA-DR$^+$ cells in the marrow and their spillover into the bloodstream. Continuation of G-CSF with a fourth dose apparently shifted the balance in favor of differentiation over self-renewal; HLA-DR$^+$ cells further increased in the marrow and blood and the HLA-DR$^-$ component reverted to baseline levels. Within the framework of this proposed mechanism several questions arise. Was there an initial drop in the HLA-DR$^-$ compartment during the first 12–24 hours that went undetected in this study? Would the continuation of G-CSF beyond 4 doses, a common situation when attempting to mobilize progenitors, result in further increases in CD34$^+$HLA-DR$^+$ cells accompanied by further decreases in CD34$^+$HLA-DR$^-$ cells in the marrow, i.e., stem cell depletion?[13,14] If the growth factor signal to differentiate is suddenly withdrawn, will self-renewal continue, thus presenting a window of opportunity to harvest progenitors more than once before the marrow returns to steady state? Initial results suggest that within 16–36 hours of growth factor cessation, bone pain subsides and circulating white counts drop but marrow progenitors continue to increase.[23]

The rate of hematopoietic recovery using mobilized peripheral stem cells in our study was somewhat slower compared with other groups that reinfused more than our median of 2.2×10^6 $CD34^+$ cells/kg.[13,14] By using equivalent numbers of $CD34^+$ cells/kg, the engraftment potential of progenitor cells harvested from activated marrow proved to be superior to cells collected under steady state conditions and was equivalent to mobilized peripheral stem cells. This suggests a qualitative difference between marrow progenitors collected with, and without, prior growth factor stimulation. One would expect an increase in the %S phase,[17,18] but there may also be differences in the expression of adhesion molecules and receptors for cytokines produced by the microenvironment,[19–21] thereby rendering homing and proliferative advantages to activated cells.

Could these similarities between activated marrow and mobilized peripheral stem cells be partially explained by contamination of blood-derived progenitors in the harvested marrow? We determined the level of $CD34^+$ cells in steady state blood to be 0.86×10^4/mL so if an entire 500 mL harvest were solely blood, the yield of $CD34^+$ cells would be 4×10^6. On the contrary, our original studies demonstrated a mean of 33×10^6 $CD34^+$ cells/500 mL harvest[7], suggesting a maximum of 12% blood contamination under steady state conditions. After stimulation with 3 doses of G-CSF the level of progenitors in the bloodstream increased to 7.43×10^4/mL yielding an estimated 37×10^6 $CD34^+$ cells/500 mL. Actually, 65×10^6 $CD34^+$ cells were collected in 500 mL of marrow so the maximum contribution of blood derived progenitors could be as high as 50–60%. Analysis of $CD34^+$ subpopulations in the marrow after 3 doses of G-CSF revealed approximately 35% were $HLA\text{-}DR^-$ which is distinctly different from an average 4% $HLA\text{-}DR^-$ in mobilized peripheral stem cell pheresis products (data not shown), suggesting that blood contamination of the marrow harvest is considerably less than 50%.

In conclusion, we found that exposure of marrow to a brief stimulus such as G-CSF increases the availability and engraftment potential of marrow progenitor cells resulting in hematopoietic recovery comparable to mobilized peripheral stem cells and superior to steady state marrow.

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REFERENCES

1. Dicke KA, Spitzer G: Evaluation of the use of high dose cytoreduction with autologous marrow rescue in various malignancies. *Transplantation* 41:4, 1986.

2. Armitage JO: Bone marrow transplantation. *N Engl J Med* 330:827, 1994.
3. Janssen WE, Hiemenz JW, Fields KF et al.: Blood and bone marrow hematopoietic stem cells for transplantation: A comparative review. *Cancer Control* 1:225, 1994.
4. Janssen WE: Peripheral blood and bone marrow hematopoietic stem cells: Are they the same? *Semin Oncol* 20(S6):19, 1993.
5. Kessinger A: Is blood or bone marrow better? *Stem Cells* 11:290, 1993.
6. Ho AD, Mason JR, Corringham RE: Hematopoietic progenitor cells: Sources, applications, and expansion. *Oncology* 7:17, 1993.
7. Beyer J, Schwella N, Zingsem J et al.: Hematopoietic rescue after high-dose chemotherapy using autologous peripheral-blood progenitor cells or bone marrow: A randomized comparison. *J Clin Oncol* 13:1328, 1995.
8. Dicke KA, Hood DL, Hanks S et al.: A marrow harvest procedure under local anesthesia. *Exp Hematol* 23:1229–1232, 1995.
9. Tong J, Hoffman R, Siena S et al.: Characterization and quantitation of primitive hematopoietic progenitor cells present in peripheral blood. *Exp Hematol* 221:1016, 1994.
10. Rusten LS, Jacobsen SE, Kaalhus O et al.: Functional differences between $CD38^-$ and DR^- subfractions of $CD34^+$ bone marrow cells. *Blood* 84:1473, 1994.
11. Briddell RA, Broudy VC, Bruno E et al.: Further phenotypic characterization and isolation of human hematopoietic progenitor cells using a monoclonal antibody to the c-kit receptor. *Blood* 79:3159, 1992.
12. Visser JW, Rozemuller H, de Jong MO et al.: The expression of cytokine receptors by purified hemopoietic stem cells. *Stem Cells* 11(suppl 2):49, 1993.
13. McClanahan T, Dalrymple S, Barkett M et al.: Hematopoietic growth factor receptor genes as markers of lineage commitment during in vitro development of hematopoietic cells. *Blood* 81:2903, 1993.
14. To LB, Haylock DN, Dyson PG et al.: Progenitor threshold effects in haemopoietic reconstitution. In: Dicke KA, Keating A (eds) Autologous Marrow and Blood Transplantation: Proceedings from the 7th International Symposium. Arlington, Texas, 1994, p 511.
15. Siena S, Bregni M, Brando B et al.: Flow cytometry for clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. *Blood* 77:400, 1991.
16. Moore MA: Does stem cell exhaustion result from combining hematopoietic growth factors with chemotherapy? If so, how do we prevent it? *Blood* 80:3, 1992.
17. Hornung RL, Longo DL: Hematopoietic stem cell depletion by restorative growth factor regimens during repeated high-dose cyclophosphamide therapy. *Blood* 80:77, 1992.
18. Tanaka B, Katayama N, Oishi K et al.: Accelerated cell-cycling of hematopoietic progenitor cells by growth factors. *Blood* 86:73, 1995.
19. Roberts AW, Metcalf D: Noncycling state of peripheral blood progenitor cells mobilized by granulocyte colony-stimulating factor and other cytokines. *Blood* 86:1600, 1995.
20. Suzuki T, Muroi K, Tomizuka H et al.: Characterization of enriched $CD34^+$ cells from healthy volunteers and those from patients treated with chemotherapy plus granulocyte colony-stimulating factor (G-CSF). *Stem Cells* 13:273, 1995.
21. Dercksen MW, Gerritsen WR, Rodenhuis S et al.: Expression of adhesion molecules on $CD34^+$ cells: $CD34^+$ L-selectin$^+$ cells predict a rapid platelet recovery after peripheral

blood stem cell transplantation. *Blood* 85:3313, 1995.

22. Turner ML, McIlwaine K, Anthony RS et al.: Differential expression of cell adhesion molecules by human hematopoietic progenitor cells from bone marrow and mobilized adult peripheral blood. *Stem Cells* 13:311, 1995.
23. Dicke KA, Hood DL, Hanks S et al.: Effects of G-CSF cessation on the harvest of activated marrow progenitors under local anesthesia. *Blood* 86:982a, 1995.

QUALITATIVE ASPECTS OF THE ENGRAFTMENT POTENTIAL OF CD34+ CELLS

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INTRODUCTION

Hematopoietic stem cells (HSC) are defined as cells with pluripotent differentiating potential, including lymphoid as well as erythromyeloid lineages, and extensive self-renewal capacity. While there has been dramatic recent progress in stem cell characterization, controversy exists as to the ability to unequivocally quantitate true stem cells, particularly in man. This is due, in part, to a reliance on surrogate assays for stem cells and the ambiguity of the term extensive. Clearly, heterogeneity in self-renewal potential exists within HSC populations, and in addition, the self-renewal versus differentiation probability is stochastically determined at the single cell level, being p=0.5 at the HSC population level in steady-state hematopoiesis. Furthermore, the term self-renewal can be questioned, since, as with all somatic cells, the daughter HSC differ from their parent by a small but finite change in doubling potential associated with loss of chromosome telomere base pairs.[1] Ultimately, telomere loss dooms self-renewing populations to senescence after 20–75 population doublings—the Hayflick limit. In the absence of artificial insults, such as chemotherapy, this doubling potential is more than sufficient to sustain a lifetime of hematopoietic demand. Indeed, the transplantation of the equivalent of 1% of the total HSC population in a typical allogeneic bone marrow transplant (BMT) appears compatible with lifelong reconstitution with no evidence of late marrow failure at 20+ years.

In the adult, the HSC population is quiescent, with the majority of cells in a G_0 state. The daily demands of mature cell production are sustained by differentiation of very few HSC, with very extensive amplification within the transit population of lineage-restricted progenitor cells. The greatest loss of HSC self-renewal potential occurs during fetal life, followed by postnatal growth of the hematopoietic system. Serial passaging experiments in lethally irradiated mice indicated that, in a comparison of yolk sac, early and late fetal liver, and adult BM HSC, there was an ontogeny-related hierarchy in passaging potential.[2] Recently, a mechanistic explanation has been provided by the demonstration that hematopoietic cells in human fetal liver and umbilical cord blood (CB) have much longer telomeres than cells in adult BM.[1] Central to the concept of restriction of proliferative potential as

a function of developmental stage is the concept of stem cell migration. This theory, as originally formulated, proposed that HSC developed from a precursor (hemangioblast) at a very early stage of development in the yolk sac blood islands and all subsequent hematopoiesis was due to migration and colonization of emerging hematopoietic microenvironments, first fetal liver, then spleen and, finally, BM.[3–6] The evidence for this concept was that if vascular anastomosis was established naturally or artificially[4,6] between genetically disparate pairs of embryos at an early stage of development, lifelong stable chimerism of lymphohematopoietic tissues resulted. In some species, an early prehepatic intraembryonic source of HSC has been proposed in addition to yolk sac, for example, the paraaortic region in mice,[7] but this does not change the migration concept as it pertains to all subsequent hematopoiesis. There have been reports that HSC and stromal components of the marrow environment can arise locally from a common precursor, but in general, they have not been confirmed, or have been retracted.[8] Extension of the migration concept to adult hematopoiesis envisages an equilibrium between a marrow HSC pool and a circulating HSC population that can colonize distant sites of hematopoiesis. The ability of HSC to enter the circulation as well as their selective ability to home back to sites of hematopoiesis is central to the success of BM, and more recently, peripheral blood transplantation.

For optimal short-term (2–3 week) platelet and neutrophil recovery, $2–5\times10^6$ $CD34^+$ cells/Kg or $5\times10^4–1\times10^5$ progenitor cells/Kg are the minimum requirement, while, for long-term repopulation, HSC are required. In the murine transplant system, a single HSC may be sufficient for repopulation, and reproducible long-term multilineage engraftment can be obtained with 30 highly purified stem cells.[9] Extrapolating on a body weight basis, this would be 10^5 HSC for humans. On theoretical grounds, the only limitation in terms of numbers of HSC required is the ability of the patient to survive a prolonged period of leukopenia and thrombocytopenia, while the HSC compartment regenerates, and the doubling potential of the HSC reflecting its previous proliferative history. If HSC have proliferated extensively prior to transplantation and few HSC are engrafted, then they may reach their Hayflick limit, leading to delayed marrow failure.

STEM CELL CONTENT OF HEMATOPOIETIC CELL POPULATIONS USED FOR TRANSPLANTATION AND THEIR PROLIFERATIVE POTENTIAL AS DETERMINED BY TELOMERE MEASUREMENTS

We have determined the stem cell content of various transplant cell sources using the long-term culture-initiating cell (LTC-IC) assay (Table 1). This shows that LTC-IC comprise, on average, 0.4–0.85% of the CD34 population of adult marrow, cord blood and mobilized peripheral blood.

Based upon these data, one can calculate that an average transplant of mobilized PB at 2×10^6 $CD34^+$ cells/kg in a 75 kg recipient provides approxi-

Table 1. Long-term culture-initiating cells in CD34+ populations obtained from normal bone marrow (BM), umbilical cord blood (CB) and G-CSF plus cytoxan mobilized blood from patients with advanced breast and ovarian cancer

	CD34+		*LTC-IC/LTC-IC*		*LTC-IC/CFU*
Stromal line	*source*	*No. of cases*	*10^6CD34+*	*% of CD34+*	*Ratio*
	BM	4	4930	0.49 ± 0.10	1:27
murine	PB	2	8530	0.85 ± 0.04	1:25
M210B4	CB	4	5030	0.50 ± 0.16	1:59
	BM	2	3850	0.39 ± 0.09	1:40
human	PB	3	4770	0.48 ± 0.09	1:21
fetal spleen	CB	7	7880	0.79 ± 0.30	1:41

mately 10^6 LTC-IC, and the LTC-IC content of the total hematopoietic system (10^{12} nucleated cells) is approximately 7×10^7. Thus, only 6–7 population doublings would reconstitute the stem cell pool and 20 doublings would regenerate the total nucleated population of the marrow. HSC seeding efficiency to the marrow has been reported in the range of 4–10% in mice and may be influenced by modulation of expression of HSC cytoadhesion molecules or cycle status of the cells. A further 4–5 population doublings (PD) would be required, in addition to the above, to correct for seeding efficiency. The number of PD required to reconstitute the adult marrow stem cell pool from a CB transplant, assuming a 10% seeding efficiency, would be 15–16; well within the proliferative potential of such cells, based upon their telomere length.

In the majority of patients post-allogeneic transplantation, leukocyte mean telomere length has been reported to decrease an average of 880 base pairs.[10] Following autologous peripheral blood stem cell rescue in patients with advanced ovarian cancer undergoing high-dose chemotherapy, we have shown that the telomeric repeat fragment (TRF) of peripheral blood leukocytes obtained prior to chemotherapy was a mean of 7.74 ± 0.22 kilobases, compared with 5.71 ± 0.27 kb following short-term recovery (3.5 weeks) after transplantation.[11] At longer follow-up, mean TRF increased to 6.86 ± 0.48 kb and stabilized at later intervals. In vitro, in cytokine-stimulated Delta suspension cultures of CD34+ cells obtained from fetal liver, cord blood, adult granulocyte colony-stimulating factor (G-CSF)/cytoxan-mobilized peripheral blood and normal BM, the average telomere lengths were 11 kb, 10.4 kb, 7.4 kb and 7.6 kb, respectively, at the outset. Following expansion, an average telomere DNA

loss of 1–2 kb per week was observed.[12] This corresponded to a minimum of 21.4 PD and a maximum average base-pair loss of 73 per PD. Extrapolating from the in vitro data to the transplant situation, twelve PD were required for stable, long-term repopulation in both the allogeneic and autologous situations, but considerably more PD (28) were used in the generation of the first wave of leukocyte recovery, as shown in the autologous transplant data. A number of explanations present themselves: 1) this early recovery came from nonself-renewing, more committed progenitors with a more extensive proliferative history (shorter telomeres) than the quiescent stem cell pool that was responsible for the later recovery, 2) in the autologous transplant, the later recovery came from surviving endogenous stem cells that had not proliferated as extensively as the transplanted cells, 3) the later long-term repopulation came from cells partially protected from telomere shortening by expression of telomerase. Low levels of telomerase can be detected in primitive human hematopoietic cells. We have shown a correlation between telomerase activity in $CD34^+$ and $CD34^+CD38^-$ cells and their entry into cell cycle, as determined by BrdU incorporation and induction of a variety of cycle-associated proteins (phosphorylated RB protein, CDC2, CDK2, cyclin D1 and cyclin A).[12] Cytokine combinations inducing maximum proliferation of $CD34^+$ or $CD34^+CD38^-$ cells in culture (Table 2) were also the combinations inducing the highest levels of telomerase expression in these cells.[12] Antiproliferative cytokines, such as $TGF\beta_1$ downmodulated telomerase activity. In ex vivo expansion cultures, telomerase levels were high in the first two weeks of culture, before falling to low or undetectable levels by 3–5 weeks. Analysis of the rate of base-pair loss revealed that this was significantly lower per PD in the first two weeks of culture than subsequently, indicating that telomerase upregulation in early hematopoietic stem/progenitor cells can partially protect the cells from telomere shortening.

IMPROVED CYTOKINE ACTIVATION OF QUIESCENT STEM/PROGENITOR POPULATIONS

The $CD34^+CD38^-$ fraction of mobilized PB contains all the stem cells as measured by the LTC-IC assay and such cells are in a G_0 state at the time of purification. We have observed that a combination of KL, Flk-2 ligand, IL-3, IL-6, G-CSF and erythropoietin (Epo) was highly effective in stimulating proliferation in this stem cell–rich (~10% LTC-IC) subpopulation, as measured by direct colony formation and by 7-day expansion of cells and progenitors (Table 2).

In other studies, LTC-IC expansion was also determined using the cytokine combinations indicated in Table 3 and up to 15-fold expansion was observed at 7 and 14 days with K,F,3,6,G,E, at the doses indicated, with even greater expansion noted with increased concentrations of KL and Flk-2 ligands.

Table 2. Comparison of mobilized PB $CD34^+CD38^+$ and $CD34^+CD38^-$ populations in 7-day Delta assay

			*7-Day Delta**			
CD34⁺	*Cytokines*	*Cloning efficiency %*	*Cells×10⁵*	*Fold increase*	*CFU*	*Fold increase*
$CD38^+$	K,3,6,G,E	13.4	26.3	65.6	129,150	21.7
$CD38^-$	K,3,6,G,E	12.4	22.7	56.7	55,830	10.1
$CD38^+$	F,3,6,G,E	9.0	17.5	43.8	90,000	14.1
$CD38^-$	F,3,6,G,E	6.8	9.3	23.3	21,950	4.0
$CD38^+$	K,F,3,6,G,E	14.8	36.3	90.6	223,300	37.5
$CD38^-$	K,F,3,6,G,E	14.4	37.7	91.6	112,882	20.5
$CD38^+$	K,F,3,6,G,E,TGFβ	1.0	11.6	29.0	12,400	2.1
$CD38^-$	K,F,3,6,G,E,TGFβ	1.2	8.3	20.6	5,610	1.0

**$CD34^+$ and $CD38^-$ cells were isolated using Miltenyi immunomagnetic particle separation technology. Cells were cultured in 24 well plates in 1 mL of Iscove's Modified Dulbecco's Medium + 20% fetal calf serum, at plating densities of 4×10^4/mL in the presence of combinations of cytokines, including c-kit ligand (K, 20 ng/mL), Flk-2/Flt-3 ligand (F, 20 ng/mL), IL-3 (50 ng/mL), IL-6 (20 ng/mL), G-CSF (10 ng/mL), erythropoietin (E, 5 U/mL) and transforming growth factor β (100 ng/mL). After 7 days, total cell recovery and secondary colony forming capacity was determined. Fold increase in CFU was calculated, based upon primary colon-forming efficiency and divided into the total number of secondary colonies generated in 7 days. Both primary and secondary CFU were measured in 14-day semisolid agarose cultures stimulated by KL, IL-6, IL-3, G-CSF and Epo. The addition of both KL and FL to the cytokine mix achieved maximum primary cloning, and 7-day expansion of both cells and progenitors in both $CD34^+38^+$ and $CD34^+38^-$ cultures, whereas addition of TGFβ significantly suppressed both primary cloning and Delta expansion achieved with the optimal combination of stimulatory cytokines.*

THE INFLUENCE OF PRIOR CHEMOTHERAPY AND MOBILIZING REGIMENS ON THE EX VIVO EXPANSION POTENTIAL OF PERIPHERAL BLOOD $CD34^+$ CELLS

The adequacy of an apheresis collection for autologous transplantation is usually defined by the numbers of $CD34^+$ cells or of CFU-GM, and in general, there is a linear relationship between these two. However, there is growing evidence that neither CD34 nor CFU-GM measurements necessarily predict the degree of mobilization of more primitive populations, including stem cells. We have shown that a much weaker correlation exists between $CD34^+$ cells and pre-CFU, in an assay in which in vitro 4-hydroperoxy cyclophosphamide purged $CD34^+$ cells were cultured for 7 days with cytokines, and the capacity to generate secondary CFU was used as an indirect measure of "pre-CFU".[13] This lack of correlation was particularly evident in heavily pretreated breast cancer patients. In a modification of the pre-CFU assay, we have evaluated the ex vivo expansion capacity of $CD34^+$ cells in the presence of a combination of cytokines (KL, IL-3, IL-6, G-CSF and Epo) where, at weekly intervals, the cells are replated at the

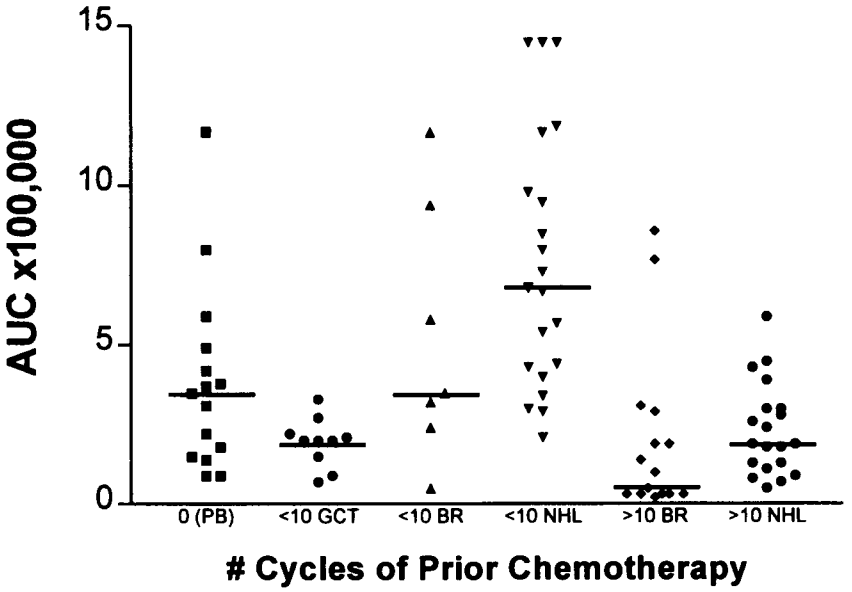

Figure 1. *Area under the curve (AUC) of cumulative progenitor cell expansions in Delta assays of CD34⁺ cells isolated from mobilized peripheral blood from patients with untreated ovarian cancer (0), or from moderately (<10 cycles of prior chemotherapy) or heavily (>10 cycles) pretreated patients with germ cell tumor (GCT), Stage 4 breast cancer or non-Hodgkin's lymphoma (NHL). Each point is an assay of CD34 cells harvested from a single apheresis.*

starting cell concentration (10^4–4×10^4 cells/mL), with fresh media and cytokines and passaging is continued until there is no further cellular expansion. At weekly intervals, the number of CFU generated are determined in secondary clonogenic agarose assay. The results of this Delta assay are expressed as cumulative progenitor numbers. The shape of the curve of cumulative progenitor numbers provides qualitative and quantitative information.[14] In general, with mobilized $CD34^+$ cells from previously untreated patients (e.g., ovarian cancer), a 100- to 200-fold increase in progenitors is seen, peaking by 3 weeks and subsequently declining to baseline levels by 4–5 weeks. In heavily pretreated patients, expansion may be normal for 1–2 weeks and then fall to baseline by 3 weeks, suggesting a deficit of early precursors that generate secondary progenitors at later stages of the culture. These early precursors may include true self-renewing stem cells (LTC-IC), a proportion of which do enter cycle during the course of Delta culture and may, under appropriate cytokine stimulation, expand. Other candidate primitive

precursors include cells with high proliferative potential and cells the human equivalent of murine day 8 and day 12 colony-forming unit-spleen (CFU-S). To provide a proliferative index of $CD34^+$ cells or a measure of their proliferative potential, we calculate the area under the curve (AUC) of progenitor cell expansion using the trapezoidal rule. Using A as the number of progenitors at day 0, B at day 7, C at day 14, D at day 21 and E at day 28, the formula is 1/2A+B+C+D+1/2E. Representative Delta expansion AUC data is shown for $CD34^+$ cells from aphereses of various patients in Figure 1. Ovarian cancer patients (0) had received no prior chemotherapy and a mobilization regimen of G-CSF (10 μg/kg/day) + cytoxan. It is of note that, even without prior chemotherapy, considerable patient-to-patient variation is seen in the expansion potential and progenitor-generating capacity of the $CD34^+$ cells. A similar mobilization regimen in germ cell tumor (GCT) patients who had received less than 10 cycles of prior chemotherapy resulted in CD34 cells with a lower overall expansion potential than seen with $CD34^+$ from untreated patients. In Stage 4 breast cancer, a relationship was seen between the extent of prior chemotherapy and the expansion potential of the $CD34^+$ cells. Those receiving >10 cycles of prior chemotherapy had very low CD34 expansion, compared with those receiving <10 cycles, with the bulk of the difference attributed to 7 patients whose $CD34^+$ cells were severely impaired in Delta expansion. A similar difference, determined by extent of prior chemotherapy, was seen in mean AUC of CD34 cells mobilized by G-CSF or G-CSF plus stem-cell factor in non-Hodgkin's lymphoma. The greater mean AUC seen in the lymphoma cases with <10 cycles of prior chemotherapy than seen with untreated ovarian cancer can be attributed to the addition of stem cell factor (SCF) to the G-CSF for mobilization. Even with extensive prior chemotherapy, there was better expansion than seen with the heavily pretreated breast cancer patient CD34 cells, probably attributable to the inclusion of lymphoma patients, whose $CD34^+$ cells were mobilized with G-CSF+SCF. Further evidence that the combination of SCF (20 μg/kg) with G-CSF (5 μg/kg) was superior to the latter alone in mobilization of primitive "pre-CFU" was provided by studies in previously untreated ovarian cancer patients where a 5.8-fold increase in LTC-IC mobilization was observed when SCF was combined with G-CSF.[15]

CONCLUSION

The development of a "birth and death" model for self-renewal of stem cells was initially based on data generated from analysis of CFU-S content of single spleen colonies. The numbers of CFU-S per colony varied greatly, following a skewed "gamma" type of distribution. This heterogeneity can be attributed to the existence of functionally distinct stem cell subsets, but equally, and more plausibly, by the stochastic process, which accounts for heterogeneous kinetic behavior of

otherwise homogeneous populations. In steady-state hematopoiesis, the probability of HSC self-renewal versus differentiation approximates p 0.5. At a single cell level, this would mean that in an otherwise homogeneous population of HSC, after two divisions, 12.5% of HSC would exclusively self renew, while 60% of clones would contain no stem cells and one HSC in a thousand would, by chance, self renew ten times, generating a further thousand stem cells.[16] This latter cell would probably be read out as a long-term repopulating cell and, if retrovirally or genetically marked, would dominate hematopoiesis. One of the prevailing concerns of current transplantation biology is that, if highly purified stem cells were to be transplanted, time to platelet and neutrophil recovery would be greatly extended, since more committed progenitors would be absent. The above stochastic considerations would argue that the bulk of transplanted stem cells would rapidly differentiate to progenitors, and the only reason pure stem cells would be slower to engraft is that the numbers engrafted would be low.

Rigid application of the stochastic theory would argue that, at least in the adult, stem cell expansion must be modest, if indeed it occurs at all, either in vivo or in vitro, since the probability that a stem cell generates another stem cell upon division is 0.5. As we have indicated, a minimum of 6 and a maximum of 11 self-renewing population doublings would be required to regenerate a normal stem cell population, following an autologous or allogeneic transplant. Either the stem cell population is never fully reconstituted, or a significant change in the self-renewal probability of the stem cell occurs upon transplantation. Both appear likely from current observations, with the latter probably dependent upon upregulation of specific cytokines, the most important being c-*kit* and Flk-2/Flt-3 ligands.

REFERENCES

1. Vaziri H, Dragowka W, Allsopp RC, Thomas TE, Harley CB, Lansdorp PM: Evidence for a mitotic clock in human hematopoietic stem cells; loss of telomeric DNA with age. *Proc Natl Acad Sci USA* 91:9857, 1994.
2. Metcalf D, Moore MAS: Haemopoietic Cells. Frontiers of Biology Series. Amsterdam: North-Holland Publishing Company, 1971, pp 172–271.
3. Moore MAS, Owen JJT: Stem cell migration in developing myeloid and lymphoid systems. *Lancet* ii:658–659, 1967.
4. Moore MAS, Owen JJT: Chromosome marker studies on the development of the haemopoietic system in the chick embryo. *Nature* 208:986–990, 1965.
5. Moore MAS, Metcalf D: Ontogeny of the haemopoietic system. Yolk sac origin of in vivo and in vitro colony forming cell in the developing mouse embryo. *Br J Haematol* 18:279–296, 1970.
6. Moore MAS, Owen JJT: Experimental studies on the development of the thymus. *J Exp Med* 126:715–726, 1967.
7. Medvinsky AL, Samoylina NL, Muller AM, Dzierzak EA: An early pre-liver intra-embry-

onic source of CFU-S in the developing mouse. *Nature* 364:64–66, 1993.

8. Waller EK, Olweus J, Lund-Johansen F, Huang S, Nguyen M, Guo G-R, Terstappen L: The "common stem cell" hypothesis reevaluated: Human fetal bone marrow contains separate populations of hematopoietic and stromal progenitors. *Blood* 85:2422–2435, 1995.
9. Spangrude GJ, Brooks DM, Tumas DB: Long-term repopulation of irradiated mice with limiting numbers of purified hematopoietic stem cells: In vivo expansion of stem cell phenotype but not function. *Blood* 85:1006–1016, 1995.
10. Notaro R, Cimmino A, Tabarini D, Rotoli B, Luzzatto L: Hematopoietic cells lose telomere length after bone marrow transplantation (BMT). *Blood* 88(suppl 1):456a, 1996.
11. Shapiro F, Engelhardt M, Ngok D, Han W, Moore MAS: Telomere length shortening and recovery following high-dose chemotherapy with autologous peripheral stem cell rescue. *Blood* 88(suppl 1):601a, 1996.
12. Engelhardt M, Albanell J, Kumar R, Han W, Moore MAS: Telomerase upregulation and telomere length in ex vivo expansion cultures of hematopoietic progenitor cells. *Blood* 88(suppl 1):341a, 1996.
13. Schneider JG, Crown JP, Wasserheit C, Kritz A, Wong G, Reich L, Norton L, Moore MAS: Factors affecting the mobilization of primitive and committed hematopoietic progenitors into the peripheral blood of cancer patients. *Bone Marrow Transplant* 14:877–884, 1994.
14. Shapiro F, Yao T-J, Raptis G, Reich L, Norton L, Moore MAS: Optimization of conditions for ex vivo expansion of $CD34^+$ cells from patients with stage IV breast cancer. *Blood* 84:3567–3574, 1994.
15. Weaver A, Ryder D, Crowther D, Dexter TM, Testa NG: Increased numbers of long-term culture-initiating cells in the apheresis product of patients randomized to receive increasing doses of stem cell factor administered in combination with chemotherapy and a standard dose of granulocyte colony-stimulating factor. *Blood* 88:3323–3328, 1996.
16. Gordon MY, Blackett NM: Routes to repopulation—A unification of the stochastic model and separation of stem-cell subpopulations. *Leukemia* 8:1068–1073, 1994.

PBPC MOBILIZATION IN MULTIPLE MYELOMA: HIGH-DOSE CHEMOTHERAPY PLUS G-CSF VERSUS G-CSF IN STEADY-STATE

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ABSTRACT

Dose-escalated cytotoxic therapy with peripheral blood progenitor cell (PBPC) support results in a 5–10-fold increase of a stringently defined response rate compared with standard treatment in patients with multiple myeloma (MM). In 12 patients with MM we administered granulocyte colony-stimulating factor (G-CSF) during steady-state hematopoiesis as well as after cytotoxic chemotherapy for the mobilization of PBPC. We compared both mobilization modalities: G-CSF 1200 µg/d s.c. during steady-state versus high-dose cyclophosphamide plus G-CSF 300 µg/d s.c. To support a high-dose conditioning regimen including total-body irradiation (TBI), we considered an autograft containing $\geq 2.0 \times 10^6$ $CD34^+$ cells/kg bodyweight (BW) to be necessary. It was possible to harvest this number with two leukaphereses (LP) in 8/12 patients during steady-state mobilization and in 9/12 patients post chemotherapy. FACS analysis showed that harvests post chemotherapy contained a similar number of hematopoietic progenitor cells (2.3 versus 2.4×10^6 $CD34^+$ cells/kg) as harvests collected during steady-state. In contrast, the content of potentially malignant B cells (6.1 versus 1.35×10^6 $CD19^+$ cells/kg BW) was significantly lower in harvests post chemotherapy.

In order to examine these leukapheresis products for tumor cells, we designed antisense oligonucleotide primers for 5 patients With the ASO-PCR we could detect low quantities of patient-specific clonal B cells (sensitivity $1:10^5$). We found cells of the tumor clone in 12 of 17 leukapheresis products. Aliquots from ASO-PCR-positive products were FACS-sorted for the CD34 and CD19 antigen. In no case did the $CD34^+$ sample contain cells of the tumor clone. On the other hand, all $CD19^+$ samples were PCR-positive, indicating that tumor cells were present in these fractions. Therefore, the harvesting of PBPC following high-dose chemotherapy appears to be superior due to the higher $CD34^+/CD19^+$ ratio compared with steady-state mobilization.

INTRODUCTION

Peripheral blood progenitor cell (PBPC) autografting after high-dose therapy including total-body irradiation (TBI) is increasingly used for the treatment of multiple myeloma.[1] A sustained and rapid engraftment after myeloablative therapy depends primarily on the number of hematopoietic progenitor cells reinfused.[2] In the case of multiple myeloma (MM), cyclophosphamide (CY) in combination with hematopoietic growth factors is an effective regimen for mobilizing PBPC.[3] It has been assumed that the frequency of tumor cells in the blood of MM patients is less than that in bone marrow (BM). But the level of circulating B-cells in PB as well as the percentage of tumor cells in these B cell populations is controversial. Bergsagel et al.[4] showed that percentages of up to 50% of the circulating late stage $CD19^+$ B cells are involved in the malignant clone. On the other hand, Chen and Epstein[5] found that the B-lymphocytes constitute, on average, 6% of blood mononuclear cells. Only a minor fraction of these B cells were clonally related to the myeloma cells. The results of both groups indicated the involvement of B cells in the myeloma clone.

Mobilization strategies should be evaluated for their efficacy to mobilize PBPC and to lower the number of malignant cells in the autografts. We present here our data comparing the amounts of $CD34^+$ progenitor cells and $CD19^+$ B cells in leukapheresis products mobilized in steady-state and post chemotherapy using granulocyte colony-stimulating factor (G-CSF). Furthermore, we investigated highly purified $CD34^+$ and $CD19^+$ cells for the presence of the tumor specific IgH rearrangement.

METHODS AND MATERIALS

Patients

Twelve patients were enrolled into the study comparing the two mobilization regimens (Table 1). There were 6 males and 6 females with a median age of 52. Two patients had stage II, while 10 patients had stage III disease. The patients received G-CSF (Filgrastim, Amgen-Roche, München, Germany) in steady state (1200 μg/day) for collecting PBPC-harvest I and high-dose cyclophosphamide (7 g/m^2) and G-CSF with 300 μg/day for collecting PBPC harvest II.

Collection of PBPC and assessment of hematopoietic progenitor and B cells

The blood-derived progenitor cells were harvested as soon as a distinct population of $CD34^+$ cells (>10 $CD34^+$ cells/μL) could be identified by direct immunofluorescence flow cytometry analysis. Harvesting was performed with a Fenwal CS 3000 (Baxter Deutschland, München, Germany). For each leukapheresis, 10 liters of blood were processed at a flow rate of 50–70 mL/min. To support this high-dose conditioning regimen with melphalan and TBI, an autograft

Table 1. Patient characteristics

males/females	6/6
age (median, range)	52 (42–64)
stage II/III	2/10
paraprotein	
G	6
A	2
BJ	2
D	2
no. of previous therapy cycles (median, range)	5 (3–21)
previous radiotherapy	5 patients

containing more than 2.0×10^6 $CD34^+$ cells/kg body weight (BW) was considered necessary for sustained engraftment.

Blood-derived hematopoietic progenitors and B cells were assessed using direct immunofluorescence analysis as described in detail elsewhere.[6]

Purification of $CD34^+$ and $CD19^+$ cells from leukapheresis products

Mononuclear cells (5×10^7) from leukapheresis products were incubated with anti-CD34 FITC and anti-CD19 PE. The cells were washed once in PBS + 1% BSA and sorted on a FACStar^{Plus} (Becton Dickinson, Mountain View, CA). The cell sorter was aligned and set up using MNC from healthy donors. Cells were analyzed after appropriate gating at a rate of 5000 cells/sec. The $CD34^+$ and the $CD19^+$ populations were sorted in the normal-R mode with a sort rate of 7 cells/sec upwards, depending on the size of the populations. The sorted $CD34^+$ and $CD19^+$ cells were then reanalyzed and resorted with the aim to achieve a purity of over 95%.

Myeloma CDR3 sequencing

The myeloma CDR3 region was sequenced as previously described.[7] Briefly, cDNA was synthesized using random hexamers and total RNA from BM taken at diagnosis or prior to mobilization therapy. Reverse transcription was performed with Moloney-murine leukemia virus reverse transcriptase (Perkin Elmer, Weiterstadt, Germany) at 42°C for 15 minutes. For amplification the whole cDNA synthesized was used with CDR3 consensus primers described by Yamada et al.[8] elongated by so-called cloning tails in order to proceed with the following cloning steps. The amplification was performed with Klentaq polymerase (Clontech, Heidelberg, Germany) for 15 initiating cycles with denaturation, annealing and

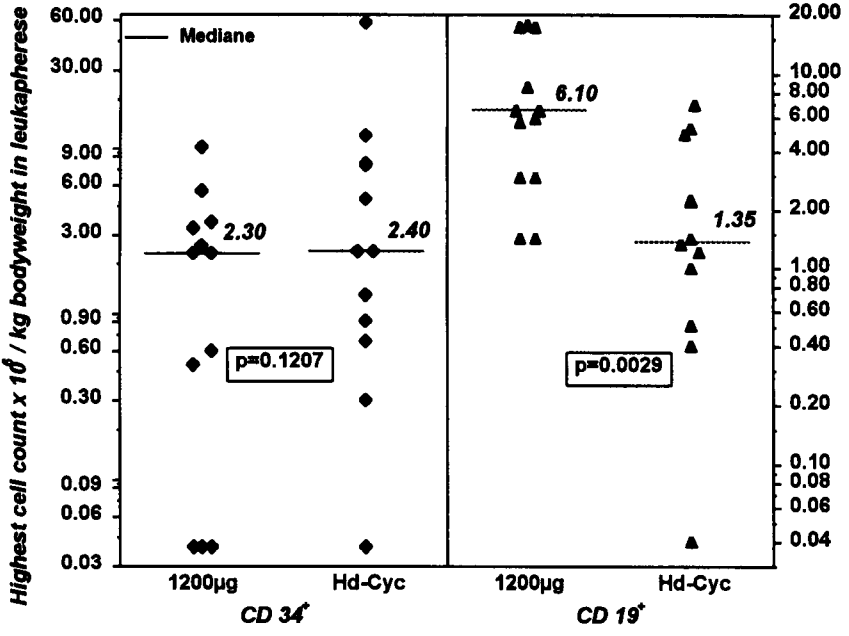

Figure 1. *Comparison of CD34 and CD19 content in leukapheresis products after steady state and post chemotherapy mobilization with G-CSF.*

extension at 94°C, 55C and 72°C, respectively, followed by 25 cycles with an increased annealing temperature of 60°C. The appropriately sized PCR products were then excised from ethidium bromide–stained agarose gels. If the CDR3 PCR revealed a distinct band, sequencing was done directly using the fmol sequencing kit (Promega, Heidelberg, Germany). Otherwise, the excised PCR products were cloned using the PCR direct cloning system (Clontech). Plasmid-DNA was cycle-sequenced after lysis of bacterial clones with 10 mM Tris-Cl pH 7.5, 1 mM EDTA and 100 µg/mL of Proteinase K (Boehringer, Mannheim, Germany). Sequencing was done with the Sequitherm kit (Biozym, Göttingen, Germany) on an automated sequencer (ALFexpress, Pharmacia, Freiburg, Germany). For the identification of identical clones, initially only one base was sequenced ("C-tracks"). The pattern of C-tracks obtained was compared with the fragment manager program. Identical C-tracks were then sequenced in both orientations.

DNA was extracted from leukapheresis products using standard techniques.[9] $CD34^+$ cells obtained after preparative fluorescence activated cell sorting (FACS) were lysed directly in PCR-buffer with the addition of Pretaq (Gibco, BRL, Eggenstein, Germany). Aliquots were used for PCR. In the case of small cell

numbers (<20,000 cells), one-third of the lysate was used to prove whether the DNA was amplifiable followed by the specific assay done twice. For each patient, at least two allele-specific oligonucleotides from the individual CDR3 sequences were designed. The PCR assays were done with the ASO primer as sense and the JH consensus primer as antisense primer. The optimal annealing temperatures were tested for each primer. For the patient-specific primer used here, an annealing temperature of 60°C turned out to be optimal. PCR was then performed for 40 cycles. Products were separated on 5% agarose gels stained with ethidium bromide.

RESULTS

PBPC mobilization and harvesting

The 12 patients with multiple myeloma presented here differ with respect to stage, previous therapy cycles and previous radiation therapy. In all patients, PBPC were collected during G-CSF enhanced leukocyte recovery. It was possible to harvest $\geq 2.0 \times 10^6$ $CD34^+$ cells/kg BW with two leukaphereses in 8 of the 12 patients during steady state mobilization and in 9 of 12 patients post chemotherapy.

Analysis of the $CD34^+$ and $CD19^+$ cell contents of the leukaphereses obtained with both mobilization regimens using immunofluorescence staining revealed that the $CD34^+$ cells differed only slightly with median values of 2.3 in steady state versus 2.4×10^6cells/kg BW post chemotherapy (Figure 1). In contrast, the content of $CD19^+$ cells as potentially malignant cells was significantly lower in harvests post chemotherapy with median values of 6.4 in steady-state versus 1.35×10^6 cells/kg BW post chemotherapy ($p=0.0029$; Figure 1).

Tumor cell contamination of PBPC harvests

In dilution experiments with cells of a myeloma cell line with normal buffy coat cells, sensitivities of 1 myeloma cell in 10^5 normal cells using a cell line specific ASO-primer were routinely reached (data not shown). In order to examine the PBPC harvests for tumor cell contamination the tumor specific CDR3 regions of 5 patients could be identified using the bone marrow sample obtained at diagnosis or prior to mobilization therapy. Up to 4 ASO primers were designed for each patient and tested with the bone marrow DNA sample used for the identification of the tumor clone and with normal buffy coat DNA. For each patient, the specificity of at least one ASO primer could be demonstrated. With the optimized ASO-PCR assay optimized for one ASO-primer, we could detect tumor cells in 12 of 17 LP products.

Assessment of $CD34^+$ cells and $CD19^+$ cells

$CD34^+$ cells were purified with a double sorting assay from ASO-PCR positive leukapheresis products of the 5 patients. Purities between 96.9% and 98.6% were achieved (Table 2). In these $CD34^+$ cell concentrates, tumor cells

Table 2. PCR results of samples from FACS-sorted leukapheresis products (LP) of patients with multiple myeloma

Patient	*Fraction*	*Cell number*	*Purity %*	*PCR result*
1	$CD34^+$	350,000	98.6	negative
	$CD19^+$	20,000	93.5	positive
2	$CD34^+$	25,000	96.9	negative
	$CD19^+$	600,000	92.0	positive
3	$CD34^+$	300,000	98.5	negative
	$CD19^+$	600,000	91.7	positive
4	$CD34^+$	250,000	98.0	negative
	$CD19^+$	17,000	91.7	positive
5	$CD34^+$	160,000	98.1	negative

Samples of the LP-products were sorted for the CD34 antigen present on hematopoietic progenitor cells and for the B cell antigen CD19. PCR results were obtained with patient-specific complementary-determining-region 3 primers. All $CD34^+$ fractions were PCR-negative, while the $CD19^+$ were PCR-positive.

could not be detected with PCR using allele-specific primers. In contrast, tumor-specific rearrangement of the heavy chain locus could be detected in the $CD19^+$ cell fraction of the transplant product in four cases. All were ASO-PCR positive using between 20,000 and 600,000 cells.

DISCUSSION

In this study we demonstrated that a high-dose CY-based mobilization regimen was able to reduce significantly the amount of potentially malignant B cells in PBPC harvests of patients with MM in comparison to the steady state situation. That the $CD19^+$ cells measured in LP aliquots include tumor cells was shown with an ASO-PCR assay of preparatively obtained $CD19^+$ cell fractions. The presence of tumor cells in LP of patients with multiple myeloma has been demonstrated by various groups,[10–13] recently also quantitatively with a median value of 0.0024%.[14,15] To minimize the amount of tumor cells transplanted, one may also consider positive selection of $CD34^+$ cells for patients with MM. The involvement of the $CD34^+$ progenitor cell in the malignant process in MM is controversial. With the results presented here, we could demonstrate that highly purified $CD34^+$ cell fractions from LP products of patients do not contain the tumor clone. This is in agreement with data of Vescio et al. and Willems et al.,[15,16] both demonstrating that CD34 is not expressed on the malignant cells of MM. In contrast, Belch et al.[17] showed that $CD34^+$ B cells in the blood of patients with MM express clonotypic IgH sequences. Using CD34 for positive selection, Schiller et al.[18] could demonstrate the feasibility of transplanting $CD34^+$ PBPC after high-dose chemotherapy for patients with MM accompanied by a 2.7 to 4.5-log reduction in contaminating MM cells. Randomized

studies with CD34 selection will give answers about the impact of reducing tumor cells in the autografts of patients with MM.

REFERENCES

1. Vesole DH, Tricot G, Jagannath S et al.: Autotransplants in multiple myeloma: What have we learned? *Blood* 3:838–847, 1996.
2. Haas R, Moehle R, Fruehauf S et al.: Patient characteristics associated with successful mobilizing and autografting of peripheral blood progenitor cells in malignant lymphoma. *Blood* 83:3787–3794, 1994.
3. Goldschmidt H, Hegenbart U, Haas R, Hunstein W: Mobilization of peripheral blood progenitor cells with high-dose cyclophosphamide (4 or 7 g/m^2) and granulocyte colony-stimulating factor in patients with multiple myeloma. *Bone Marrow Transplant* 17:691–697, 1996.
4. Bergsagel PL, Smith AM, Szczepek A et al.: In multiple myeloma, clonotypic B lymphocytes are detectable among CD19$^+$ peripheral blood cells expressing CD38, CD56, and monotypic Ig light chain. *Blood* 85:436–447, 1995.
5. Chen BJ, Epstein J: Circulating clonal lymphocytes in myeloma constitute a minor subpopulation of B cells. *Blood* 87:1972–1976, 1996.
6. Hohaus S, Goldschmidt H, Ehrhardt R et al.: Successful autografting following myeloablative conditioning therapy with blood stem cells mobilized by chemotherapy plus rhG-CSF. *Exp Hematol* 21:508–514, 1993.
7. Moos M, Schulz R, Cremer et al.: Detection of minimal residual disease by polymerase chain reaction in B cell malignancies. *Stem Cells* 13(suppl 2):42–51, 1995.
8. Yamada M, Hudson S, Tournay O et al.: Detection of minimal disease in hematopoietic malignancies in the B-cell lineage by using third-complementary-determining region (CDR-III)-specific probes. *Proc Natl Acad Sci USA* 86:5123–5127, 1989.
9. Sambrook J, Fritsch EF, Maniatis T: Isolation of high molecular weight DNA from mammalian cells, 9.16–9.23. In: Sambrook J, Fritsch EF, Maniatis T: (eds) Molecular Cloning—A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989.
10. Deane M and Norton JD: Immunoglobulin gene "fingerprinting": An approach to analysis of B lymphoid clonality in lymphoproliferative disorders. *Br J Haematol* 77:274–281, 1991.
11. Corradini P, Voena C, Astolfi M et al. High dose sequential chemoradiotherapy in multiple myeloma: Residual tumor cells are detectable in bone marrow and peripheral blood cell harvests and after autografting. *Blood* 85:1596–1602, 1995.
12. Bird JM, Bloxham D, Samson D et al.: Molecular detection of clonally rearranged cells in peripheral blood progenitor cell harvests from multiple myeloma patients. *Br J Haematol* 88:110–116, 1994.
13. Mariette X, Fermand JP, Brouet JC: Myeloma cell contamination of peripheral blood stem cell grafts in patients with multiple myeloma treated by high-dose therapy. *Bone Marrow Transplant* 14:47–50, 1994.
14. Vescio RA, Han EJ, Lee JC et al.: Quantitative comparison of multiple myeloma tumor contamination in bone marrow harvest and leukapheres autografts. Abstracts of the 37th annual American Society of Hematology meeting. Dec 1–5, 1995 regarding multiple

myeloma: 29(P-924), 1995.

15. Vescio RA, Han EJ, Schiller GJ et al.: Quantitative comparison of multiple myeloma tumor contamination in bone marrow harvest and leukapheres autografts. *Bone Marrow Transplant* 18:103–110, 1996.
16. Willems P, Croockewit A, Raymakers R et al.; CD34 selections from myeloma peripheral blood cell autografts contain residual tumor cells due to impurity, not to $CD34^+$ myeloma cells. *Br J Haematol* 93:613–622, 1996.
17. Belch AR, Szczepek A, Bergsagel PL et al.: Circulating $CD34^+$ cells from peripheral blood in multiple myeloma include B cells with patient-specific IGH CDR3 sequences and CD34 mRNA as well as DNA hyperdiploidy and N-ras mutation. *Blood* 86:1089, 1995 [abstr].
18. Schiller G, Vescio R, Freytes C et al.: Transplantation of $CD34^+$ peripheral blood progenitor cells after high-dose chemotherapy for patients with advanced multiple myeloma. *Blood* 86:390–397, 1995.

LOW-DOSE CYCLOPHOSPHAMIDE FOLLOWED BY SEQUENTIAL GM-CSF AND G-CSF MOBILIZES LARGE NUMBERS OF PROGENITOR CELLS WITH A MINIMUM OF LEUKAPHERESIS SESSIONS

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ABSTRACT

The successful mobilization and collection of peripheral blood progenitor cells (PBPC) has allowed more rapid engraftment and reduced overall toxicities following high-dose chemotherapy (HDCT). The optimum type of mobilization remains unknown. Growth factor alone or chemotherapy plus growth factor regimens have proliferated. The use of growth factor alone is less toxic but may yield lower numbers of progenitors, necessitating additional pheresis sessions. Chemotherapy plus growth factor regimens, however, are associated with significant toxicities including febrile neutropenia, as well as additional toxicity to the patient. In addition, they require close monitoring of serial white blood cell or CD34 count sequentially to allow pheresis at the optimum time. In the current work, low-dose cyclophosphamide (1.5 g/m^2) was administered, followed by sequential granulocyte macrophage colony-stimulating factor (GM-CSF) 250 $\mu g/m^2$, and granulocyte colony-stimulating factor (G-CSF) 5 $\mu g/kg$. This regimen did not cause significant neutropenia (mean nadir white blood cell count 1700) and was associated with a very low risk of febrile neutropenia (1 of 48 patients, 2%). A single 3-hour, 10-liter leukapheresis yielded a minimum of 2.5×10^6 CD34 cells/kg in 26 of 28 patients with solid tumors and in 16 of 20 patients with non-Hodgkin's lymphoma. Engraftment following infusion of a minimum of 2.0×10^6 CD34 cells was rapid (absolute neutrophil count [ANC] $>500/mm^3$ at 10 days, platelet transfusion independence at 12 days). This technique for the mobilization of PBPC is nontoxic and produces high numbers of cells in patients with both solid tumors and lymphomas. Therefore, it suggests itself as a very cost-effective method of progenitor cell collection.

INTRODUCTION

The use of peripheral blood progenitor cells (PBPC) in place of autologous bone marrow to provide hematopoietic support following myeloablative chemotherapy and/or radiation therapy has led to substantial benefits. The overall time to

engraftment and transfusion independence is shorter in most clinical studies.[1,2] In addition, overall toxicities associated with the treatment seem to be reduced.[3] This has facilitated reduction in hospital length of stay and in some cases has allowed outpatient transplantation to be performed.[4,5] In general, when a given threshold of progenitor cell number is exceeded, rapid and reliable engraftment follows. Despite the widespread use of PBPC, many questions remain regarding their use; among them, the optimum method of "mobilization." Many studies have found that when using growth factor alone, mobilization regimens yield lower numbers of PBPC than do chemotherapy plus growth factor regimens.[6] In some cases this translates into extra leukapheresis and cryopreservation procedures, which raise costs. Growth factor alone regimens, however, have the advantage of being relatively nontoxic and predictable in the sense that the initiation date of growth factor and the pheresis date are predetermined so that serial measurement of either the white blood cell count or CD34 count is not required. Moreover, the need for weekend pheresis is eliminated. This makes it easier to schedule pheresis sessions in busy pheresis units and may increase the efficiency of such leukapheresis and cryopreservation facilities.

Although chemotherapy plus growth factor regimens may yield higher numbers of cells, they can cause toxicity in patients who may have already received considerable chemotherapy and who will receive much more. Chief among these toxicities is febrile neutropenia, the incidence of which is 30–50% or higher.[7] Further, some chemotherapy mobilization regimens use agents that are not active against a particular tumor type. Thus, the patient is subjected to the toxicities of chemotherapy solely for the purpose of mobilizing progenitor cells, even though the tumor burden is unlikely to be substantially reduced.

The current work assesses the ability of a low-dose cyclophosphamide and growth factor regimen to mobilize a large number of PBPC. We postulated that a low-dose cyclophosphamide and growth factor regimen could still mobilize a large number of cells while imparting minimal toxicities. Moreover, cyclophosphamide is a broadly active chemotherapy agent and would be expected to have significant activity in a variety of tumor types. Sequential GM- and G-CSF were utilized to take advantage of the potential synergy of these two independently active hematopoietic growth factors.

PATIENTS AND METHODS

Forty-eight consecutive patients who met institutional requirements for HDCT were enrolled. Table 1 shows the characteristics of these patients. All patients signed an informed consent document approved by the institutional Human Subjects Committee. Patients underwent placement of a central venous catheter into the superior vena cava capable of supporting pressures of 100–150 mmHg. Leukapheresis was performed using a COBE Spectra cell separator (COBE Laboratories, Lakewood,

Table 1. Patient characteristics

median age (range)		47 (26–69)
median Karnofsky pos. (range)		90% (60–100)
diagnoses		
Hodgkin's disease	4	chemoresponsive relapse (4)
Non-Hodgkin's lymphoma	16	1st high-risk remission (3)
		chemoresponsive relapse (11)
		chemorefractory relapse (2)
stage IV breast cancer	14	chemoresponsive (14)
high-risk primary	9	1st remission consolidation (9)
ovarian cancer	4	1st remission consolidation (1)
		chemosensitive 1st relapse (2)
		chemosensitive 2nd relapse (1)
testicular cancer	1	chemosensitive relapse

CO) utilizing a continuous collection technique for 4 hours. An average of 10–11 liters of whole blood was processed by this technique. The mobilization regimen consisted of cyclophosphamide 1.5 g/m^2 administered over 1 hour with appropriate prehydration and antiemetic therapy. Forty-eight hours later, granulocyte macrophage colony-stimulating factor (GM-CSF) (Leukine®, Immunex Corp., Seattle, WA) was administered at a dose of 250 μg/m^2 subcutaneously once a day. After 5 days of GM-CSF, G-CSF (Neupogen®, Amgen, Thousand Oaks, CA) was administered at a dose of 10 μg/kg subcutaneously once a day. Leukapheresis was commenced on the tenth day after cyclophosphamide. G-CSF was continued until the leukapheresis was completed and a target CD34 number was obtained. Neither white blood cell counts nor CD34 cell counts were serially monitored prior to the pheresis. The leukapheresis was continued until 4×10^6 CD34 cells/kg were obtained for patients undergoing single-cycle HDCT regimens. Some patients were enrolled on double transplant protocols which required $6\text{-}8\times10^6$ CD34 cells/kg[8].

Chemotherapy treatment supportive care

Patients received appropriate prehydration, antiemetics and then disease-specific chemotherapy regimens (Table 2). PBPC were thawed to body temperature and reinfused 48 hours following completion of chemotherapy on day +1. G-CSF was commenced on day +1 at a dose of 5 μg/kg subcutaneously once a day and continued until the absolute neutrophil count was 2000/mm^3. Single donor platelets were administered for a morning platelet count <15,000/mm^3 and red blood cells were transfused for a morning hemoglobin of <8.0 g/dL. All transfusions were leuco-depleted and irradiated with 2000 cGy.

Table 2. Chemotherapy regimens

	Cycle 1	*Cycle 2*
lymphomas		
	cyclophosphamide 6000 mg/m^2	Thiotepa 600 mg/m^2
	VP-16 1500–1800 mg/m^2	Mitoxantrone 50–60 mg/m^2
	cisplatin or carboplatin 180 or 1200 mg/m^2	
solid tumors		
	cyclophosphamide 6000 mg/m^2	Melphalan 140 mg/m^2
	thiotepa 500 mg/m^2	Carboplatin 1200 mg/m^2
	cisplatin or carboplatin 180 or 1200 mg/m^2	

Measurement of progenitor cells

A standard blood count was done to determine the nucleated cell content. CFU-GM were measured after dilution in IMDM media and plating in methylcellulose cultures. CD34 content was performed within 1 hour of collection using FACS analysis of cells incubated with HPCA-1 (Becton Dickinson, Mountain View, CA) directed against the CD34 antigen. All FACS analyses were interpreted by a single immunopathologist, and all CFU-GM plates read by a single technologist.

RESULTS

Toxicity

The mobilization regimen was well tolerated. One of 48 patients (2%) developed febrile neutropenia requiring intravenous antibiotics and 3 days of hospitalization. Mucositis, hemorrhagic cystitis and hemorrhagic myocarditis were not seen. The antiemetic regimen given as prophylaxis varied by physician preference and patient requirement. Only 1 of 48 patients (2%) had nausea and vomiting lasting 3 days or more.

Outcomes of the pheresis

Table 3 shows the nadir white blood cell count and the white blood cell count on the day of pheresis for both the solid tumor patients and the lymphoma patients. The median CD34 and CFU-GM yields for each day of leukapheresis are shown in Table 3 as well for both groups of patients. Engraftment data is shown in Table 4 for both groups of patients following the first cycle of high-dose chemotherapy.

Table 3. Progenitor cell yields after mobilization

	Median nadir WBC (cells/mm³)	*Median WBC on 1st day of pheresis (cells/mm³)*	*CFU-GM × 10⁴/kg median (range)*	*CD34 × 10⁶/kg median (range)*
lymphomas (n=20)	1200	7600		
leukapheresis #1			14.6 (2–117)	2.0 (0.1–13)
leukapheresis #2			36.0 (6–127)	3.8 (0.8–8.0)
solid tumors (n=28)	1800	11,500		
leukapheresis #1			47.0 (17–217)	4.4 (1–18)
leukapheresis #2			75.0 (12–167)	6.1 (1–22)

DISCUSSION

These data demonstrate low-dose cyclophosphamide (1.5 g/m^2) followed by sequential GM-CSF and G-CSF is capable of producing high numbers of CD34 cells, adequate for engraftment with a minimal number of pheresis sessions. Over 90% of both lymphoma patients and solid tumor patients were able to complete mobilization in a single session, achieving a commonly used CD34 threshold value of 2.5×10^6/kg. Because some of these patients were enrolled in double transplant programs, additional pheresis sessions were required for some patients. The virtual absence of significant toxicity makes this a very favorable regimen when compared to other high-dose regimens that are associated with an incidence of febrile neutropenia that exceeds 30%.[7] These data show that the beneficial effects of combination chemotherapy and growth factor can be obtained without the intensive chemotherapy that can produce pancytopenia lasting almost as long as the myeloablative regimen itself. Furthermore, cyclophosphamide is a broadly active chemotherapy agent that would be expected to have cytotoxic effects on a wide variety of tumors in addition to its benefits for mobilization. This is not true of all chemotherapy mobilization programs.[9]

One of the most important benefits of this regimen is that it is highly predictable and reliable. Unlike other higher-dose chemotherapy and growth factor regimens, it is not necessary to serially monitor the white blood cell count or CD34 content. The unpredictable nature of white blood cell count recovery and the requirement to monitor the white blood cell count and/or CD34 count serially is cumbersome and expensive. Although it is possible and even likely that we missed the peak of the yield in individual patients, the results nevertheless are quite sufficient for adequate engraftment, as demonstrated in Table 4. Further, in managed care settings, the mobilization chemotherapy regimen is often delivered by the primary oncologist or primary care medical group. In these types of situations the treatment of febrile

Table 4. Time to hematologic recovery following each dose of high-dose therapy

Median (range)	*CD34 infused 10^6/kg*	*Days to ANC >500*	*Days to PLT T.F. indep.*	*# PLT trans.*	*RBC T.F. indep.*	*# RBC trans.*
lymphomas*						
cycle 1 n=20	3.5 (1.1–6.4)	10 (12)	14 (9–20)	4 (1–6)	15 (10–20)	3 (0–6)
cycle 2 n=16	3.1 (1.0–6.0)	10 (9–12)	14 (8–18)	3 (1–2)	14 (8–22)	4 (2–8)
solid tumors						
cycle 1 n=28	4.5 (3.6–9.6)	10 (9–11)	13 (8–16)	3 (1–4)	15 (9–18)	3 (1–6)
cycle 2 n=20	4.1 (2.8–5.6)	10 (9–12)	14 (8–18)	3 (2–6)	15 (9–16)	4 (2–8)

**3 patients receiving 1–1.5×10^6 CD34 cells/kg required infusion of back-up bone marrow or PBPC for delayed recovery.*

neutropenia, should it occur, may not be permitted to proceed at the transplant institution, thus limiting the access to the leukapheresis machines.

Sequential use of GM-CSF and G-CSF was designed to take advantage of the potential synergy of these two agents, which are independently active in the mobilization of PBPC. From the design of the study, it is not possible to determine the contribution of each individual component of the mobilization regimen.

In summary, the use of lower dose cyclophosphamide (1.5 g/m^2) followed by sequential GM-CSF and G-CSF was highly successful in the efficient mobilization of large numbers of PBPC. This regimen has the advantage of reliability and predictability and is associated with minimal toxicity. Ongoing studies are currently examining the use of large-volume leukapheresis to further augment yields of PBPC. This mobilization regimen and the rapid engraftment that follows the high-dose chemotherapy set a high standard for investigational cytokines, which are just entering clinical trials.

REFERENCES

1. Chao NJ, Schriber JR, Grimes K et al.: Granulocyte colony-stimulating factor "mobilized" peripheral blood progenitor cells accelerate granulocyte and platelet recovery after high-dose chemotherapy. *Blood* 81:2031–2035, 1993.
2. Stadtmauer EA, Schneider CJ, Silberstein LE: Peripheral blood progenitor cell generation and harvesting. *Semin Oncol* 22:291–300, 1995.
3. Peters WP, Rosner G, Ross M et al.: Comparative effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) on priming peripheral blood progenitor cells for use with autologous bone marrow after high-dose chemotherapy. *Blood* 81:1709–1719, 1993.
4. Peters WP, Ross M, Vredenburgh JJ et al.: The use of intensive clinic support to permit outpatient autologous bone marrow transplantation for breast cancer. *Semin Oncol* 21:25–31, 1994.

5. Meisenberg BR, Miller WE, McMillan R et al.: Outpatient high-dose chemotherapy with autologous stem cell rescue for hematologic and non-hematologic malignancies. *J Clin Oncol* (in press).
6. Teshima T, Harada M, Takamatsu Y et al.: Cytotoxic drug and cytotoxic drug/G-CSF mobilization of peripheral blood stem cells and their use for autografting. *Bone Marrow Transplant* 10:215–220, 1992.
7. To LB, Shepperd KM, Haylock DN et al.: Single high doses of cyclophosphamide enable the collection of high numbers of hemopoietic stem cells from the peripheral blood. *Exp Hematol* 18:442–447, 1990.
8. Meisenberg B, McMillan R, Miller W: Sequential cycles of high-dose chemotherapy (HDC) with autologous peripheral blood progenitor cells (PBPC). *Proc Am Soc Clin Oncol* 14:318, 1995.
9. Pettengell R, Testa NG, Swindell R et al.: Transplantation potential of hematopoietic cells released into the circulation during routine chemotherapy for non-Hodgkin's lymphoma. *Blood* 82:2239–2248, 1993.

FEASIBILITY AND TOXICITY OF DOUBLE CYCLES OF HIGH-DOSE CHEMOTHERAPY AND AUTOLOGOUS PROGENITOR CELL RESCUE

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ABSTRACT

The ability to harvest large numbers of peripheral blood progenitor cells (PBPC) has allowed the testing of multiple cycles of high-dose chemotherapy (HDCT) for treatment of neoplastic diseases. In this study we enrolled 106 consecutive patients in a trial of tandem cycles of high-dose treatment. Chemotherapy consisted of cyclophosphamide 6000 mg/m^2, thiotepa 500 mg/m^2 and cisplatin 180 mg/m^2 or carboplatin 1200 mg/m^2. Some patients received etoposide 1800 mg/m^2 instead of thiotepa. A second cycle of therapy consisting of thiotepa 500 mg/m^2 and mitoxantrone 50–60 mg/m^2 or melphalan 140 mg/m^2 and carboplatin 1200 mg/m^2 was planned 6 weeks after the first cycle. PBPCs were harvested using growth factor alone or chemotherapy plus growth factor regimens. Of the 106 patients, 39 (37%) did not receive the second cycle of HDCT. The most common reason was refusal among patients who were already in complete remission after HDCT cycle 1. The toxicities of the tandem program were acceptable with 3 treatment-related deaths, 1 after cycle 1, and 2 more than 100 days after cycle 2. Further chemotherapy responses were documented among the 36 patients with persistent measurable disease after HDCT cycle 1. Eight patients developed complete responses and 18 patients had further partial responses after HDCT cycle 2. We conclude that tandem cycles are feasible, toxicity is limited and there is some evidence of increased efficacy. A randomized trial of this concept is appropriate with disease-free survival and overall survival as endpoints.

INTRODUCTION

High-dose chemotherapy (HDCT) has become an increasingly utilized treatment for a variety of neoplastic conditions. To date, most treatment plans call for a single cycle of HDCT to consolidate a previous response to lower-dose chemotherapy. This single-treatment regimen was adopted, not because of proven optimum efficacy, but rather because the significant toxicities of HDCT made contemplation of more than one treatment inappropriate. With the ability to harvest large numbers of blood progenitor cells and the reduction in overall

toxicities due to the use of progenitor cells and other supportive care measures, it becomes reasonable to contemplate the use of more than one cycle of HDCT. The current work describes the outcomes of a double-cycle program of HDCT supported with mobilized blood progenitor cells.

PATIENTS AND METHODS

One-hundred-and-six consecutive metastatic or high-risk patients were enrolled in the program. All patients signed an informed consent document approved by the institutional Human Subjects Committee. No patient was excluded from the double-cycle program based on pretransplant performance status. All patients met minimal baseline physiologic criteria for treatment. The chemotherapy regimens were designed to be delivered 6 weeks apart, assuming resolution of all serious toxicities. For patients with breast and ovarian cancer, the first cycle consisted of cyclophosphamide 6000 mg/m^2, thiotepa 500 mg/m^2 and cisplatin 180 mg/m^2 or carboplatin 1200 mg/m^2 in divided doses over 4 days. Patients with non-Hodgkin's lymphoma or Hodgkin's disease received etoposide 1800 mg/m^2 in divided doses over 4 days instead of thiotepa. The second cycle for patients with breast and ovarian cancer consisted of either melphalan 140 mg/m^2 plus carboplatin 1200 mg/m^2, or thiotepa 600 mg/m^2 plus mitoxantrone 50–60 mg/m^2 for patients with lymphomas. One patient with testicular cancer received a single cycle of carboplatin 1800 mg/m^2 and etoposide 2000 mg/m^2.

SUPPORTIVE CARE

Patients received peripheral blood progenitor cell (PBPC) rescue. PBPCs were mobilized with either growth factor alone or chemotherapy plus growth factor regimens. Initially, autologous bone marrow was collected as a "backup." This practice was discontinued after the first 6 months of the study. A target of 6-8$\times$10^6 CD34 cells/kg was used to determine adequacy of progenitor cell collection. Patients were allowed to receive cycles of HDCT, however, with a minimum threshold of 1$\times$10^6 CD34 cells/kg per cycle. The PBPCs were collected following induction chemotherapy prior to the initiation of the first cycle of HDCT. Cells were split into roughly two equal aliquots for subsequent reinfusion with each cycle. Following HDCT, stem cells were reinfused 48 hours later (on DOT +1). Granulocyte colony-stimulating factor (G-CSF) 5.0 μg/kg was administered subcutaneously beginning on DOT +1 and continued until the ANC reached 2000/mm^3. Red blood cells were transfused for hemoglobin <8.0 g/dL. Single-donor pheresed platelets were transfused for a morning platelet count <15,000/mm^3.

Table 1. Patient Characteristics (n=106)

median age (range)	48 (26–69)
gender (M:F)	36:70
ECOG performance status	
0	18
1	67
2	21
diagnoses	
lymphoma/Hodgkin's disease	51
1st complete remission	6
1st partial remission	5
sensitive relapse	35
resistant relapse	5
breast cancer	44
inflammatory	5
chemoresponsive metastasis	39
ovarian cancer	8
1st remission	2
sensitive relapse	6
other	3

RESULTS

One-hundred-and-six consecutive patients meeting institutional criteria for HDCT were enrolled. Table 1 shows the patient characteristics. Of this number, 39 (37%) did not receive the second cycle of HDCT. Reasons for not receiving the second cycle included: patient refusal, 19; insurer refusal, 3; toxicities of the first cycle, 7; progressive disease or lack of response after cycle 1, 5; and inadequate mobilization or poor engraftment, 5. Of the 19 patients who refused the second cycle, most did so citing lingering constitutional toxicities and a strong desire to return to work or usual activities. All but one of these patients were already in complete clinical remission from either the induction therapy or cycle 1 of HDCT. No patient refused a second cycle when offered, if they still had persistent measurable disease.

The median interval between cycles of HDCT was 56 days, range 38–100 days, among the 67 patients (63%) who received a second cycle.

Toxicities of the tandem cycles

Eleven percent of patients in cycle 1 had grade IV or V toxicities, including 1 patient who died of respiratory failure from disseminated pulmonary aspergillus 9

days after chemotherapy. In cycle 2, 6% of patients had grade IV (life-threatening) toxicity but there were no deaths. Two additional patients had unexplained deaths several weeks after the second cycle was completed. The first patient, a 45-year-old woman with recurrent lymphoma, died of acute heart failure of undetermined etiology 110 days after the second cycle. Endomyocardial biopsy was not definitive. A 61-year-old man with recurrent non-Hodgkin's lymphoma died of idiopathic ARDS approximately 100 days after the second cycle of therapy.

Hematologic toxicity

Engraftment was rapid after both cycles of HDCT so long as a minimum threshold of 2×10^6 CD34 cells/kg were infused. Median time to ANC >500/mm^3 was 10 days after cycle 1 and 11 days after cycle 2. Median time to platelet transfusion independence was 11 days after cycle 1 and 11 days after cycle 2. There were no differences in the number of transfusions required after each cycle.

Chemotherapy responsiveness

Patients were routinely reassessed after cycle 1 of HDCT and prior to cycle 2. Patients were assessed again after cycle 2 to determine additional responses (Table 2). Of the initial 106 patients, 84 patients were evaluable, the other patients having been treated in the adjuvant setting or with nonevaluable disease. Twelve patients (14%) entered into complete remission with induction therapy prior to HDCT cycle 1. Following HDCT cycle 1, 36 patients had persistent measurable disease that could be evaluated for response to cycle 2. Of these 36 patients, 8 patients (22%; 6 with lymphoma, 2 with breast cancer) entered complete remissions after HDCT cycle 2. Fifteen patients (42%) had additional responses qualifying as partial responses (reduction in bi-perpendicular dimensions of tumor by >50%). Nine patients had no further response and 4 patients had progressive disease at the time of reevaluation after cycle 2.

DISCUSSION

These data show that with modern techniques of PBPC mobilization, rapid engraftment can be seen after each of two cycles of HDCT. The toxicities of this program were limited and the feasibility of such tandem cycle programs is now well established. One of the more interesting findings of the study is that 37% of patients did not receive the second cycle for a variety of reasons. This differs from other tandem-cycle programs where 80–100% of patients received cycle 2.[1–4] The reason for the high dropout rate in our study may have been that patients in this program were carefully reevaluated after cycle 1 and given the option of not proceeding with

Table 2. Response to each phase of therapy: Number of patients and percentage of 84 evaluable patients

	CR	*PR*	*NR*	*PD*	*NE*	*ADJ*	*Early death*
prior to HDC #1	12 (14%)	67 (80%)	2 (2%)	3 (4%)	11	11	0
after HDC #1	40 (48%)	36 (42%)	3 (4%)	5 (6%)	11	11	
after HDC #2	48 (57%)	24 (23%)	3 (4%)	9 (11%)	11	11	

Abbreviations: CR=complete response; PR=partial response; NR=no response; PD=progressive disease; NE=not evaluable; ADJ=adjuvant therapy.

cycle 2 at several points in their therapy. These patients were made to understand that the role of the second cycle was exploratory and that proof of benefit was not at hand. Therefore, many patients who were already in complete remission opted not to proceed with cycle 2. The fact that no patient with persistently measurable disease declined the second cycle is noteworthy by way of contrast.

The toxicities of cycle 2 were lower than with the first cycle. This may be because patients vulnerable to treatment-related toxicities were screened out after cycle 1. In addition, the second cycle regimens consisted of only two drugs and thus may have been less than maximally dose-intensive. The median interval to the initiation of cycle 2 was 56 days, which was approximately 2 weeks longer than anticipated. This median 14-day postponement was not due to specific organ toxicity but rather due to the constitutional effects of the therapy such as lingering nausea, fatigue and weakness. This finding has implications for model high-dose programs which seek to deliver multiple cycles of intensive therapy on an every 4- to 5-week basis. This finding is in distinction to the work of Ayash et al.[2] in which high-dose melphalan was followed at a median of 25 days by cyclophosphamide, thiotepa and carboplatin. In that study, however, the less intensive regimen was given first, unlike our program where it was given second.

Other studies of HDCT have served to document the feasibility and acceptable toxicity of this approach. Only a few studies, however, have systematically followed response after each portion of the treatment: induction therapy, HDCT chemotherapy cycle 1 and HDCT chemotherapy cycle 2. Thus, in only a few studies is the contribution of cycle 2 to the total antineoplastic effort subject to critical analysis. In this study we did see further responses in tumor size, including 8 of 36 patients (22%) who obtained complete remissions. These data are similar to those of Broun et al.[5] who also documented additional responses following the

second cycle of HDCT in patients with breast cancer. In our data, most of the additional complete responses (6 of 8) were seen among patients with non-Hodgkin's lymphomas and thus some of these complete remissions may represent slow resolution of residual fibrotic masses rather than actual antineoplastic effects.

The feasibility of this approach has now been well documented by our study and by others. We feel it is now time to consider a randomized trial with survival as the endpoint of tandem cycles of HDCT versus a single cycle. In such a trial, patients would be stratified based on response to the first cycle, complete remission versus partial remission, and then followed for disease progression and overall survival.

The second cycle might consist of the initial therapy repeated or a different regimen using potentially noncross-resistant agents.

REFERENCES

1. Broun ER, Nichols CR, Kneebone P et al.: Long-term outcome of patients with relapsed and refractory germ cell tumors treated with high-dose chemotherapy and autologous bone marrow rescue. *Ann Intern Med* 117:124–128, 1992.
2. Ayash LJ, Elias A, Wheeler C et al.: Double dose-intensive chemotherapy with autologous marrow and peripheral-blood progenitor-cell support for metastatic breast cancer: A feasibility study. *J Clin Oncol* 12:37–44, 1994.
3. Ghalie R, Williams SF, Valentino LA et al.: Tandem peripheral blood progenitor cell transplants as initial therapy for metastatic breast cancer. *Biology of Blood and Marrow Transplantation* 1:40–46, 1995.
4. Rodenhuis S, Westermann A, Holtkamp MJ et al.: Feasibility of multiple courses of high-dose cyclophosphamide, thiotepa, and carboplatin for breast cancer or germ cell cancer. *J Clin Oncol* 14:1473–1483, 1996.
5. Broun ER, Sridhara R, Sledge GW et al.: Tandem autotransplantation for the treatment of metastatic breast cancer. *J Clin Oncol* 13:2050–2055, 1995.

CHAPTER 9
Treatment After BMT

INTERLEUKIN-2 (IL-2) AFTER AUTOLOGOUS BLOOD OR MARROW TRANSPLANTATION

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ABSTRACT

The success of ASCT for advanced hematologic malignancy is limited largely by a high relapse rate. Therapy with IL-2 + lymphokine-activated killer (LAK) cells has induced clinical responses in patients with refractory acute myelogenous leukemia (AML) and non-Hodgkin's lymphoma (NHL). It is postulated that this approach might reduce relapses if it is used early after ASCT as consolidative immunotherapy against minimal residual disease. A phase I trial with IL-2 (Hoffman LaRoche) administered early after ABMT identified a tolerable regimen with immunomodulatory effects. It consisted of IL-2 at 3.0×10^6 Roche U/m^2/day $\times$ 5 by continuous IV infusion (CIV), 6 days of rest and then IL-2 at 0.9×10^6 Roche U/m^2/day $\times$ 10 by CIV. Autologous LAK cells obtained during the rest period were also infused into some patients. Of 17 AML patients beyond CR1 (3 in Rel1, 11 in CR2, 3 > CR2) who underwent ABMT followed, 21 to 85 days later, by IL-2 (12 patients) or IL-2 + LAK cells (5 patients), 1 died of sepsis, 6 relapsed and 10 remain in CR at 4 to 7.6 years (median 6.5). These and other encouraging results (e.g., with NHL) stimulated the design of phase III trials.

However, because Roche IL-2 became unavailable, a phase I/II trial was performed to identify an equitoxic regimen of IL-2 provided by Chiron to be used in subsequent randomized trials. Patients age 1–63 (median 44) with hematologic malignancies underwent ASCT with blood (34), marrow (23) or both (10) and began IL-2 therapy a median of 46 days later.

Cohorts of 3–4 patients received 9, 10 or 12×10^6 International U/m^2/day of induction IL-2 by CIV for 4 or 5 days as an inpatient, followed by 4 days of rest as an outpatient, and then 1.6×10^6 IU/m^2/d$\times$10 of maintenance IL-2 by CIV as an outpatient. The maximum tolerated dose (MTD) of induction IL-2 identified as 9×10^6 IU/m^2/d $\times$ 4. In the Phase II study, 52 patients received the MTD. Most patients exhibited some degree of capillary leak. One patient died of CMV pneumonia and one of ARDS. Maintenance IL-2 was well tolerated. IL-2–induced reversible neutrophilia

and thrombocytopenia (median nadir of 50,000), lymphocytosis with an increased number of cells expressing CD3, CD8, CD56 and p75, and the appearance of cells which lysed NK-resistant tumor without exposure to IL-2 in vitro. In the Phase II study, 14/24 NHL patients, 4/16 AML patients, 2/6 Hodgkin disease patients and 3/3 ALL patients remain in CR. Two of 3 myeloma patients remain in PR. Although the IL-2 regimen had significant side effects, it was well tolerated in the majority of patients. Phase III prospectively randomized trials are in progress to determine if this IL-2 regimen will decrease the relapse rate after ASCT for NHL and AML.

INTRODUCTION

The major unresolved problem in autologous stem cell transplantation using blood or marrow (ASCT) is the high relapse rate.[1–4] The relapses may represent progeny of residual host cells and/or of tumor cells contaminating the infused stem cells. As other problems associated with transplantation are diminished, e.g., with the use of appropriate growth factors, the centrality of tumor recurrence is further highlighted. The effectiveness of more intense conditioning regimens is limited by shared and cumulative toxicities and/or by their inability to affect transferred tumor cells. It is postulated that immunotherapy with IL-2, administered early after ASCT at a time of minimal residual disease, might reduce the relapse rate by eradicating the residual tumor as well as any transferred tumor cells and might thereby represent a form of in vivo purging. This hypothesis is based on laboratory data, animal model data and small sporadic clinical trials in which IL-2 therapy with or without infusion of exogenous lymphokine-activated killer (LAK) cells induced regression of some hematologic malignancies in some patients refractory to conventional and unconventional therapies.[5–8] Moreover, IL-2 administered after ASCT can induce a syndrome consistent with GVHD[9] and, therefore, might induce or augment a GVL effect. This paper summarizes some of our results from the laboratory and from phase I and II trials that have recently led us to initiate prospectively randomized phase III clinical trials of IL-2 versus no IL-2 after ASCT for patients at high risk for relapse of non-Hodgkin's lymphoma (NHL) and AML.

MATERIALS AND METHODS

These are detailed in previous publications.[9–15]

RESULTS

Sequence of studies

Ideally, immunotherapy should be administered a) early enough after ASCT before the malignancy is likely to recur, b) late enough for the patient to have

recovered from the toxicities of the conditioning regimen and c) late enough for IL-2-responsive lymphocytes to have been reconstituted so that the patient would have the capability of responding to exogenous IL-2. Accordingly, our sequence of studies was as follows: a) the reconstitution of IL-2-responsive lymphocytes after ASCT, b) a phase I trial of IL-2 (provided by Hoffman-LaRoche) administered early after ASCT, c) a trial to determine the feasibility of generating and infusing autologous LAK cells in the context of the MTD of IL-2 identified, d) another phase I/II trial of IL-2 provided by Chiron Therapeutics and e) phase III trials of IL-2 versus observation after ASCT for hematologic malignancies.

Reconstitution of IL-2-responsive lymphocytes

Responsiveness of lymphocytes to IL-2 was assessed by incubating them with IL-2 in vitro for 5 days, then measuring their ability to lyse the NK-resistant Daudi tumor cell line. Such LAK precursor activity was detected in the peripheral blood of patients as early as 2 weeks after transplantation of autologous marrow,[12] or peripheral blood stem cells,[14] suggesting that patients would be capable of responding to in vivo IL-2 as early as that.

Phase I trial of Roche IL-2 after ABMT[13]

Sixteen patients with hematologic malignancies underwent ABMT and 14 to 91 (median 33) days later received a course of IL-2. The eligibility criteria for initiation of IL-2 therapy included a neutrophil count $\geq 500/mm^3$, platelet count $\geq 20{,}000/mm^3$ with no more than 1 transfusion per day, adequate renal, hepatic, pulmonary and cardiac function, and absence of infection off antibiotic therapy. Cohorts of patients received 0.3 to 4.5×10^6 Roche U/m^2/day by continuous intravenous infusion (CIV) on days 1 to 5 of the IL-2 protocol. After a 6-day rest period, a low dose of maintenance IL-2 (3×10^5 Roche U/m^2/day) were administered by CIV in the outpatient clinic on days 12 to 21. The toxicities were the same as those associated with IL-2 in non-BMT settings and included fever, nausea, diarrhea, rash and fluid retention secondary to the vascular leak syndrome. The dose-limiting toxicities were hypotension and thrombocytopenia. All toxicities were rapidly reversible by decreasing or stopping IL-2. A marked rebound lymphocytosis was observed at the end of induction IL-2 and reflected primarily a marked increase in $CD3^+CD8^+$ T-cells, as well as $CD3\text{-}CD56^+$ lymphocytes—the latter being associated with activated NK cells.

Feasibility trial of IL-2 plus LAK cells[11]

Patients who received the MTD induction IL-2 underwent leukapheresis at the time of maximum lymphocytosis, i.e., on days 7 to 9 after IL-2 was initiated. Their cells were incubated for 5 days in IL-2 and reinfused on days 12 to 14, with a concurrent outpatient maintenance dose of IL-2 (as established above) on days

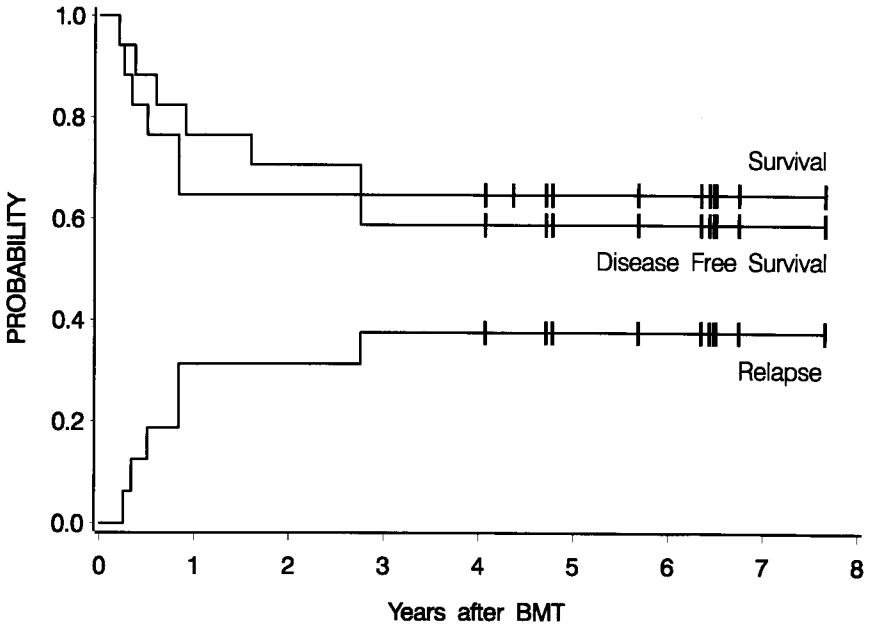

Figure 1.

12 to 21. A total of 21 patients received a median total of 1.4×10^{11} cells, which were predominantly $CD3^+CD8^+$ and $CD3\text{-}CD56^+$ and exhibited potent LAK activity in vitro. However, LAK cell infusions were associated with additional toxicities in the form of fever, chills and dyspnea, with more severe thrombocytopenia during pheresis, especially in patients with AML.[10] The additional side effects coupled with the additional hospitalization and expense led us to discontinue LAK cell generation and infusion in all subsequent patients.

Clinical outcomes of therapy with IL-2 + LAK cells after ABMT

The overall results obtained on 22 patients with malignant lymphoma[11] compare favorably with results obtained in historical controls without IL-2 at our institution.

However, particularly encouraging results were obtained in 17 patients who underwent ABMT for AML beyond first CR, followed by IL-2 (12 patients) or IL-2 + LAK cells (5 patients).[10,16,17] Their median age was 39, the median duration of their CR1 was 12 months (range 5 to 37); 8 patients received marrow purged with 4HC while 9 received unpurged marrow. At the time of ABMT 11 patients were in relapse 1, 3 in CR2 and 3 beyond CR2. IL-2 therapy was begun a median of 54 days after ABMT (range 21 to 91). The conditioning regimen was busulfan,

Table 1. IL-2 regimen

*IL-2 Rx day**	*IL-2 dose*
1–4	9×10^6 IU/m^2/day (as inpatient)
5–8	rest (as outpatient)
9–18	1.6×10^6 IU/m^2/day (as outpatient)

**"Day" refers to day of IL-2 protocol, not to day after ASCT.*

cytoxan and TBI in 13 patients, and busulfan and cytoxan in 4. Detailed clinical characteristics[10] and preliminary results have been reported.[10,16,17] Updated results are depicted in Figure 1. Of the 17 patients treated, 1 died of *Pseudomonas cepacae* sepsis, 6 relapsed (5 at 4 to 10 months and 1 at 32 months), while 10 remain in continuous CR for a minimum of over 4 years, a maximum of 7.6 and a median of 6.5 years. These results on this small heterogeneous group of patients compare rather favorably with results reported from this institution as well as those reported from others with autologous ABMT for AML beyond CR1.[4] Indeed, these results are better than those that have been reported in similar patients after allogeneic BMT. The results stimulated the design of phase III trials.

Phase I/II trial of Chiron IL-2 after ASCT for hematologic malignancies

Before a phase III trial could be initiated, however, Roche IL-2 become unavailable. A phase I/II trial was, therefore, performed to identify an equitoxic regimen of IL-2 (provided by Chiron Therapeutics), and to gain experience with it prior to embarking on a phase III trial. Between November 1992 and April 1995, 67 patients with a median age of 44 (range 1 to 63) were treated with high-dose chemotherapy with or without fractionated TBI plus an infusion of autologous stem cells from peripheral blood (PB, 34 patients), bone marrow (BM, 23) or PB plus BM (n=10), followed by IL-2 therapy. Thirty-one patients were treated for NHL, 17 for AML, 8 for Hodgkin's disease, 6 for multiple myeloma and 5 for ALL. A brief summary of the regimen and the data has been published as an abstract,[18] while detailed results of the IL-2 regimen, the clinical characteristics of these patients and their clinical outcomes are in press.[15] A phase I trial identified the MTD of induction IL-2 in this post-transplant setting as 9×10^6 IU/M^2/d$\times$4d, all administered by CIV as an inpatient. This was followed by 4 days of rest outside the hospital and 1.6×10^6 IU/M^2/d$\times$10d of self-administered maintenance IL-2 by CIV as an outpatient. All patients received only a single cycle of the IL-2 regimen presented in Table 1.

Toxicities[15]

In the phase II study, 52 patients who underwent ASCT for hematologic malignancies and met the eligibility criteria for IL-2 received a single cycle of the

IL-2 regimen shown in Table 1. The principal toxicities of induction IL-2 consisted of fever, malaise, fatigue, rash, capillary leak syndrome with weight gain, and mild and reversible hypotension responsive to IV fluids, and bacteremia in 10%. There were 2 treatment-related deaths, both in AML patients with pulmonary complications: 1 patient with a prior history of Hodgkin's disease treated with mantle radiation died of ARDS, and 1 died of CMV pneumonia 53 days after ASCT. Although all patients on the phase II trial received prophylactic antibiotics, 5 still developed bacteremia—4 with coagulase negative staphylococcal species, and 1 with JK diphtheroides, which resolved.

The clinical toxicities associated with maintenance therapy were primarily fatigue and malaise. In addition, 6 patients developed hypotension, which responded to IV fluids, 25% of patients had fever, and 4 patients developed bacteremia—3 gram positive and 1 gram negative.

Hematologic and immunomodulatory effects[15]

In 21 patients whose median pre-IL-2 platelet count had been 40,000, the platelet count fell to 20,000 or less, while among the rest of the patients whose pre-IL-2 count had been 118,000, no platelet counts below 50,000 were observed. All platelet counts recovered totally by the end of maintenance therapy.

Lymphocytosis was observed after induction IL-2 (median 5300 versus 1200 before IL-2 therapy was begun). It reflected a significant rise in the number of cells expressing CD3, CD8, CD56 and P75 and was associated with very vigorous lytic anti-tumor activity by PBL from all 18 patients tested, without any exposure to IL-2 in vitro.

Clinical outcomes[15]

The most interesting clinical outcome results in the phase II trial are the relatively poor results obtained in AML and the relatively good results obtained in NHL. Of 16 patients treated for AML (all but 2 beyond CR1), one (with prior Hodgkin's disease) died due to treatment, one died in CR of interstitial pneumonitis at 11 months, one died in CR at 7 months with pulmonary complications of unknown cause, nine relapsed, while 4 remain in CR at 12+ to 25+ months. These results are similar to those reported without IL-2.[4] However, the regimen and source of IL-2 used in this trial was different from that used in the past, as was the stem cell source (only marrow in past trials versus largely peripheral blood or peripheral blood plus BM in the present trial). The possibility that relapses of AML might be more frequent after PBSCT than after marrow, as raised by some reports,[19,20] might also explain or contribute to the discrepant results. However, the patient groups in the various trials were small, heterogeneous and not necessarily clinically comparable.

Among the 24 treated for NHL, of 11 low-grade NHL, three of whom were treated in first relapse or CR2 and eight beyond CR2, 4 relapsed while seven

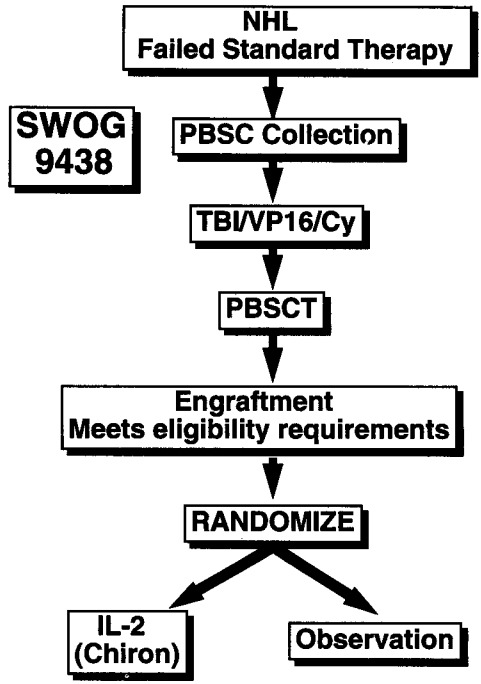

Figure 2.

remain in continuous CR at 13+ to 37+ (median 20+) months; of 13 patients with intermediate or high-grade NHL, nine of whom were treated in first relapse or CR2 and four beyond CR2, six relapsed and seven remain in continuous CR at 12+ to 26+ (median 22+) months. Although conclusions about the efficacy of this approach cannot be drawn from this study on a small and heterogeneous group of patients, the experience with this regimen of IL-2 and the results obtained have been sufficiently encouraging to lead us to initiate two prospectively randomized phase III trials to test the hypothesis that IL-2, administered at a time of minimal residual disease, will reduce the relapse rate and improve overall survival.

A phase III trial of IL-2 versus observation after ASCT for AML in Rel1 or CR2, i.e., SWOG 9328, was initiated but turned out to be not feasible, largely because harvesting and storing stem cells in CR1, then carefully following patients for relapse, and then to have a transplant bed available was logistically complex for referring physicians and for many transplant centers. For patients in relapse, too little time was available, while attempts to induce a CR2 are often unsuccessful. Therefore, accrual to this study was poor and the trial was closed. The possibility of a different protocol for AML is being considered.

A phase III trial of IL-2 versus observation after PBSCT for NHL in relapse, i.e., SWOG 9438, was initiated in mid-1995. The design is outlined in Figure 2. The IL-2 regimen is the one shown in Table 1. There will be stratification for intermediate and high-grade versus low-grade disease, and for chemotherapy sensitive versus resistant disease. The endpoints of the study will be overall survival and disease-free survival. The goal is to detect an increase in overall survival from an estimated 40% without IL-2 to 54% with IL-2 in randomized patients (power >80%, $p<0.05$). It is calculated that 206 patients will have to be randomized and, therefore, that 275 patients will have to be transplanted. To date approximately 100 patients have been entered onto the study from 25 SWOG transplant centers.

DISCUSSION

If IL-2 turns out to be detectably effective as consolidative immunotherapy, the results will have major implications for BMT, and, by providing proof of principle, might stimulate the wider application of this approach to other marrow transplant settings, as well as to the treatment of other hematologic malignancies without BMT. For example, this same IL-2 regimen is already being tested after autologous PBSCT for a variety of hematologic malignancies as a single-armed study by Massumoto in Brazil (C. Massumoto, personal communication), and after autologous PBSCT for AML in first CR in a single-armed pilot trial at the City of Hope (S. Forman, personal communication). In addition, the Children's Cancer Study Group has just completed a pilot trial of this IL-2 regimen for children with AML in CR1—in a nontransplant setting with little toxicity (Eric Sievers, personal communication), and has just embarked on a phase III trial of this IL-2 regimen versus no IL-2 in children with AML in CR1 (Stephen Feig, personal communication).

REFERENCES

1. Attal M, Blaise D, Marit G et al.: Consolidation treatment of adult acute lymphoblastic leukemia: A prospective, randomized trial comparing allogeneic versus autologous bone marrow transplantation and testing the impact of recombinant interleukin-2 after autologous bone marrow transplantation. BGMT Group. *Blood* 86:1619–1628, 1995.
2. Petersen FB, Appelbaum FR, Hill R et al.: Autologous marrow transplantation for malignant lymphoma: A report of 101 cases from Seattle. *J Clin Oncol* 8:638–647, 1990.
3. Philip T, Guglielmi C, Hagenbeek A et al.: Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma [see comments]. *N Engl J Med* 333:1540–1545, 1995.
4. Petersen FB, Lynch MH, Clift RA et al.: Autologous marrow transplantation for patients with acute myeloid leukemia in untreated first relapse or in second complete remission.

J Clin Oncol 11:1353–1360, 1993.

5. Meloni G, Foa R, Vignetti M et al.: Interleukin-2 may induce prolonged remissions in advanced acute myelogenous leukemia. *Blood* 84:2158–2163, 1994.
6. Maraninchi D, Blaise D, Viens P et al.: High-dose recombinant interleukin-2 and acute myeloid leukemias in relapse. *Blood* 78:2182–2187, 1991.
7. Gisselbrecht C, Maraninchi D, Pico JL et al.: Interleukin-2 treatment in lymphoma: A phase II multicenter study [see comments]. *Blood* 83:2081–2085, 1994.
8. Benyunes M, Fefer A: Interleukin-2 in the treatment of hematologic malignancies. In: Atkins M, Mier J (eds). Therapeutic Applications of Interleukin-2. New York: Marcel Dekker, 1993, pp 163–175.
9. Massumoto C, Benyunes MC, Sale G et al.: Close simulation of acute graft-versus-host disease by interleukin-2 administered after autologous bone marrow transplantation for hematologic malignancy. *Bone Marrow Transplant* 17:351–356, 1996.
10. Benyunes MC, Massumoto C, York A et al.: Interleukin-2 with or without lymphokine-activated killer cells as consolidative immunotherapy after autologous bone marrow transplantation for acute myelogenous leukemia. *Bone Marrow Transplant* 12:159–163, 1993.
11. Benyunes MC, Higuchi C, York A et al.: Immunotherapy with interleukin 2 with or without lymphokine-activated killer cells after autologous bone marrow transplantation for malignant lymphoma: A feasibility trial. *Bone Marrow Transplant* 16:283–288, 1995.
12. Higuchi CM, Thompson JA, Cox T et al.: Lymphokine-activated killer function following autologous bone marrow transplantation for refractory hematological malignancies. *Cancer Res* 49:5509–5513, 1989.
13. Higuchi CM, Thompson JA, Petersen FB et al.: Toxicity and immunomodulatory effects of interleukin-2 after autologous bone marrow transplantation for hematologic malignancies. *Blood* 77:2561–2568, 1991.
14. Neubauer MA, Benyunes MC, Thompson JA et al.: Lymphokine-activated killer (LAK) precursor cell activity is present in infused peripheral blood stem cells and in the blood after autologous peripheral blood stem cell transplantation. *Bone Marrow Transplant* 13:311–316, 1994.
15. Robinson N, Benyunes MC, Thompson JA et al.: Interleukin-2 after autologous stem cell transplantation for hematologic malignancy: A phase I/II study. *Bone Marrow Transplant* (in press).
16. Fefer A, Benyunes MC, Massumoto C et al.: Interleukin-2 therapy after autologous bone marrow transplantation for hematologic malignancies. *Semin Oncol* 20(suppl 9): 41–45, 1993.
17. Fefer A, Benyunes MC, York A et al.: Use of interleukin-2 after bone marrow transplantation. In: Champlin R, Gale RP (eds) Advances and Controversies in Bone Marrow Transplantation, 1995.
18. Robinson N, Benyunes MC, York A et al.: Interleukin-2 after autologous stem cell transplantation for hematologic malignancy: A phase I/II study. *Blood* 86(suppl 1):962a , 1996 [abstr 3837].
19. Mehta J, Powles R, Singhal S et al.: Peripheral blood stem cell transplantation may result in increased relapse of acute myeloid leukaemia due to reinfusion of a higher number

of malignant cells [letter; comment]. *Bone Marrow Transplant* 15:652–653, 1995.
20. Demirer T, Petersen FB, Bensinger WI et al.: Autologous transplantation with peripheral blood stem cells collected after granulocyte-colony stimulating factor in patients with acute myelogenous leukemia. *Bone Marrow Transplant* 18:29–34, 1996.

OUTPATIENT INTERLEUKIN-2–ACTIVATED NK IMMUNOTHERAPY AFTER AUTOLOGOUS TRANSPLANTATION

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ABSTRACT

A graft-versus-leukemia (GVL) effect can prevent and treat relapse in chronic myelogenous leukemia (CML) after allogeneic marrow transplantation, but the possibility of inducing a similar effect in the autologous setting is unknown. One type of lymphocyte that may contribute to this effect is the natural killer (NK) cell, which, following activation with interleukin-2 (IL-2), exhibits a broad range of cytolytic activity against tumor targets. Although autologous transplant can induce complete remissions in patients with lymphoma, advanced breast cancer and CML, nearly 50, 80 and 100%, respectively, will ultimately relapse. We propose that IL-2-activated NK (ANK) cells may be efficacious in preventing relapse after autologous transplantation. In support of this, in vitro studies suggest that ANK cells, but not IL-2–activated $CD4^+$ or $CD8^+$ T cells, mediate lytic activity against lymphoma and breast cancer cell lines. For CML, we evaluated the effect of autologous ANK cells on normal and malignant primary progenitors in a long-term bone marrow culture assay. Autologous ANK cells did not suppress normal committed and primitive progenitors. In contrast, autologous ANK cells from CML patients with potent cytotoxicity against NK-sensitive (K562) and NK-resistant (Raji) tumor targets exhibited an ANK dose-dependent suppression of both committed and primitive progenitors. ANK suppression of primary CML progenitors was not mediated by soluble factors and was absolutely dependent on direct cell-cell contact. Based on these preclinical data, clinical trials have been initiated using low-dose, outpatient subcutaneous IL-2 therapy after recovery from autologous transplantation. A phase I study using subcutaneous IL-2 has been completed and no medically serious toxicity was observed. Although this approach is relatively safe, several studies suggest that maximal in vivo immune activation measured by in vitro assays cannot be achieved by these doses of subcutaneous IL-2 alone. Therefore, current and future trials will focus on ex vivo cell manipulations to further activate immune effectors.

INTRODUCTION

Allogeneic bone marrow transplantation (alloBMT) results in an approximately 40–60% disease-free survival (DFS) in patients with acute or chronic leukemias and myelodysplastic syndromes. The effectiveness of alloBMT depends in part on ablation of the malignant clone with a preparative regimen and reinfusion of benign stem cells to restore normal hematopoiesis. However, it is now well established that the success of allogeneic transplantation is in part mediated by a graft-versus-leukemia (GVL) effect induced by donor-derived T lymphocytes or natural killer (NK) cells. The GVL effect is usually associated with acute and/or chronic graft-versus-host disease (GVHD), which is also mediated by donor-derived lymphocytes. In patients with leukemia there is a statistically significant correlation between the occurrence of acute GVHD and freedom from relapse.[1–3]

A GVL effect has also been demonstrated for lymphoma.[4] However, its role in breast cancer and other solid tumors is uncertain. Immune activation can be obtained in the autologous setting [cyclosporine withdrawal,[5] IL-2,[6] IL-1,[7] etc.], yet it is not known whether this immune activation correlates with an anti-tumor effect. The main advantage of autologous cellular immunotherapy is that there are no allogeneic differences since the patients themselves serve as the cell donors. Therefore, morbidity and mortality from GVHD and other complications due to allogeneic disparity can be avoided. The goal of immunotherapy after autologous transplantation is to safely induce lymphocyte activation and establish the efficacy of this therapy in a minimal residual disease setting.

Chronic myelogenous leukemia (CML) as a model to study immunotherapy

CML is a lethal, malignant disease of the hematopoietic stem cell. Marrow transplant therapy using hematopoietic cells obtained from either related or unrelated donors can result in prolonged DFS and may be curative.[8–11] The success of alloBMT for CML is in part derived from an immunologic disparity between the host and recipient that results in a GVL effect. Probably the best evidence in support of this immune antileukemia effect is demonstrated by the four- to five-fold increase in 2-year relapse rates for patients receiving lymphocyte-depleted donor grafts.[1,3] Additional results from patients receiving syngeneic transplantation where there is no immunologic disparity supports the hypothesis that residual leukemic clones after transplantation are suppressed or ablated by an immune response.[12] The mechanisms of this response are not yet known but may involve effectors such as cytotoxic T-lymphocytes,[13,14] NK cells[15,16] or lymphokine-activated killer cells.[17]

Several therapeutic approaches to induce a GVL effect have been successfully applied in patients who relapse after allogeneic transplantation.[18,19] Higano et al.[18] treated relapsed patients with interferon-α, which controlled elevated leukocyte

counts in all cases and seven of 18 patients achieved complete cytogenetic remission. However, seven patients achieved only partial or no cytogenetic remission. Despite the possible benefit in some patients, four of the 18 patients in this series eventually progressed to accelerated disease or blast crisis while on therapy. Alternative approaches using donor leukocyte infusions have been more promising. There has now been a large clinical experience, including our own at the University of Minnesota, showing that infusion of donor leukocytes can return a patient in relapse to donor-derived Ph-negative hematopoiesis without cytotoxic therapy.[20–22] Taken together, these data strongly implicate an antileukemic effect of the immune system in CML.

CML disease progression correlates with NK functional defects

Human NK cells are a subset of peripheral blood mononuclear cells that express the $CD56^+/CD3^-$ phenotype and have the appearance of large granular lymphocytes. These cells have highly specialized functions associated with the recognition and destruction of infected or viral tumor targets.[23–29] Over the past several decades, research has focused on ways to manipulate these functions for therapeutic benefit. Several recombinant IL-2-activated killer cell populations generated in humans and animals are cytolytic for either fresh or cultured tumor targets both in vitro and in vivo. The lytic activity in these populations resides predominantly in activated NK cells.[30,31] Selective depletion of rat NK cells in vivo abolishes the antitumor effect against subsequently inoculated tumor, supporting a role for NK cells in immune surveillance.[32]

We have been interested in the role of expanded and highly activated NK cells in CML. Based on a previously described method that depends on the propensity of activated NK cells to adhere tightly to plastic in the presence of IL-2 (adherent lymphokine-activated killer cells [A-LAK]), we evaluated peripheral blood mononuclear cells from CML patients for their ability to support activated NK cell generation.[16,25] The $CD56^+/CD3^-$ NK phenotype was present on $79 \pm 9\%$ of CML A-LAK after the 14-day culture period. These cells had high proliferative activity and exhibited potent cytotoxic activity against both NK-sensitive and NK-resistant targets when derived from patients in chronic phase of CML. Cytogenetic evaluation of the CML-activated NK cell population revealed that 119/120 metaphases were Philadelphia chromosome negative. In seven cases, Southern blot analysis failed to demonstrate a *BCR-ABL* gene rearrangement in NK cells even though the myeloid population contained the *BCR-ABL* gene rearrangement, suggesting that these cells are not derived from the malignant clone.

CML is usually diagnosed in chronic phase with eventual progression to accelerated phase, then to blast crisis. The mechanism of this progression is unknown, but correlation with immune function may be involved. Although different methods of obtaining NK-enriched populations have been utilized, few

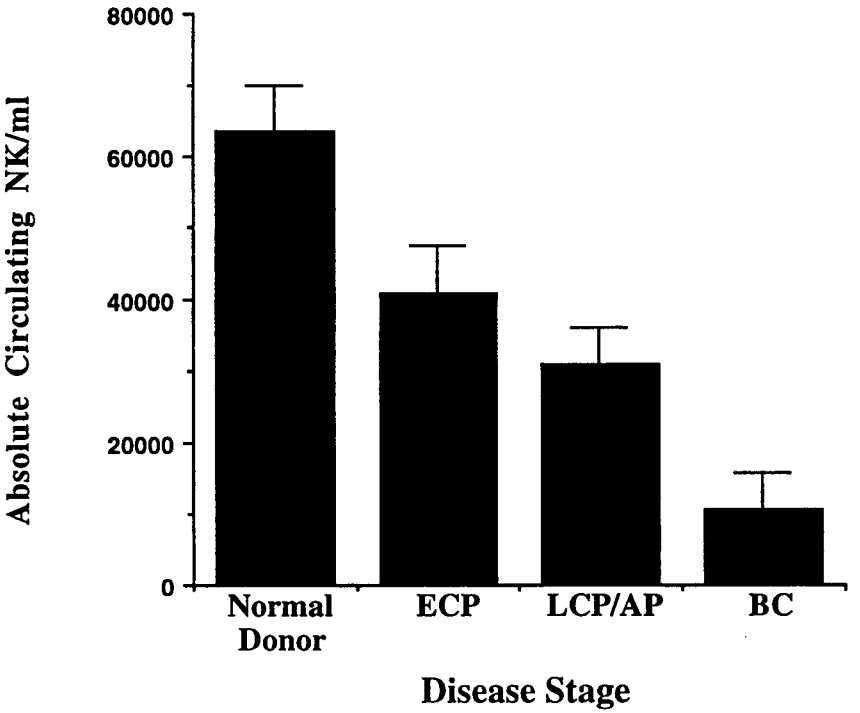

Figure 1. *Circulating NK cells diminish in the blood of CML patients as their disease progresses. Peripheral blood mononuclear cells were enriched by immunomagnetic bead depletion to remove contaminating myeloid cells, B cells and T-lymphocytes.[35] The percent NK cells was determined by the CD56$^+$/CD3$^-$ population compared to results with isotype control antibodies. The absolute circulating NK cells was calculated and expressed per mL of peripheral blood processed from normal donors, early chronic phase (ECP) CML patients, late chronic phase or accelerated phase (LCP/AP) CML patients or patients in blast crisis (BC).*

have used pure NK starting populations. Therefore, definitive conclusions cannot be made about NK activity and proliferative capacity in the absence of contaminating malignant cells. To avoid these confounding variables, FACS-purified CD56$^+$/CD3$^-$ NK cells with a well-defined nonmalignant accessory cell source (M2-1OB4[33,34]) were used to further investigate NK in patients with CML.[35]

Patients with CML have reduced numbers of both circulating bulk CD56$^+$/CD3$^-$ NK and the highly proliferative CD56$^{+bright}$ NK subset (data not shown) compared with normal controls (Figure 1). CML NK cells also demonstrate specific abnormalities in NK responses to contact and soluble stimuli. We demonstrated that

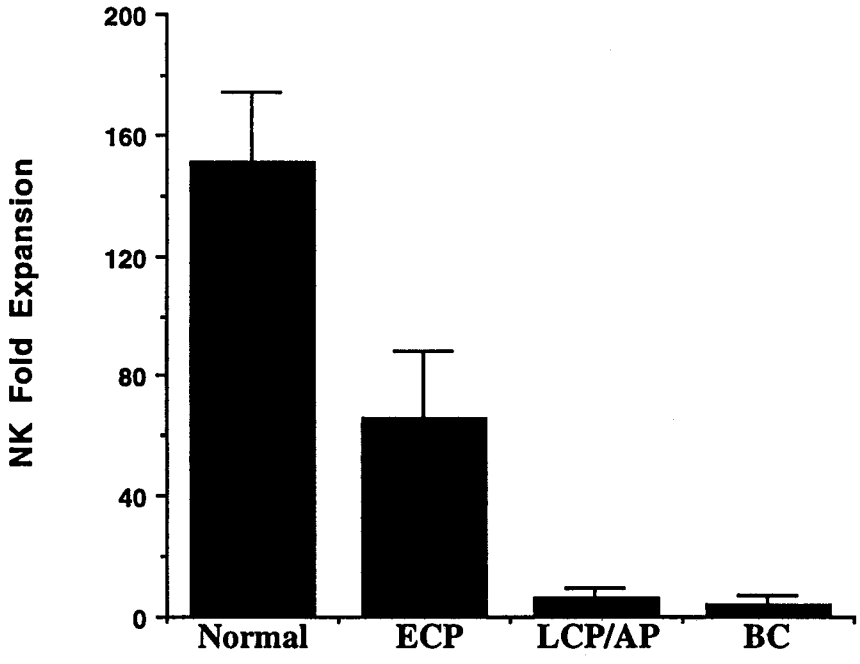

Figure 2. *NK cells from CML patients exhibit progressive proliferative defects as their disease progresses. FACS purified CD56^{+}/CD3^{-} NK (1×10^{5})were cocultured in 1 mL medium in 24-well plates in direct contact with irradiated M2-10B4 (a murine fibroblast stromal cell line) as described.[35] At day 7, culture volumes were doubled to 2.0 mL and subsequently half medium changes were performed every 34 days. Contents of culture wells were split 1:2 as needed to maintain cell concentrations below 2–3$\times10^{6}$ cells/mL. Cultures were terminated after 28 days and cells were enumerated with a hematocytometer. NK-fold expansion was determined as (total cultured cells at day of harvest)/(initial NK cells plated at day 0).*

the bulk CD56^{+}/CD3^{-} population (Figure 2) and the CD56$^{+bright}$ NK subset lose their ability to expand in response to contact and soluble factors as CML progresses. A detailed evaluation was also done using a limiting dilution assay to assess NK clonogenic frequency and proliferation per cell of the main NK subsets: CD56$^{+bright}$ and CD56^{+dim} NK. Even though clonogenic frequency of the CD56$^{+bright}$ and CD56^{+dim} NK fraction remains unchanged from normal controls compared with the NK population from CML patients in early chronic phase (ECP), four significant therapy-independent defects could be observed: 1) the absolute number of

circulating NK cells is reduced; 2) the absolute number of $CD56^{+bright}$ NK cells is reduced; 3) bulk NK cell expansion is reduced; and 4) proliferation per NK cell in all NK subsets is reduced.[35] Taken together, these data suggest that modulation of the NK compartment begins early in CML and becomes more pronounced as disease progresses. Further investigation of these defects may illuminate the mechanism by which CML gradually escapes from immune control.

In vitro IL-2 corrects NK cytotoxic defects in patients with CML

While several laboratories have reported decreased NK function in patients with CML, the potential for dilution of NK activity by the vast myeloid compartment has confounded the results and it is unclear whether NK cells are inherently less active or just diminished in number. We utilized freshly purified $CD56^{+}/CD3^{-}$ NK cells in standard chromium release assays against K562 tumor targets to address this question. Even purified resting NK cells, utilized to correct for differences in circulating NK cell number, demonstrate a significant decrease in cytolytic activity in early chronic phase and late chronic phase or accelerated phase patients and nearly complete loss of cytolytic function in patients with blast crisis. Analysis of resting NK function by prior cytotoxic therapy demonstrated that NK cells from patients "off hydroxyurea" were not different from normal controls. In contrast, patients "on hydroxyurea" or after "extensive therapy" exhibited significantly reduced function. However, short-term (18 hours) incubation with high-dose IL-2 restores cytotoxic activity to normal levels in both the "on hydroxyurea" and "extensive therapy" groups, demonstrating a reversible defect in NK function that may be of therapeutic value.[35]

In vitro models of immunotherapy in CML

Restoring or enhancing NK function in patients with CML by infusion of autologous activated NK cells or purging of leukemic bone marrow cells by ex vivo treatment would only have importance if coexisting, benign hematopoietic progenitors are spared. To test this hypothesis, experiments were performed using autologous activated NK cells and highly purified CD34 positive progenitors from CML patients in chronic phase and normal donors. Previously, studying the effect of ANK on CML primitive progenitors in vitro has been problematic because these cytotoxic effectors rapidly kill marrow stroma and thus cannot be added to classic "Dexter" type long-term marrow cultures. The destruction of stroma is mediated by direct NK cell contact with stromal components and not by interleukin-2 alone.[36] Furthermore, addition of ANK to long-term cultures that contain hydrocortisone results in inhibition of ANK function.[37] To overcome these technical problems, a "stroma noncontact culture" described by our group[38] was modified to test the role of autologous IL-2-activated NK cells on CML and normal progenitors (Figure 3).

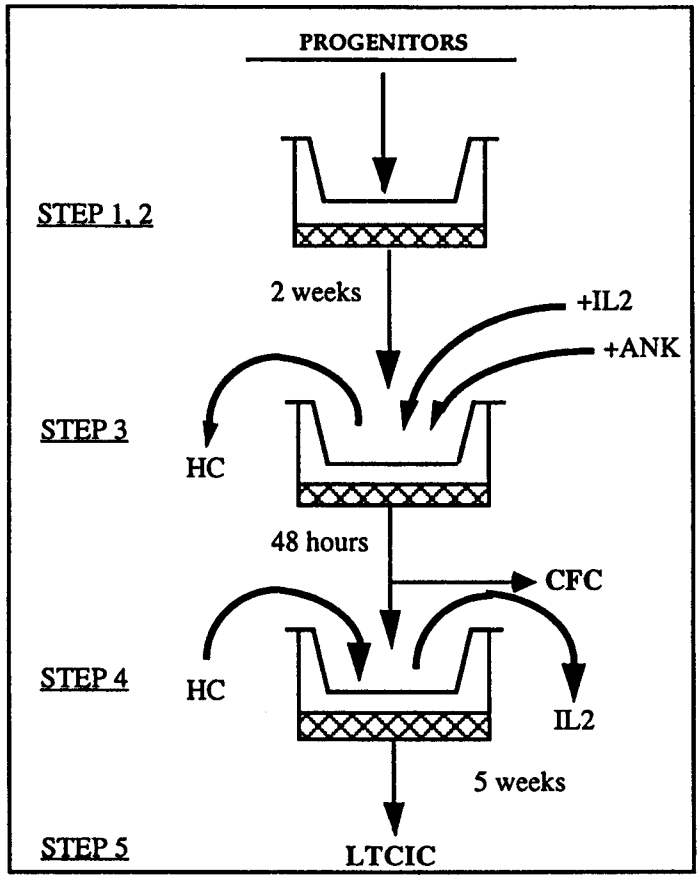

Figure 3. *Autologous activated NK cell co-incubation in long-term culture. Confluent, irradiated (6000 cGy) M2-10B4 monolayers were established in the bottom of 24-well plates (Step 1). A collagen treated Transwell insert is placed above the stromal layer and FACS sorted progenitors are placed in the Transwell insert in long-term bone marrow culture (LTBMC) medium (Step 2). After 14 days, LTBMC medium containing hydrocortisone (HC) was removed from the Transwell insert without loss of progenitor cells by capillary drainage induced by touching the bottom of the Transwell membrane to a piece of sterile gauze. LTBMC medium without HC supplemented with 1000 U/mL IL-2 was added to the bottom well and ANK in the same medium were added to the Transwell insert at the indicated effector-to-target ratio based on the number of progenitors inoculated on day zero (Step 3). After 48 hours, the medium was removed from the Transwell insert, replaced with standard LTBMC medium containing hydrocortisone and the Transwell insert was then placed on a fresh stromal layer containing the same medium (Step 4). Long-term culture-initiating cells (LTC-IC) were assayed in stroma noncontact culture with weekly half medium changes as previously described (Step 5).*[37,38]

After a 48-hour preincubation, coculture of normal $CD34^+$ cells with autologous ANK did not affect the numbers of colony forming cells (CFC) (data not shown) or long term culture-initiating cells (LTC-IC) (Figure 4) at any of the effector-to-target ratios tested. In marked contrast, when ANK from CML patients, which exhibited significant killing of K562 and Raji tumor target cells, were incubated with CD34 positive, HLA-DR positive cells, a dose-dependent suppression of the growth of both CFC (data not shown) and LTC-IC (Figure 4) was observed.[37] Effector populations that failed to lyse the Raji cell line also failed to suppress the growth of autologous CML progenitors. This inhibitory effect of ANK on CML progenitors was dependent on direct NK-progenitor contact. The role of soluble factors in this inhibition appears to be minimal since ANK supernatants failed to inhibit progenitors and neutralizing antibodies against interferon-γ, TNF-α or TGF-β failed to block the inhibitory effect of ANK on malignant progenitors. This model offers advantages to other preclinical studies, specifically, the use of primary tumor cells rather than cell lines.

Safety of subcutaneous IL-2 therapy after autologous transplantation

The above review of some of the laboratory and clinical data in CML supports the notion of testing immunotherapy in the autologous setting. These data suggest that immune activation of NK cells sufficient to lyse the NK-resistant Raji target will also suppress autologous primary CML progenitors and may be of therapeutic benefit. In breast cancer and lymphoma, there are less data with primary cells given the difficulty of obtaining primary tissue and technical problems with reliable killing assays. As an alternative, we have performed in vitro studies that demonstrate that IL-2 activated NK cells, but not resting NK or IL-2 activated $CD4^+$ or $CD8^+$ T cells, mediate lytic activity against three lymphoma and five breast cancer cell lines. Clinical trials at the University of Minnesota are now focusing on safe methods of immune activation that can be applied after autologous transplantation and delivered on an outpatient basis.

Patients were eligible for IL-2 between 30 and 180 days post-transplant after engraftment defined as independence from transfusion support and growth factors with a platelet count greater than 50,000/μL, hemoglobin greater than 9 gm/dL and absolute neutrophil count greater than 1000/μL. Patients were required to have adequate organ function (creatinine <1.5 mg/dL, liver function tests <2 times normal, pulmonary function tests >60% of predicted, no evidence of cardiac disease). All patients were free of active infection, off IV antibiotics and tolerating oral intake. Those patients with planned post-transplant radiation must have completed therapy and patients on any immunosuppression medications (steroids) were not eligible for IL-2. IL-2 was manufactured and provided by Amgen Inc. (Thousand Oaks, CA) as R-met HuIL-2 (ala-125) in a buffered aqueous excipient at a protein concentration of 0.4 mg/mL with a specific activity of 4.3 to 10.3×10^6

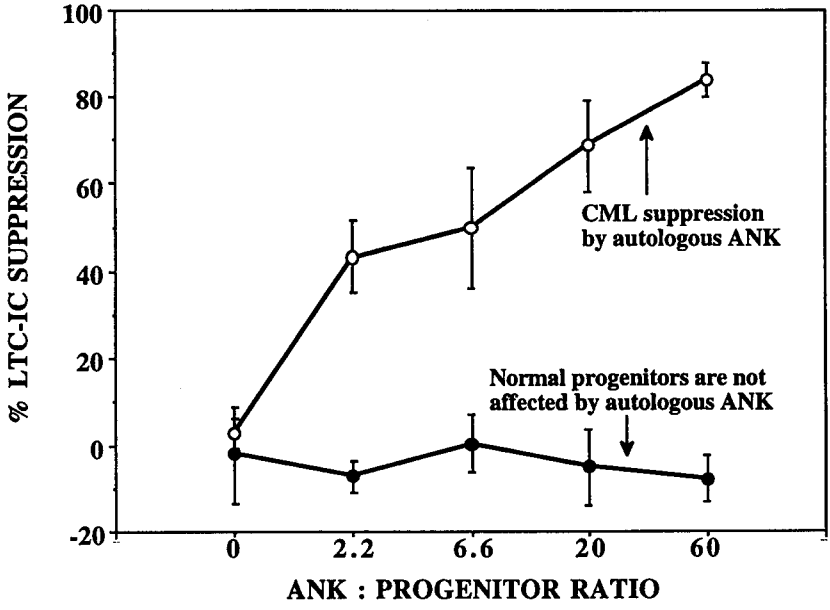

Figure 4. *Autologous activated NK cells suppress malignant progenitors but not benign progenitors. Activated NK cells were incubated with normal CD34⁺ (closed circles) or CML CD34⁺/DR⁺ progenitors (open symbols) cultured for 14 days in stroma noncontact culture as described in Figure 3. ANK were added to autologous progenitors in medium without hydrocortisone and with IL-2 for 48 hours. To evaluate the effect of ANK on LTC-IC, LTBMC medium (+HC-IL-2) was added after the 48-hour incubation and maintained for 5 weeks and then evaluated for the number of secondary CFC. LTC-IC were analyzed in an autologous setting from normal donors (closed circles, n=6) and CML patients with good ANK function (open circles, n=8). CML ANK with good function significantly suppressed autologous LTC-IC at all effector-to-target ratios (p=<0.001 for each E:T ratio) compared with normal ANK and autologous progenitors.*[37]

U/mg protein according to specific lot. They were told to inject IL-2 in the early evening after premedication with oral acetaminophen (650 mg), Benadryl (25–50 mg) and ibuprofen (200–400 mg) and to time injections such that a second dose of medication could be given before going to sleep. Depending on the degree of constitutional symptoms, patients were instructed on how to adjust these medications to individual comfort and tolerance.

Eleven evaluable patients (five lymphoma, six breast cancer) were enrolled on a phase I dose escalation study after autologous transplantation (median day + 94, range 50–166). IL-2 (Amgen) was self-administered subcutaneously at 0.25×10^6 (n=5) or 0.5×10^6 (n=6) U/m^2 daily for 84 consecutive days. The best-tolerated

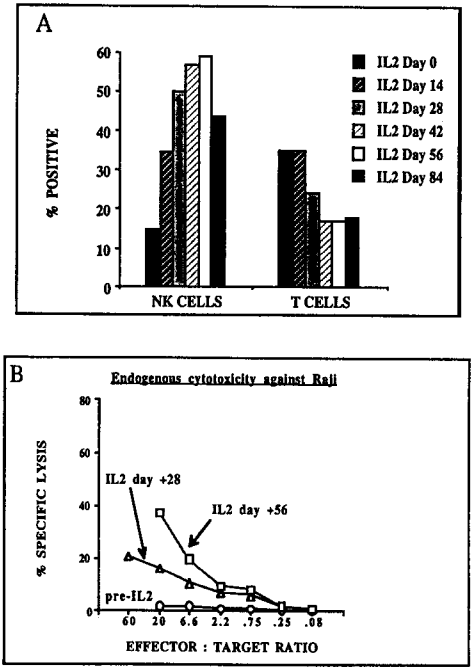

Figure 5. *Subcutaneous IL-2 after autologous transplantation expands NK cell number and function in vivo. A representative patient with lymphoma was started on subcutaneous IL-2 after recovery from autologous transplantation. IL-2 (0.5×10^6 U/m^2/day) was self-administered daily for 84 consecutive days. Peripheral blood lymphocytes were analyzed every 14 days for CD56$^+$/CD3$^-$ NK and CD3$^+$ T-lymphocytes and are presented as percent of total lymphocytes* ***(A)****. Cytotoxicity in a 4-hour chromium release assay of peripheral blood mononuclear cells obtained after density centrifugation against the NK-resistant Raji target is shown pre-IL-2 and after 28 and 56 days of IL-2 therapy without further* in vivo *activation* ***(B)****.*

dose was 0.25×10^6 U/m^2 (78% of planned doses given versus 56% at the higher dose). Dose-limiting toxicity occurred in six patients (2 at 0.25×10^6, 4 at 0.5×10^6). Four of the six toxicities were hematologic (three thrombocytopenia, one mild neutropenia). Despite this toxicity, all symptoms resolved within a week off IL-2. No patient required hospitalization for toxicity related to IL-2.

Low dose subcutaneous IL-2 was safely administered to patients after intensive therapy with autologous transplant in the outpatient setting. This therapy consistently gave local skin reactions at the injection site and constitutional symptoms including fatigue, malaise, sweats and fever. These constitutional symptoms were dose-limiting in two patients (both receiving the higher

IL-2 dose). All constitutional symptoms resolved within one week off therapy in all patients. Although one patient required hospitalization for treatment of herpes zoster, no other patients required hospitalization during the study period. No patient developed weight gain or evidence of the vascular leak syndrome.[39] Liver function tests, coagulation studies, renal function and pulmonary and cardiac function were not notably affected by IL-2. Patients who met eligibility for IL-2 with a platelet count below 80,000 developed thrombocytopenia on IL-2. This thrombocytopenia resolved by stopping the drug and no patient's platelet count dropped below 20,000 requiring platelet transfusions.

Immune activation with subcutaneous IL-2

IL-2 induces a lymphocytosis comprised mostly of $CD56^+/CD3^-$ NK cells (Figure 5A). Patients receiving at least 28 days of IL-2 exhibited a >10-fold increment in absolute circulating NK cells. This increase in NK number corresponded to an increase in cytotoxic activity. Peripheral blood lymphocytes collected after 56 days of low dose subcutaneous IL-2 killed better than at 28 days while there was virtually no cytolytic activity of blood lymphocytes prior to starting IL-2 (Figure 5B). In addition to in vivo expansion of NK cells, lytic function could be further enhanced against Raji (lymphoma) and MCF-7 (breast cancer) by ex vivo activation in 1000 U/mL IL-2 overnight.

We conclude that low dose subcutaneous IL-2 based immunotherapy is feasible, relatively safe and able to be administered in an outpatient setting. Furthermore, since in vivo activation with subcutaneous IL-2 alone is submaximal, we hypothesize that additional ex vivo incubation in IL-2 may be used to generate NK cells with potent anti-tumor effects in vivo. At the University of Minnesota, ex vivo activation and cell infusion is currently being tested to maximize NK function in an attempt to prevent relapse after autologous transplantation for lymphoma, breast cancer and CML.

REFERENCES

1. Marmont AM, Horowitz MM, Gale RP et al.: T-cell depletion of HLA-identical Transplants in leukemia. *Blood* 78:2120, 1991.
2. Sullivan KM, Weiden PL, Storb R et al.: Influence of acute and chronic graft-versus-host disease on relapse and survival after bone marrow transplantation from HLA-identical siblings as treatment of acute and chronic leukemia. *Blood* 73:1720, 1989.
3. Wagner JE, Zahurak M, Piantodosi S et al.: Bone marrow transplantation of chronic myelogenous leukemia in chronic phase: Evaluation of risks and benefits. *J Clin Oncol* 10:779, 1992.
4. Ratanatharathorn V, Uberti J, Karanes C, Abella E, Lum LG, Momin F, Cummings G, Sensenbrenner LL: Prospective comparative trial of autologous versus allogeneic bone marrow transplantation in patients with non-Hodgkin's lymphoma. *Blood* 84:1050,

1994.

5. Ruvolo P, Bright E, Kennedy MJ, Morris L, Fischer A, Vogelsang G, Jones R, Hess A: Cyclosporine-induced autologous graft versus host disease: Assessment of cytolytic effector mechanisms and the v-beta T-cell receptor repertoire. Transplantation Proceedings 27:1363, 1995.
6. Soiffer RJ, Murray, C, Cochran, K et al.: Clinical and immunologic effects of prolonged infusion of low-dose recombinant interleukin-2 after autologous and T-cell depleted allogeneic bone marrow transplantation. *Blood* 79:517-526, 1992.
7. Weisdorf D, Katsanis E, Verfaillie C, Ramsay NK, Haake R, Garrison L, Blazar BR: Interleukin-1 alpha administered after autologous transplantation: A phase I/II clinical trial. *Blood* 84:2044, 1994.
8. Enright H, Davies SM, DeFor T, Shu X, Weisdorf D, Miller W, Ransay NKC, Arthur D, Verfaillie C, Miller JS, Kersey J, McGlave P: Relapse following non-T-depleted allogeneic bone marrow transplant for CML: Early transplant, the use of an unrelated donor and chronic graft-versus-host disease are protective. *Blood* 88:714–720, 1996.
9. McGlave P, Bartsch G, Anasetti C et al.: Unrelated donor marrow transplantation therapy for chronic myelogenous leukemia: Initial experience of the National Marrow Donor Program. *Blood* 81:543, 1993.
10. McGlave PB: The biology and therapy of chronic myelogenous leukemia. In: Champlin R (ed): Bone Marrow Transplantation. Boston, Martin Nijhoff Press; 1990, pp 235–258.
11. Thomas ED, Clift RA: Indications for marrow transplantation in chronic myelogenous leukemia. *Blood* 73:861, 1989.
12. Fefer A, Cheever MA, Greenberg PD: Identical twin (syngeneic) marrow transplantation for hematologic cancers. *JNCI* 76:1269, 1986.
13. Falkenburg JHF, Goselink HM, van der Harst D et al.: Growth inhibition of clonogenic leukemia precursor cells by minor histocompatibility antigen-specific cytotoxic T lymphocytes. *J Exp Med* 174:27, 1991.
14. Jiang Y-Z, Kanfer EJ, Macdonald D: Graft-versus-leukaemia following allogeneic bone marrow transplantation: Emergence of cytotoxic T lymphocytes reacting to host leukemia cells. *Bone Marrow Transplant* 8:253, 1991.
15. Mackinnon S, Bungey J, Chase A et al.: Origin and function of adherent lymphokine activated killer cells in patients with chronic myeloid leukaemia who relapse following bone marrow transplantation. *Br J Haematol* 77:60, 1991.
16. Verfaillie C, Kay N, Miller W, McGlave P: Diminished A-LAK cytotoxicity and proliferation accompany disease progression in chronic myelogenous leukemia. *Blood* 76:401, 1990.
17. Mackinnon S, Hows JM, Goldman JM: Induction of in vitro graft-versus-leukemia activity following bone marrow transplantation for chronic myeloid leukemia. *Blood* 76:2037, 1990.
18. Higano CS, Raskind WH, Singer JW: Use of alpha interferon for the treatment of relapse of chronic myelogenous leukemia in chronic phase after allogeneic bone marrow transplantation. *Blood* 80:1437, 1992.
19. Steegman JL, Perez M, Vazquez L et al.: Interferon alpha treatment of accelerated-

phase chronic myeloid leukemia in relapse after bone marrow transplantation: A case with complete cytogenetic and molecular remission. *Bone Marrow Transplant* 7:65, 1991.

20. Kolb HJ, Mittermuller J, Clemm CH et al.: Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood* 76:2462, 1990.
21. Cullis JO, Jiang YZ, Schwarer AP et al.: Donor leukocyte infusions for chronic myelogenous leukemia in relapse after allogeneic bone marrow transplantation. *Blood* 79:1379, 1992.
22. Drobyski WR, Roth MS, Thibodeau SN, Gottschall JL: Molecular remission occurring after donor leukocyte infusions for the treatment of relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation. *Bone Marrow Transplant* 10:301, 1992.
23. Miller JS, Verfaillie C, McGlave P: Adherent lymphokine-activated killer cells suppress autologous human normal bone marrow progenitors. *Blood* 77:2389, 1991.
24. Miller JS, Oelkers S, Verfaillie C, McGlave P: Role of monocytes in the expansion of human activated natural killer cells. *Blood* 80:2221, 1992.
25. Verfaillie C, Miller W, Kay N, McGlave P: Adherent lymphokine-activated killer cells in chronic myelogenous leukemia: A benign cell population with potent cytotoxic activity. *Blood* 74:793, 1989.
26. Robertson MJ. Ritz J: Biology and clinical relevance of human natural killer cells. *Blood* 76:2421, 1990.
27. Trinchieri G: Biology of natural killer cells. *Adv Immunol* 47:187, 1989.
28. Schmidt RE: Natural Killer Cells: Biology and Clinical Application. Switzerland: Karger, Basel, 1990.
29. Melder RJ, Balachandran R, Rinaldo CR et al.: Cytotoxic activity against HIV-infected monocytes by recombinant interleukin 2-activated natural killer cells. *AIDS Research and Human Retroviruses* 6:1011, 1990.
30. Schwarz RE, Felgar RE, Hiserodt JC: Successful adoptive immunotherapy of established lung and liver metastases with highly purified IL-2-activated natural killer cells (A-LAK Cells). In: Schmidt RE (ed) Natural Killer Cells: Biology and Clinical Significance. Basel: Karger, 1990, p 275.
31. Hercend T, Farace F, Baume D et al.: Immunotherapy with lymphokine-activated natural killer cells and rIL-2: A feasibility trial in metastatic renal cell carcinoma. *J Biol Response Mod* 9:546, 1991.
32. van den Brink MRM, Hunt LE, Hiserodt JC: In vivo treatment with monoclonal antibody 3.2.3. selectively eliminates natural killer cells in rats. In: Schmidt RE (ed) Natural Killer Cells: Biology and Clinical Significance. Basel: Karger, 1990, p 303.
33. Pierson BA, McGlave PB, Hu WS, Miller JS: Natural killer cell proliferation is dependent on human serum and markedly increased utilizing an enriched supplemented basal medium. *J Hematother* 4:149–158 1995.
34. Pierson BA, Gupta K, Hu W-S, Miller JS: Human natural killer cell expansion is regulated by thrombospondin-mediated activation of TGF-01 and independent cell-derived

contact and soluble factors. *Blood* 87:180, 1996.

35. Pierson BA, Miller JS: $CD56^{+bright}$ and $CD56^{+dim}$ natural killer cells progressively decrease in patients with chronic myelogenous leukemia, respond less to stimuli which recruit clonogenic NK, and exhibit decreased proliferation on a per cell basis. *Blood* (in press), 1996.
36. Miller JS, Verfaillie C, McGlave P: The generation of natural killer cells from $CD34^{+}$/DR-primitive progenitors in human long-term bone marrow culture. *Blood* 80:2182–2187, 1992.
37. Cervantes F, McGlave PB, Verfaillie CM, Miller JS: Autologous activated natural killer cells suppress primitive chronic myeloid leukemia progenitors in long term culture. *Blood* 87:2476–2485, 1996.
38. Verfaillie CM: Direct contact between human primitive hematopoietic progenitors and bone marrow stroma is not required for long-term in vitro hematopoiesis. *Blood*, 79:2821, 1992.
39. Rosenberg SA, Lotze MT, Munl LM et al.: A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med* 316:889, 1987.

POST-TRANSPLANT IMMUNOTHERAPY BY INDUCTION OF AUTOLOGOUS GVHD

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ABSTRACT

Relapse of disease remains the most common cause of treatment failure after high-dose chemotherapy (HDCT) with autologous stem cell rescue. Post-transplant immune therapy may be noncross-resistant with chemotherapy and is increasingly being evaluated for its ability to prevent recurrence. A syndrome similar to graft-versus-host disease (GVHD) can be induced in rodents following syngeneic transplant by treatment with cyclosporine A (CsA). This syndrome is associated with the elaboration of cytotoxic T-lymphocytes, which recognize the MHC Class II antigen on target cells and can be transferred into irradiated syngeneic recipients. Cytotoxicity may be increased by upregulation of MHC Class II antigen on the target cells by treatment with interferon (IFN) or by expansion of the effector cells with interleukin-2 (IL-2). Further studies have indicated that this syndrome can be induced in humans and is associated with the elaboration of autocytotoxic lymphocytes. Serial trials in patients with resistant lymphoma suggest an improvement in event-free and overall survival for patients treated with CsA and γ-IFN (n=16) after high-dose therapy compared with historical controls (n=48). A lesser effect may be observed in similar patients treated with CsA and α-IFN (n=24). In a retrospective review of women with poor risk metastatic breast cancer treated on serial studies of high-dose therapy and induction of autologous GVHD, no impact of CsA treatment has been noted on time to progression. However, in vitro studies in these patients indicate that lytic T-lymphocytes are being generated against autologous lymphoblasts and MHC Class II expressing breast cancer cells lines. This activity is greater in patients who develop biopsy proven cutaneous GVHD than in those who do not. Clinical antitumor activity against breast cancer may only be feasible in less advanced disease (i.e., the adjuvant setting), or with amplification of lytic cells by agents such as IL-2, strategies that are currently under evaluation. T-lymphocytes elaborated by autologous GVHD may specifically recognize a peptide from the Class II invariant chain termed "CLIP." Pretreatment with anti-human CLIP antibodies will inhibit lysis by GVHD effector T-lymphocytes and

force loading CLIP onto tumor targets can increase lysis. Strategies for augmenting the anti-tumor activity of autologous GVHD via immunomodulation of CLIP are also currently under evaluation.

INTRODUCTION

High-dose chemotherapy (HDCT) with autologous stem cell rescue has an established role in the treatment of lymphomas and leukemias, and is the subject of ongoing clinical trials in select solid tumors, notably breast and ovarian cancer. Relapse remains the major cause of clinical failure, however, notably in patients with chemoinsensitive disease and particularly in women with metastatic breast cancer. It is clear that therapeutic strategies are required that will augment the cytotoxic activity of HDCT with autologous stem cell support in these clinical situations.[1]

Detailed clinical research of allogeneic transplantation conducted over the past two decades has confirmed the importance of the immunologic antitumor effect associated with the evolution of graft-versus-host disease (GVHD).[2,3] In addition, the capacity of donor T-lymphocytes to induce remissions in patients who have relapsed after allogeneic transplantation has been well documented.[4] This antitumor effect is lost in the context of autologous transplantation, which may at least partially account for the increased risk of recurrence associated with this therapy. The allogeneic graft-versus-tumor effect is clearly a potent one. Immunologic therapy administered after HDCT supported with autologous stem cells might also be capable of improving clinical outcome.

It has been reported that rodents treated with cyclosporine A for at least 30 days following syngeneic marrow transplantation will develop a syndrome that clinically and histologically resembles acute GVHD.[5] This syndrome develops after discontinuation of CsA and its evolution is dependent on the dose and duration of therapy with CsA and is mediated by $CD4^+$ and $CD8^+$ T-lymphocytes.[6] CsA-induced autologous GVHD can be transferred by these T-lymphocytes into syngeneic recipients who have received thymic irradiation. This fact has greatly facilitated the study of antitumor effects associated with the elaboration of autologous GVHD. A series of transfer experiments has indicated that there is an antitumor effect associated with the evolution of autologous GVHD and that this cytotoxicity is directed in a promiscuous fashion at cells that bear the MHC Class II or HLA-DR antigen.[7] This antitumor effect can be augmented in animal models by cotreatment with γ-interferon, which potently upregulates expression of MHC Class II antigens on target tissues. The addition of low dose IL-2 also appears to augment the cytotoxic activity of CsA-induced autologous GVHD against MHC Class II expressing CRL1662 cells in these rodent systems. CsA-induced GVHD appears to result in about 1–2 logs of tumor cell kill against CRL1662 cells, but

this can be increased by another 1–2 logs without major toxicity by co-treatment with γ-interferon and IL-2.

These laboratory data have prompted the investigation of CsA induced GVHD in patients with a variety of malignancies undergoing HDCT with autologous stem cell rescue. It has been reported previously in an initial phase I study that CsA administered at a dose of 1 mg/kg daily for 28 days produced biopsy-proven grade II GVHD of the skin with a characteristic rash in 5 patients with resistant lymphoma undergoing autologous BMT.[8] There was no evidence of visceral or chronic GVHD in any patient on this initial study. GVHD resolved spontaneously or with corticosteroids within 3 weeks of its first appearance. Autoreactive lymphocytes were detected in patients treated on this initial study and produced lytic activity against pretransplant lymphoblasts which was MHC Class II restricted.

A proportion of breast cancer cells also bear the MHC Class II antigen and may be a target for any anti-tumor activity associated with the induction of GVHD by CsA. In an initial phase I study it was determined that higher doses of CsA were required to reproducibly cause biopsy-proven grade II GVHD in patients with breast cancer.[9] A dose of 2.5 mg/kg/day produced biopsy evidence of grade II GVHD in more than 50% of patients with metastatic breast cancer when administered following an autologous BMT. The reasons for this difference are not clear, but may be due to more intact immunoregulatory mechanisms in patients with breast cancer, due to less exposure to prior chemotherapy. As γ-interferon potently upregulates expression of MHC Class II antigens on breast cancer cell lines, subsequent to this study, a phase I trial of γ-interferon to augment CsA-induced GVHD was undertaken in a patient population with metastatic breast cancer. γ-interferon, 0.025 mg/M2/qod/s.c. days 7–28, was administered in conjunction with CsA 2.5 mg/kg/day days 0–28 in 36 women with advanced breast cancer responding to therapy. The incidence of GVHD was similar to that seen with CsA alone at the same dose in the prior phase I study, but the intensity of the phenomenon appeared greater.[10] An extensive rash occurred in over 30% of patients who were treated with CsA and γ-interferon, compared with only 3% of patients who received CsA alone. Cotreatment with CsA and with both α- and γ-interferon has also been evaluated in phase II studies in patients with chemotherapy-resistant lymphoma and leukemia. Updated information on these studies and on studies of the impact of peripheral blood progenitor cell support on the induction of GVHD by CsA will be presented.

CLINICAL STUDIES

The possible impact of CsA-induced GVHD on time to relapse and death has been evaluated in a series of phase II trials in patients with refractory aggressive hematopoietic malignancies, predominantly lymphoma. After a median follow-up

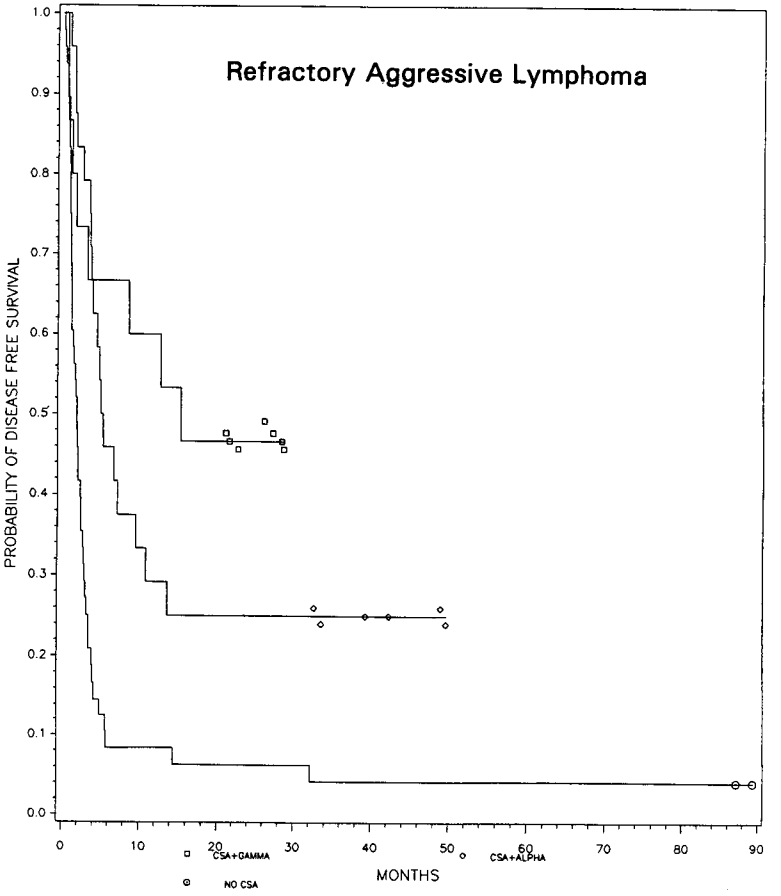

Figure 1. *Disease-free survival (DFS) for patients with refractory aggressive hematopoietic malignancies treated on serial studies of high-dose therapy alone (⊙), or high-dose therapy followed by CsA and α-interferon (◇) or CsA and γ-interferon (□).*

of around 2 years the rate of relapse and death appears less in the 16 patients who were treated with CsA and γ-interferon when compared with 48 historical controls treated with autologous transplantation alone (Figure 1). Twenty-four patients who received CsA and α-interferon had an intermediate outcome. These encouraging preliminary results should be approached with caution due to the historical nature of the controls. In contradistinction to these data, a retrospective review of time to progression and survival for patients treated on 3 sequential trials of ABMT for women with metastatic breast cancer has shown no impact of treatment with CsA or CsA and γ-interferon on time to progression or death after 5 years of follow-up

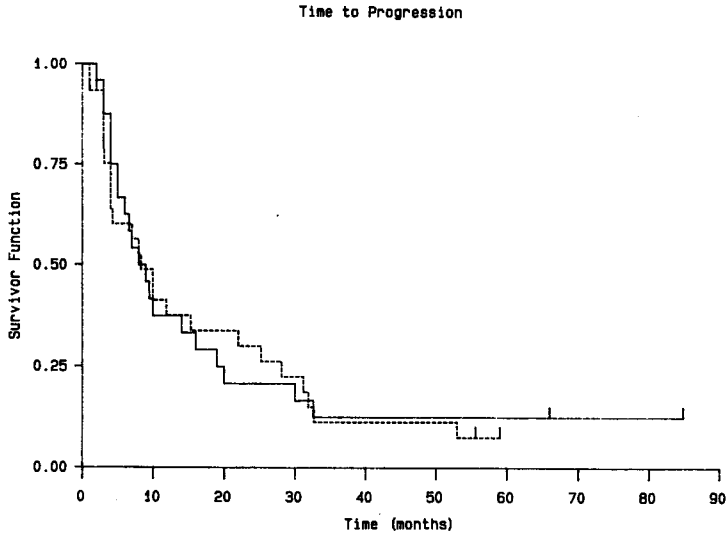

Figure 2. *DFS for women with sensitive metastatic breast cancer treated with HDCT alone (—) or HDCT followed by CsA and γ-interferon (---).*

Table 1A. Pretreatment of autologous PHA lymphoblasts with anti-human CLIP antibody inhibits lysis mediated by autologous GVHD effector T cells

	Percent specific lysis after pretreatment of target cells	
	Prebleed IgG	*Anti-human CLIP IgG*
patient 1	45.2[a]	6.3
patient 2	24.2	1.7
patient 3	28.3	7.4

Table 1B. CLIP loading increases lysis mediated by autologous GVHD effector T cells[a]

	Specific lysis after CLIP loading of target cells	
	Concentration of CLIP	
	0	1.0 μM
patient 1	19.4[b]	43.6
patient 2	16.2	32.3

[a]*Percent specific 51Cr release at a 30:1 effector:target ratio using lymphocytes harvested from each patient during autologous GVHD.*

[b]*PHA lymphoblasts from each patient were preloaded with CLIP for 6 hours.*

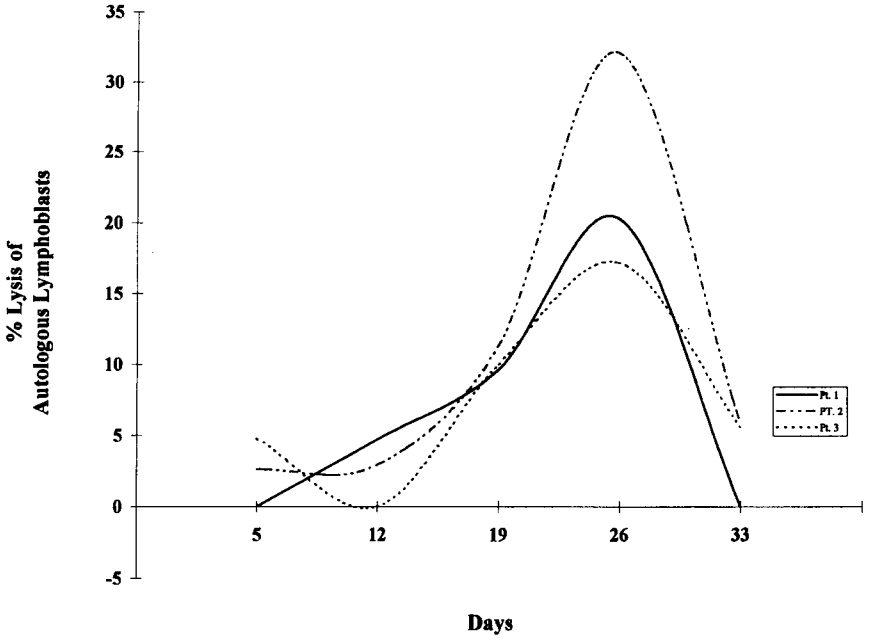

Figure 3. *Elaboration of autocytotoxic activity with time following HDCT (with stem cell infusion on day –1) in 3 women with advanced breast cancer. CsA was administered days 0–28 and γ-interferon days 7–28.*

(Figure 2). The volume of cancer present in women with metastatic breast cancer, and the variable level of expression of MHC Class II antigens in those tumors, may possibly explain the apparent lack of clinical efficacy seen to date in women with breast cancer. Studies of GVHD induction in a high-risk adjuvant population, and trials evaluating the possible impact of IL-2 on GVHD induction in women with metastatic breast cancer, are currently underway.

We have also evaluated the impact of peripheral blood progenitor cell (PBPC) infusions on the incidence of CsA-induced autologous GVHD in women undergoing HDCT for advanced breast cancer. PBPC contain large numbers of T-lymphocytes and might inhibit the induction of autologous GVHD by CsA. In a cohort of 50 women with advanced breast cancer who were treated with CsA and γ-interferon after identical HDCT and supported with PBPC or PBPC and bone marrow, the incidence of grade II GVHD on biopsy was 36%. This incidence is close to being statistically significantly lower than that seen with bone marrow alone in prior studies. Extent of prior therapy, numbers of T-lymphocytes in the PBPC

product infused or observer variation in the pathologic diagnosis of GVHD may all contribute to this observed trend. These possibilities are currently being evaluated.

LABORATORY STUDIES

The mechanisms that account for the novel and promiscuous specificity of the autoreactive T cells of autologous GVHD for the MHC Class II antigen are unclear. Preliminary data attempting to dissect the specificity of the effector T cells suggest that the promiscuous recognition of class II is due, in part, to recognition of a peptide from the class II invariant chain that regulates class II assembly and cell surface expression. This peptide, termed "CLIP" (Class II Invariant chain Peptide), has a supermotif for binding to virtually all class II molecules. Pretreatment of autologous lymphoblasts with anti-human CLIP antibody inhibits lysis mediated by autologous GVHD effector T cells (Table 1A). Conversely, preliminary data also suggest that force-loading of CLIP will increase expression of CLIP on target cells and may result in a significant increase in lysis mediated by the syngeneic GVHD effector T cells (Table 1B). Strategies to target CLIP as a mechanism for augmenting the antitumor activity of autologous GVHD are underway in the laboratory.

We have serially prospectively evaluated all patients with breast cancer undergoing treatment with CsA for the elaboration of T-lymphocytes cytotoxic to pretransplant lymphoblasts and to T47D breast cancer cells, which abundantly express the MHC Class II antigen. The evolution of lytic activity over the course of the 28 days post infusion of stem cells during which CsA is administered is shown for 3 representative patients in Figure 3. Lytic activity tends to peak as marrow engraftment evolves around days 15–20 and then disappears as therapy ends. It is worth noting that maximal lytic activity is higher in patients who develop GVHD compared with those who do not for autologous targets (mean maximum activity: 18 versus 11%, p=0.03), and against T47D breast cancer cells (32 versus 19%, p=0.004). These data suggest that lytic activity can be used as an intermediate endpoint, which will facilitate study of methods for augmenting this strategy and for evaluating its clinical impact in further studies.

SUMMARY

A syndrome that appears clinically identical to mild GVHD can be induced in patients with a variety of malignancies after HDCT and autologous stem cell transplantation by the use of CsA. T-lymphocyte–mediated cytotoxic activity directed against cells that bear the MHC Class II antigen evolves with this clinical syndrome. γ-interferon, which upregulates the MHC Class II antigen, appears to make this clinical phenomenon more potent. Clinical studies in patients with resistant hematopoietic cancers, predominantly lymphoma,

suggest an improvement in duration of remission and survival associated with this therapy when compared with historical controls. Analysis of serial studies of women with metastatic breast cancer, who had induction of GVHD by CsA after high-dose therapy, have shown no improvement in time to progression or survival. The elaboration of T-lymphocyte–mediated MHC Class II dependent lytic activity against autologous lymphoblasts and T47D breast cancer cell lines is associated with the evolution of this phenomenon. Studies of the augmentation of any antitumor activity which may be associated with the induction of autologous GVHD by cotreatment with IL-2 and the evaluation of this therapy in the adjuvant treatment of breast cancer are ongoing.

REFERENCES

1. Antman KH, Souhami RL: High-dose chemotherapy in solid tumors. *Ann Oncol* 4(suppl 1)29–44, 1993.
2. Weiden PL, Fluornoy N, Thomas ED et al.: Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. *N Engl J Med* 300:1068–1073, 1979.
3. Weiden PL, Sullivan KM, Fluornoy N et al.: Antileukemic effect of graft-versus-host disease. Contribution to improved survival after allogeneic marrow transplantation. *N Engl J Med* 304:1529–1533, 1981.
4. Kolb HJ, Mittermuller J, Clemm CH et al.: Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood* 76: 2462–2465, 1990.
5. Glazier A, Tutschka PJ, Farmer ER et al.: Graft-versus-host disease in cyclosporine A-treated rats after syngeneic and autologous bone marrow transplantation. *J Exp Med* 158:1–8, 1983.
6. Hess AD, Fischer AC: Immune mechanisms in cyclosporine-induced syngeneic graft-versus-host disease. *Transplantation* 48:895–900, 1989.
7. Geller GB, Esa AH, Beschorner WE et al.: Successful in vitro graft-versus-tumor effect against an Ia-bearing tumor using cyclosporine-induced syngeneic graft-versus-host disease in the rat. *Blood* 74:1165–1171, 1989.
8. Jones RJ, Vogelsang GB, Hess AD et al.: Induction of graft-versus-host disease after autologous bone marrow transplantation. *Lancet* 1:754–757, 1989.
9. Kennedy MJ, Vogelsang GB, Beveridge RA et al.: Phase I trial of intravenous cyclosporine to induce graft-versus-host disease in women undergoing autologous bone marrow transplantation for breast cancer. *J Clin Oncol* 11:478–484, 1993.
10. Kennedy MJ, Vogelsang GB, Jones RJ et al.: Phase I trial of interferon-gamma to potentiate cyclosporine A-induced graft-versus-host-disease in women undergoing autologous bone marrow transplantation for breast cancer. *J Clin Oncol* 12:249–257, 1994.

CYTOKINE AND ALLOGENEIC CELL-MEDIATED IMMUNOTHERAPY FOLLOWING SYNGENEIC/ AUTOLOGOUS BONE MARROW TRANSPLANTATION IN MICE AND MAN

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ABSTRACT

The feasibility of inducing cytokine-mediated anti-tumor responses utilizing recombinant human IL-2 (rIL-2) and particularly a combination of rIL-2 and recombinant alpha interferon (IFN) as well as induction of potent graft-versus-leukemia (GVL) effects with allogeneic T cells in recipients of autologous bone marrow transplantation (BMT) was studied initially in a murine model of human B-cell leukemia/lymphoma (BCL1). Allogeneic cell therapy (alloCT) induced by infusion with peripheral blood lymphocytes (PBL), a mixture of allogeneic spleen and lymph node cells and allogeneic activated cell-therapy (alloACT) induced by in vitro rIL-2-activated allogeneic bone marrow cells (BMC) in tumor-bearing mice, prevented disease development in adoptive BALB/c recipients. Concomitant in vivo activation of allogeneic lymphocytes with rIL-2 prevented even more effectively the development of leukemia in secondary adoptive recipients of spleen cells obtained from treated mice. In contrast, in vivo administration of rIL-2 after syngeneic BMT with or without equal numbers of syngeneic lymphocytes led to disease development in secondary recipients. In man too, tumor cells can be eliminated effectively by allogeneic lymphocytes similarly to the reactivity of allogeneic lymphocytes against allografts since both normal and malignant hematopoietic cells present host-type cell surface alloantigens equally well. Alloreactive potential of donor lymphocytes may thus be used for allogeneic cell therapy (alloCT) even against tumor cells that are resistant to conventional or high dose chemoradio-therapy. Following autologous BM or blood stem cell transplantation (autoSCT) at the stage of minimal residual disease (MRD) where no further tumor cytoreduction can be accomplished by conventional modalities, alloCT may represent an approach for elimination of MRD. For alloCT to be effective, host must be sufficiently immunosuppressed and donor lymphocytes sufficiently matched with the patient to

enable donor lymphocytes to react to residual tumor cells. Allogeneic interaction with normal host tissues may result in graft-versus-host disease (GVHD) which also frequently accompanies GVL effects following allogeneic BMT. The intensity of alloreactivity against tumor cells as well as against normal host tissues may be amplified by activation of donor-derived effector cells with rIL-2 given in vivo or in vitro activated donor lymphocytes or by combining both of the above. The intensity of alloreactivity, which may correspond to the frequency of host-reactive donor T lymphocytes, may also be affected by the degree of mismatching across minor and certainly major histocompatibility barriers. Thus, haploidentical donor lymphocytes may also be used effectively against tumor cells of host origin under conditions where GVHD can be prevented or adequately controlled.

INTRODUCTION

Allogeneic and autologous blood stem cells or bone marrow transplantation (BMT) represent the treatment of choice for a large number of leukemias and lymphomas resistant to conventional chemotherapy. Allogeneic bone marrow transplantation (alloBMT) is the most effective treatment for both prevention and treatment of relapse, due to the graft versus leukemia (GVL) effect exerted by allogeneic donor-derived immunocompetent lymphocytes.[1,2] Unfortunately, severe acute and/or chronic graft versus host disease (GVHD), which frequently develops in BMT recipients still is a major cause of morbidity and mortality following alloBMT. Mild and self-limiting GVHD may be the ultimate goal in treating hematologic malignancies because of the concomitantly occurring GVL effects.[1] In comparison with alloBMT, autologous bone marrow or peripheral blood stem cell transplantation (autoSCT) carry a low risk of procedure-related mortality but at a cost of a substantially higher risk of relapse. Considering the fact that alloBMT is not feasible for the large majority of patients with malignant hematologic diseases due to age limitations, refusal by the treating physician or the patient himself due to the risks associated with GVHD and especially in situations where no matched donor is available, autoSCT may offer many advantages. It is therefore important to understand the role and mechanisms by which GVL can be induced against residual tumor cells, especially in recipients with a fully matched marrow donor available.

We describe here the successful prevention of leukemia development in mice inoculated with murine B cell leukemia (BCL1) after syngeneic BM transplantation (SBMT) in an experimental model simulating minimal residual disease (MRD) post autoSCT. Post-transplantation, at the stage of MRD, mice were given resting allogeneic lymphocytes or BM cells activated in vitro with rIL-2, with or without concomitant in vivo activation of anti-leukemia effector cells with recombinant interleukin-2 (rIL-2). Measurement of the anti-leukemia effects was done by adoptive

transfer in secondary BALB/c recipients in order to prevent distortion of the message by harmful GVHD. We could show that MRD in primary recipients may be kept under control by infusion of allogeneic lymphocytes, suggesting that GVL effects may be successfully induced and be of clinical benefit once donor T cells can be effectively eliminated or controlled in case of severe GVHD.

MATERIALS AND METHODS

Mice

BALB/c (BALB), C57BL/6 (C57), (BALB/c × C57BL/6)F1 (F1) mice, 2–6 months old, were purchased from the breeding colony of the Hebrew University-Hadassah Medical School, Jerusalem. Mice were kept under standard conditions, with acidic water (pH 2.7) and no special protective measures. Mice were given 0.5% neomycin sulfate in their drinking water for 2 weeks post-transplantation.

Murine B cell leukemia (BCL1)

BCL1, a spontaneous, transplantable B-cell leukemia/lymphoma of BALB origin[3,4] is characterized by marked (up to 30-fold) splenomegaly, accompanied by extreme peripheral blood lymphocytosis (>200,000/mm^3) and results in death of all mice inoculated with ≤10–100 tumor cells. BCL1 was maintained in vivo in BALB mice by IV passage of 10^6–10^7 peripheral blood lymphocytes (PBL) obtained from tumor-bearing mice. Mice with marked lymphocytosis in the blood were subsequently used as BCL1 cell donors for experimental mice. PBL counts for all experimental groups were carried out weekly. Leukemia was defined as PBL counts exceeding 20,000/mm^3. At the peak of disease PBL counts usually reached >100,000/mm^3.

Conditioning with radiation and cyclophosphamide (CY) prior to BMT

Mice were either irradiated with a single dose of 750 cGy total body irradiation (TBI) by a Philips X-ray unit (250 kV, 20 mA) with a focus to skin distance of 70 cm at a dose rate of 60 cGy/min, or conditioned with freshly dissolved CY (300 mg/kg) (Taro, Israel) given intraperitoneally (IP). Twenty-four hours later, mice received 10^7 syngeneic marrow cells via the lateral tail vein.

Preparation of BM cells (BMC)

BMC were obtained from the femora, tibiae and humeri of syngeneic mice. Mononuclear cells containing 10^7 BMC in 0.25 mL Hank's medium were injected into the lateral tail vein of recipients 24 hours post-radiation.

Recombinant human interleukin-2 (rIL-2)

rIL-2 provided as 1 mg Proleukin (3×10^6 Cetus Units equivalent to 18×10^6 International Units) was kindly provided by Dr. S.L. Aukerman, Cetus/Chiron, CA

and Mr. C. Mudde, Chiron, Amsterdam. rIL-2 was initially diluted with water for injection and subsequently rediluted with dextrose 5%. International units (IU) are used throughout this paper.

Activation of BMC by rIL-2

BMC were cultured in 225 cm^3 flasks (Corning 25160-225, Corning Glass, Corning, NY) in RPMI 1640 medium (Biological Industries, Beit Haemek, Israel) containing L-glutamine, nonessential amino acids, pyruvate, 10% bovine calf serum (BCS) and rIL-2 (6000 IU/mL) for 4 days in a humidified incubator with 5% CO_2 at 37°C. Viability of harvested cells was determined by the trypan blue exclusion method.

Simulation of MRD following SBMT

In order to simulate MRD quantitatively, a fixed number of 10^5 BCL1 cells was added to the marrow inoculum at SBMT concomitantly with immunotherapy.

Immunotherapy by immunocompetent allogeneic lymphocytes

Immunotherapy by allogeneic lymphocytes, alloCT, was initiated by adoptive transfer of immunocompetent allogeneic lymphocytes (PBL or a mixture of donor spleen and lymph node cells) as detailed in each experiment. Allogeneic activated cell therapy (alloACT) consisted of adoptive transfer of allogeneic lymphocytes pre-activated in vitro with rIL-2 (allogeneic "LAK" cells).

Detection of residual clonogenic BCL1 by adoptive transfer experiments

To determine whether or not residual BCL1 cells were present after various treatments, 10^5 spleen cells obtained from treated mice were adoptively transferred to untreated secondary syngeneic (BALB) recipients. Absence of leukemia (≥100 days) in secondary recipients was indicative of elimination of BCL1 since as few as 1–10 cells were previously shown to cause disease.[4]

Assay for chimerism

Chimerism was documented by testing spleen cells (or PBL) by an in vitro complement-dependent microcytotoxicity assay with specific alloantisera and rabbit complement, to determine the percent of host or donor-type cells. Specific alloantisera (BALB-anti-C57 and C57-anti-BALB) were prepared by cross-immunization with full-thickness skin allografts followed by 6 intraperitoneal injections of 30×10^6 donor-type spleen cells at intervals of 1–2 weeks. Mice were bled and sera stored at –70°C until use. Chimerism was confirmed by typing spleen lymphocytes with both antisera. Lymphocytes obtained from F1 recipients showed 100% cytotoxicity with both BALB-anti-C57 and C57-anti-BALB antisera, whereas lymphocytes obtained from chimeras were lysed by BALB-anti-

C57 and not by C57-anti-BALB antisera. Percent chimerism was therefore calculated by the formula: % donor type (C57) cells = % cells lysed following treatment with BALB-anti-C57 antiserum minus % cells lysed with C57-anti-BALB antiserum minus % cells lysed with complement only.

Pilot clinical studies

Six patients with leukemia (4 AML, 1 not in CR; 2 CML in chronic phase) with matched sibling donor available who refused to undergo alloBMT received donor-derived peripheral blood lymphocytes (PBL) as soon as their blood counts recovered and stabilized (2–3 months post autoSCT). Ten patients with non-Hodgkin's lymphoma (NHL) were included: six with high grade (two in 2nd CR; two in 2nd PR; two with refractory disease); two with intermediate grade (one in 1st PR; one in 2nd PR) and two with low grade disease (two in 2nd CR). Six patients with breast cancer with documented metastases, not expected to be cured by autoSCT alone, were also treated with matched donor PBL as soon as disease progression was documented or as soon as their condition stabilized. Additional patients received donor PBL earlier post autoSCT on day +1, either graded increments or a single larger dose of PBL, but they were not included in this analysis.

Statistical analysis

The significance of differences between treated and untreated mice was calculated by the independent statistical *t*-test.

RESULTS

Induction of AlloCT and AlloACT following SBMT in mice

Lethally irradiated (750 cGy) F1 mice were grafted with 10^8 syngeneic BMC. Following inoculation with 10^5 BCL1 cells in an attempt to simulate MRD following autoSCT, varying numbers of immunocompetent C57 PBL were administered intravenously to induce GVL-like effects by alloCT. The efficacy of alloCT was monitored by eradication of residual BCL1 cells, as assayed by adoptive transfer of aliquots of 10^5 spleen cells pooled from 2–3 experimental mice to untreated secondary BALB recipients at around two weeks post-SBMT.

Summary of results obtained from three different experiments with a total of 120 mice suggests that injection of $20–30\times10^6$ PBL from normal C57 mice into F1 recipients effectively controlled residual BCL1 cells, as none of 40 secondary adoptive BALB recipients developed leukemia (>180 days). In sharp contrast, leukemia developed in all 20 secondary BALB recipients of 10^5 spleen cells from F1 recipients that had received $20–30\times10^6$ syngeneic PBL post-SBMT. Administration of rIL-2 (12×10^4 IU × 2/day for 5 days IP) post-transplant did not

improve the disease-free survival of secondary recipients of 10^5 spleen cells obtained from treated F1 mice, since all 20 secondary recipient BALB mice developed leukemia. In vivo administration of rIL-2 to recipients of 20×10^6 allogeneic PBL to additionally activate effector cells did not lead to any additional GVL effects; all 40 secondary BALB recipients remained disease-free (>180 days). Samples of spleen cells obtained from recipients of C57 PBL showed chimerism, indicating that engraftment of allogeneic lymphocytes may lead to development of GVL.

The anti-leukemic effects mediated by allo-CT were cell-dose dependent between the ranges of 30×10^6, 10×10^6, 3×10^6 or 1×10^6 with the latter two cell doses being ineffective in preventing GVL effects.

Enhancement of alloCT by in vitro activation of allogeneic lymphocytes with rIL-2 (alloACT)

We have also tested the possibility of increasing the efficacy of alloCT by in vitro pre-activation of allogeneic donor lymphocytes with rIL-2. Lethally irradiated (750 cGy TBI) F1 mice were infused with 30×10^6 C57 BMC pre-activated in vitro for 4 days with rIL-2. BMC were mixed with 10^5 BCL1 cells to simulate MRD. Results of 3 separate sets of experiments were comparable and suggested that in mice receiving no additional rIL-2 following cell therapy 10 of 33 secondary recipients of spleen cells obtained from the first experimental group remained disease-free for >150 days. Following additional in vivo activation of effector cells in the second experimental group the results were improved as 19 of 25 secondary recipients remained disease-free for an observation period >150 days ($p=0.05$).

AlloCT and alloACT following autoSCT in clinical practice

Several patients received donor PBL from a family member following autoSCT in an attempt to accomplish eradication of MRD. The longest observation of successful cell therapy with documented response was obtained in a 16 year old girl with acute promyelocytic leukemia (M3) with evidence of molecular disease (positive RT-PCR for retinoic acid receptor alpha). Following autoSCT, RT-PCR remained positive until the patient was treated with maternal activated PBL, followed by 3.7×10 cells/kg activated in vivo with rIL-2 (6×10^6 IU/m^2, subcutaneously for 3 days. For the first time RT-PCR reversed in PBL but the response lasted only 60 days. Subsequently, a durable response lasting already for more than 3 years was obtained following additional infusion of maternal PBL activated in vitro with rIL-2 for 4 days and further in vivo for 3 days as indicated above. As far as GVHD is concerned, this patient developed a mild transient skin rash that might have been compatible with a mils grade 1 GVHD. Overall, 3 of 4 patients with AML, including the one described above, are alive and well, disease-free at 33 to 41 months range post autoSCT and only 1 relapsed. But in only one

patient, the one with AML(M3) described in more detail, the evidence exists for direct response to alloCT, since the other 3 were in CR at the time of initiation of immunotherapy. Chimerism was never confirmed in any of the above cases.

Two patients with CML were also included. Both were in cytogenetic remission at the time of autoSCT. Both are alive at 41 and 40 months post autoSCT; one in continuous hematologic remission and the other in progressive disease at 26 months post autoSCT. No patient developed sustained measurable chimerism.

Of the 10 patients with non-Hodgkin's lymphoma 7 are alive and well, 3 after additional therapy following relapse at +7, +18 and +26 months post cell therapy. Four have never experienced relapse and are alive and well >31 to >51 months post autoSCT. Three of these patients developed marrow aplasia as a consequence of alloCT after administration of rIL-2. They received donor marrow infusion; 2/3 are disease-free range 33–41 months post autoSCT.

Of the 6 patients with metastatic breast cancer, 3 are alive but only one is disease-free. In one patient with metastatic liver involvement, all visible metastases disappeared transiently, although overt chimerism has never been established.

DISCUSSION

In the present report we have documented GVL-like effects induced by allo-CT or alloACT post-SBMT in mice and most likely, a similar phenomenon in pilot clinical trials. Our data suggest that MRD can be successfully controlled in vivo by cell therapy with alloreactive lymphocytes and that this anti-tumor effect can be enhanced in vivo with a short course of intermediate-dose rIL-2. GVL-like effects in this setting were also induced by infusion of BM derived allogeneic lymphocytes, pre-activated in vitro by rIL-2, without causing any gross impairment of the hematopoietic capacity of BMC in lethally irradiated recipients.

The GVL-like effects mediated by alloCT and alloACT were tested in an adoptive transfer system, instead of observing the primary recipients to avoid two possible obstacles encountered when assessing factual elimination of clonogenic BCL1 cells by post-transplant immunotherapy, namely, lethal GVHD and tumor dormancy.[5,6] Failure to develop leukemia in primary recipients, even if lethal GVHD can be prevented, does not prove elimination of all BCL1 cells, since existing tumor cells may be actively suppressed as previously documented following alloBMT [6] and rIL-2 therapy.[5]

We have previously documented that elimination of tumor cells by alloreactive donor-derived T cells may last 2–3 weeks.[7] Hence, *temporary* engraftment of allogeneic effector cells may be sufficient to induce beneficial GVL effects against MRD, without the need for permanent residence of allogeneic effector cells, which may cause severe GVHD across MHC barriers.

It was thus anticipated that following SBMT in mice and autoSCT in man, the immunosuppressive state induced by the conditioning may lead to transient engraftment of donor PBL for a limited period of time, thus permitting induction of aggressive, yet short lasting GVL effects in vivo, which may or may not be accompanied by overt GVHD. It should be kept in mind that inoculation of randomly matched blood lymphocytes into immunosuppressed recipients may lead to fatal GVHD.[8] On the other hand, such an approach might be safely considered in conjunction with infusion of graded increments of matched allogeneic blood lymphocytes.[9,10] Indeed, in one patient with NHL marrow aplasia resulted, which could be life threatening without an allogeneic marrow rescue with marrow cells obtained from the PBL donor. Whether or not effective GVL can be obtained in the absence of GVHD remains to be seen, either because the two phenomena can be separated or due to the higher sensitivity of tumor cells to the anti-tumor effects of alloCT and alloACT above the threshold of GVHD induction.

Our data imply that in the context of SBMT or autoSCT, clinically beneficial GVL effects may be induced in immunocompromised hosts with MRD by allogeneic lymphocytes, preferably with concomitant activation of effector cells by rIL-2 in vitro and/or in vivo. However, the major problem encountered with this approach is that it seems difficult, if not impossible, to predict whether immunosuppression is adequate for acceptance of donor-derived T cells or whether durable acceptance of donor PBL may result in aplasia or other GVHD manifestations. The more effective clinical use of such an appealing therapeutic strategy in further trials should only be considered under conditions that strictly ensure adequate treatment of marrow aplasia or other unanticipated complications related to GVHD or safer, as soon as newer and more effective modalities are available for elimination of potentially harmful T cells in case of durable engraftment that may lead to severe GVHD. One possible approach that may make it possible to exploit the use of this technology involves introduction of the thymidine kinase gene into the T cells prior to cell therapy for possible induction of suicide leading to eventual rejection of allogeneic lymphocytes in case of severe GVHD. Indeed, recent data from our laboratory indicate that transient "parking" of allogeneic immunocompetent effector cells in tumor-bearing hosts may be sufficient for induction of beneficial GVL-like effects.[7] Eventually, the use of alloreactive cells could be extremely effective and sufficient for eradicating MRD in conjunction with conventional rather than high-dose chemotherapy.

Finally, our preliminary data in the small series of patients with breast cancer suggests that graft versus tumor (GVT) effects comparable to GVL in hematologic malignancies may be of therapeutic benefit.

In conclusion, our results indicate that cell-mediated immunotherapy, both alloCT and alloACT, may develop into a promising approach for eradication of

MRD. GVL effects induced by administration of allogeneic lymphocytes may be additionally augmented by administration of rIL-2 in vivo. GVL effects can be induced by allogeneic PBL that may be only transiently "parked" in the recipient, causing a "hit & run" GVL effect, with beneficial anti-leukemic effects on MRD following SBMT and most likely following autoSCT as well, but only as long as life-threatening GVHD can be prevented or at least adequately controlled. Successful induction of GVL effects by allogeneic PBL in the setting of MRD may lead to clinical application of allogeneic cell therapy for control of relapse in a variety of malignant hematologic diseases and possibly solid tumors as well.

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REFERENCES

1. Horowitz MM, Gale RP, Sondel PM: Graft versus leukemia reactions after bone marrow transplantation. *Blood* 75:555–562, 1990.
2. Ringden O, Horowitz MM: Graft-vs-leukemia reactions in humans. *Transplant Proc* 21:2989–2994, 1989.
3. Slavin S, Strober S: Spontaneous murine B-cell leukemia. *Nature* 272:624–626, 1978.
4. Slavin S, Weiss L, Morecki S, Ben Bassat H, Leizerowitz R, Gamliel H, Korkesh A, Voss R, Polliack A: (1981) Ultrastructural, cell membrane and cytogenetic characteristics of B-cell leukemia (BCL1), a murine model of chronic lymphocytic leukemia. *Cancer Res* 41:4162–4166, 1981.
5. Slavin S, Eckerstein A, Weiss L: (1988) Adoptive immunotherapy in conjunction with bone marrow transplantation—amplification of natural host defence mechanisms against cancer by recombinant IL2. *Nat Immunol Cell Growth Regul* 7:180–84, 1988.
6. Slavin S, Weiss L, Morecki S, Weigensberg M: (1981) Eradication of murine leukemia with histoincompatible marrow grafts in mice conditioned with total lymphoid irradiation (TLI). *Cancer Immunol Immunother* 11:155–158, 1981.
7. Weiss L, Lubin I, Factorowich Y, Lapidot Z, Reich S, Reisner Y, Slavin S: Effective graft vs leukemia effects independent of graft vs host disease after T-cell depleted allogeneic bone marrow transplantation in a murine model of B-cell leukemia/lymphoma. Role of cell therapy and rIL-2. *J Immunol* 153:2562–2567, 1994.
8. Anderson KC, Goodnough LT, Sayers M, Pisciotto PT, Kurtz SR, Lane TA, Anderson CS, Silberstein LE: Variation in blood component irradiation practice: Implications for prevention of transfusion-associated graft-versus-host disease. *Blood* 77:2096–2102, 1991.
9. Or R, Nagler A, Ackerstein A, Naparstek E, Samuel S, Drakos P, Kapelushnik Y, Amar

A, Slavin S: Allogeneic cell-mediated cytokine-activated immunotherapy of non-Hodgkin lymphoma for eradication of minimal residual disease in conjunction with autologous bone marrow transplantation (ABMT). *Blood* 82(suppl 1):171a, 1993.

10. Slavin S, Naparstek E, Nagler A, Ackerstein A, Drakos P, Kapelushnik Y, Brautbar C, Or R: Cell mediated immunotherapy (CMI) for the treatment of malignant hematological diseases in conjunction with autologous bone marrow transplantation (ABMT). *Blood* 82(suppl 1):292a, 1993.

EX VIVO TREATMENT OF AUTOLOGOUS GRAFTS WITH IL-2 PRIOR TO TRANSPLANTATION IN PATIENTS WITH AML IN FIRST REMISSION

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ABSTRACT

Recurrence of leukemia after autologous stem cell transplantation is believed to be related to the infusion of residual malignant cells and the lack of a graft-versus-leukemia (GVL) effect. It is therefore likely that several different approaches will be required to significantly decrease disease recurrence after autografting. The strategy we have developed with these considerations in mind involves immunological activation and purging of the autograft, combined with post-transplant immunotherapy with interleukin-2 (IL-2).

Bone marrow (BM) (n=10) and more recently, G-CSF mobilized (12 μg/kg for 5 days) peripheral blood progenitor cells (PBPC) (n=14) were obtained from AML patients with poor prognostic features (based on cytogenetics, FAB morphology or high blast count) immediately after they had entered first remission. The cells were cultured for 8 days in the presence of IL-2 at a concentration of 250 U/mL on the basis of previous experiments demonstrating that these conditions support ex vivo purging and the activation and expansion of cytotoxic cells. The cultured cells were then harvested and reinfused into the patient who, during the intervening 8 days, had received myeloablative chemotherapy (busulfan and cyclophosphamide). Immediately following the transplant, patients were given daily injections of IL-2 for 7 days to maintain the cytotoxic activity of the stimulated cells in the cultured autograft and to induce a GVL effect.

The initial 10 recipients who had received bone marrow only showed a delayed recovery of both neutrophils (absolute neutrophil count [ANC] $\geq 0.5 \times 10^9$ at 49 days) and platelets ($\geq 20 \times 10^9$/L at 98 days). The next cohort of patients (n=6) received untreated PBPC in addition to IL-2 activated cultured BM cells. In these recipients, marrow recovery was much faster.

We are now transplanting a one-day PBPC leukapheresis product that has been cultured in IL-2 for 8 days and obtain full engraftment within 4 weeks (n=8).

This regimen has been well tolerated, even in patients older than 55 years. Kaplan-Meier disease-free survival (DFS) for all patients at a median follow-up of 2 years is 36%, which is encouraging for a group of poor risk patients transplanted very early after remission induction.

INTRODUCTION

Relapse remains the biggest challenge for improving outcome after autologous bone marrow (BM) or blood stem cell transplantation for acute myeloid leukemia. When the stem cell transplant is not purged, relapse rates between 50 and 70% are observed, and most of the relapses occur soon after transplantation, i.e., within the first 6–12 months.[1,2] Evidence for the need of autograft purging has been ascertained in a trial in which unpurged marrow cells from patients with AML in first CR were marked with a retrovirus and disease recurrence was accompanied by the appearance of genetically marked leukemic cells.[3]

In addition to the possibility of infusing a contaminated stem cell product, the lack of an allogeneic graft-versus-leukemic (GVL) effect after autologous transplantation can also contribute to disease recurrence. This has been shown by comparing recurrence rates for patients with AML in first CR who received a transplant from an identical twin with those receiving an allogeneic transplant. The relapse rate after twin transplantation is about 2–3 times higher than after allogeneic transplantation.[4]

In an effort to achieve both an ex vivo purging effect and an in vivo GVL effect, we have designed a clinical study based on pre-clinical data from our laboratory[5,6] and our initial results in murine studies from other centers.[7,8] This involves the culture of autologous BM or blood stem cell preparations in interleukin-2 (IL-2) for eight days and the administration of IL-2 to the patient for the first week after transplantation. The rationale behind this approach is that the ex vivo culture in IL-2 will provide some immunopurging as well as activation of cytotoxic cells, which, after infusion into the patient, may attack residual leukemic cells in the patient. Furthermore, IL-2 has previously been shown to have some beneficial effects in patients with advanced AML, possibly by activating cytotoxic cells and releasing secondary antileukemic cytokines.[9,10]

MATERIALS AND METHODS

A total of 24 patients have been entered into this study. All patients were transplanted within three months after obtaining first remission without having received any consolidation treatment. The first 10 patients received autologous BM cultured in IL-2, and a subsequent cohort of patients either received BM

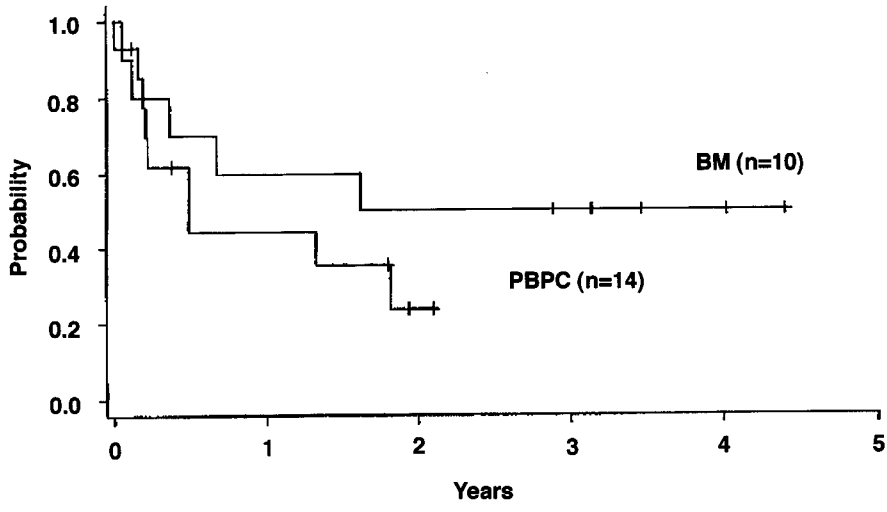

Figure 1. *Kaplan-Meier DFS curve for patients with AML who received an IL-2 cultured autograft in first remission. Ten patients were given BM (cultured in IL-2) as the only source of stem cells whereas 14 patients received a blood progenitor cell product, (PBPC). The difference in the two survival curves is not significant.*

cultured in IL-2 plus non-IL-2 treated peripheral blood progenitor cells (PBPC) (n=6), PBPC cultured in IL-2 plus noncultured PBPC (n=3) or PBPC cultured in IL-2 with no addition of non-cultured cells (n=6). All patients had one of the following poor prognostic features at the time of diagnosis: 1) Initial peripheral blast count $\geq 50 \times 10^6$/L, 2) FAB M5 morphology or 3) a karyotypic abnormality other than t(l5;17), t(8;21) or inv(16). All patients received one course of induction chemotherapy consisting of high dose ARA-C, mitoxantrone and VP16. Patients who had PBPC collected were given G-CSF at a dose of 12.5 μg/kg for 5 days and leukaphereses were performed on days 5 and 6.

The first 10 patients were given IL-2 post autograft infusion at escalating doses starting at 200,000 U/m^2/day. The dose was increased in groups of 3 patients to 400,000 and 600,000 U/m^2/day, respectively. Since these doses were well tolerated, it was decided that all subsequent patients would receive a dose of 500,000 U/m^2/day s.c. for the first seven days after autograft infusion.

RESULTS

The median age of all transplanted patients was 50 years (range, 15–63) with 6/24 patients over the age of 60. The median time from obtaining complete remission (as

documented by BM biopsy) and transplant was 37 days (range, 13–128 days), and only 4 patients required a second cycle of chemotherapy to enter first CR. In the first 10 patients who received BM cultured in IL-2, a delayed neutrophil recovery (ANC $\geq 0.5 \times 10^9$/L) of 48 days and a delayed platelet recovery were observed. As a modification, the next 6 patients received G-CSF-primed PBPC, which were added to the IL-2 cultured BM. The ANC recovery was reduced to 15 days after a transplant. In addition, the platelet recovery (maintaining a platelet count of at least 20×10^9/L without transfusion) was reduced from 98 days to 24 days. The cohort of patients who were given PBPC as the sole source of stem cells recovered 0.5×10^9/L neutrophils by day 24 and were independent of platelet transfusions by day 51 after transplant. There was no correlation between the number of CD34 cells infused and the recovery of neutrophils or platelets after grafting. Side effects of the IL-2 treatment post transplant were mild, with almost all patients experiencing fever up to 40°C and fatigue. Some patients (25%) developed a rash and about 5% had fluid retention greater than 10 percent of the baseline weight.

The disease-free survival (DFS) for the first 10 patients who received IL-2 cultured BM only continues to be 50% at 4 years. Patients who received PBPC either combined with BM or PBPC as the only stem cell source have a shorter follow-up and the DFS at 2.5 years is 24% (Figure 1).

DISCUSSION

Results from this study confirm that marrow or PBPC that have been cultured in IL-2 for 8 days can successfully provide hematopoietic reconstitution to patients with AML after myeloablative chemotherapy. Further, the IL-2 given immediately after transplant has acceptable side effects and neutrophil and platelet recovery after PBPC infusion is rapid and maintained. Patients given IL-2–cultured BM have a favorable DFS considering their poor risk features at diagnosis and the fact that no consolidation treatment was given before transplantation. In patients who received PBPC, longer follow-up is needed to see if PBPC autografts, even after culture in IL-2, are inferior to BM with respect to the post-transplant relapse rate. To address the question as to whether insufficient purging of leukemic cells from the autograft contributes to relapse, we are planning to conduct a gene marking trial in which CD34-enriched cells after IL-2 culture are marked with a neomycin resistant gene and reinfused into the patient together with the IL-2 cultured but nonmarked portion. We further believe it should be possible to furbish hematopoietic progenitor cells with an IL-2 gene so that these cells, which are expected to home to the BM, will produce IL-2 locally for some time after engraftment and activate site-specific immune effect cells. Preclinical studies to test the antileukemic effects of such targeted immunotherapy are currently underway.

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REFERENCES

1. Korbling M, Fliedner TM, Holle R, Magrin S, Baumann M, Holdermann E, Eberhardt K: Autologous blood stem cell (ABSCT) versus purged bone marrow transplantation (pABMT) in standard risk AML: Influence of source and cell composition of the autograft on hemopoietic cell survival. *Bone Marrow Transplant* 7:343, 1991.
2. Sanz MA, de la Rubia J, Sanz GF, Martin G, Martinez J, Jarque I: Busulfan plus cyclophosphamide followed by autologous blood stem-cell transplantation for patients with acute myeloblastic leukemia in first complete remission: A report from a sit. *J Clin Oncol* 11:1661, 1993.
3. Brenner MK, Rill DR, Moen RC, Krance RA, Mirro Jr J, Anderson WF, Ihle JN: Genemarking to trace origin of relapse after autologous bone-marrow transplantation. *Lancet* 341:85, 1993.
4. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, Rimm AA, Ringden O, Rozman C, Speck B, Truitt RL, Zwaan FE, Bortin MM: Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* 75:555, 1990.
5. Greenwalt DE, Lipsky RH, Ockenhouse CF, Ikeda H, Tandon NN, Jamieson GA: Membrane glycoprotein CD36: A review of its role in adherence, signal transduction, and transfusion medicine. *Blood* 80:1105, 1992.
6. Klingemann H-G, Eaves CJ, Barnett MJ, Eaves AC, Hogge DE, Nantel SH, Reece E, Shepherd JD, Sutherland HT, Phillips GL: Transplantation of patients with high risk acute myeloid leukemia in first remission with autologous marrow cultured in interleukin-2 followed by interleukin-2 administration. *Bone Marrow Transplant* 14:389, 1994.
7. Charak BS, Brynes RK, Groshen S, Chen S, Mazumder A: Bone marrow transplantation with interleukin-2-activated bone marrow followed by interleukin-2 therapy for acute myeloid leukemia in mice. *Blood* 76:2187, 1990.
8. Oshimi K, Oshimi Y, Akutsu M, Takei Y, Saito H, Okada M, Mizoguchi H: Cytotoxicity of interleukin 2-activated lymphocytes for leukemia and lymphoma cells. *Blood* 68:938, 1986.
9. Klingemann H-G, Neerunjun J, Schwulera U, Ziltener HJ: Culture of normal and leukemic bone marrow in interleukin-2: Analysis of cell activation, cell proliferation, and cytokine production. *Leukemia* 7:1389, 1993.
10. Kuhr T, Dougherty GJ, Klingemann H-G: Transfer of the tumor necrosis factor lal gene into hematopoietic progenitor cells as a model for site-specific cytokine delivery after marrow transplantation. *Blood* 84:2966, 1994.

THERAPEUTIC RATIO OF YTTRIUM-90–LABELED ANTIFERRITIN (AF) THERAPY FOR RECURRENT HODGKIN'S DISEASE (HD)

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INTRODUCTION

Ferritin is an iron storage molecule found in normal liver, spleen and bone marrow (BM) tissues. Cells contained in tumor masses consisting of HD, Kaposi sarcoma, neuroblastoma and hepatocellular carcinoma secrete ferritin in the tumor interstitium. This "tumor" ferritin usually does not contain iron, but can otherwise not be distinguished from normal ferritin.[1] Nevertheless, ferritin will function as a selective "tumor-associated" antigen for radiolabeled immunoglobulin therapy (RIT) due to the high concentration of ferritin in tumors and its better accessibility to circulating immunoglobulin in comparison with normal tissue ferritin due to the presence of tumor neovasculature.[2,3]

The first RIT study with AF in HD was reported in 1985 and utilized iodine-131.[4] Five different studies have since been performed with indium-111–labeled AF for tumor targeting followed by yttrium-90–labeled AF for therapy.[5–9] The advantages to the switch in radioisotopes appear to be that the combination of indium-111 and yttrium-90 allows for outpatient treatment and has a higher response rate than iodine-131–labeled AF. Patients with HD provide several unique opportunities for RIT studies. Allogeneic immunoglobulin will not induce a measurable humoral immune response in patients with HD. Repeated administrations of AF will not induce the complication of anti-antibodies. The radiosensitivity of HD provides the opportunity to observe clinically significant tumor responses at the relatively low tumor radiation doses that can be obtained with current RIT protocols.

In this communication we update 3 RIT studies in patients with HD performed at the M.D. Anderson Cancer Center between 1992 and 1996. We conclude that radiolabeled AF will be most helpful in the future for the control of bulky HD and that many of the principles uncovered in the application of radioimmunoconjugates to patients with HD deserved to be applied to RIT studies in patients with other malignancies.

Table 1. Rabbit antiferritin (RAF) for Hodgkin's disease (HD) at M.D. Anderson Cancer Center.

Protocol number and goal		*Protocol Schema*	*No. of patients entered*
1. escalation	weeks	RAF in-111 (0); RAF Y-90 (1); 0.3 mCi/kg; 0.4 mCi/kg; 0.5 mCi/kg	49
2. fractionation	weeks	RAF in-111 (0); RAF Y-90 (1); RAF Y-90 (2); 0.25 mCi/kg; 0.25 mCi/kg	16
3. conditioning	weeks	chemo ×2; VP-16 Ifos G-CSF; PBC collection; RAF in-111 (0); RAF Y-90 (1); RAF Y-90 (2); 0.3 mCi/kg ×2; CBV (3); PBC reinfusion (4)	22

Chemo=Idarubicin, cisplatin, Ara-C, Solu-medrol; G-CSF=human recombinant colony stimulating factor; Ifos=ifosfamide; CBV=cyclophosphamide, carmustine (BCNU), etoposide (VP-16); VP-16=etoposide; PBC=peripheral blood cells.

MATERIALS AND METHODS

Antigen

Human ferritin from a splenectomy specimen from a patient with HD was purified using techniques reported previously. Ferritin has a high molecular weight (MW 440,000), is not a membrane antigen, and is present in the interstitium and cell cytoplasm of some normal cells in liver, spleen, and bone marrow (BM) and tumor cells of different histologies.[2]

Isotopes

^{111}In and ^{90}Y were used for radiolabeling. They were obtained from New England Nuclear, Boston, MA (indium) and from Battelle Pacific Laboratories, Richland, WA (yttrium).

Radiolabeling methods

IgG was purified from sera of immunized rabbits. Immunoconjugates were prepared by reacting isothiocyanatobenzyl-DTPA chelators to the lysine residues of the immunoglobulin for the connection between the protein and the bifunctional chelator. On average, one chelate was conjugated per IgG molecule. Subsequently, chelate-immunoconjugates were complexed with radiometals. A backbone-substituted isothiocyanatobenzyl-DTPA was conjugated to the immunoglobulin and radiolabeled with indium or yttrium as described by Quadri et al.[10]

Quality control

Radioimmunoconjugates were tested for radiochemical purity 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. Radioimmunoconjugates were filtered through a 0.2 μm sterile filter. A pyrogenicity test was performed prior to injection. One percent human serum albumin was added to decrease radiolysis of the radioimmunoconjugate.

Patients

End-stage HD patients with measurable disease (n=87) were entered in three different studies. Ages ranged from 10 to 71 years. Male:female ratio was 1.5:1. Most patients were white. Black and Hispanic minorities were proportionally represented. A summary of the studies is given in Table 1. A complete response was defined as disappearance of disease on physical exam and diagnostic studies. A partial response was defined as a decrease in the product of orthogonal tumor diameters by more than 50%. Patients with tumors that did not show a complete or partial response were scored as stable disease or progressive disease.

Table 2. Eligibility criteria, prior treatment and inevaluable patients for RAF studies in HD patients.

Protocol	*Eligibility*	*Prior treatment*		*Inevaluable patients*
1.	• HD histology • HIV negative	multi-agent chemotherapy ×2 or more	100%	5/49
	• second or later relapse	radiation	80%	
	• platelets $>100\times10^9/L^{-1}$	BMT	75%	
	• neutrophils $>1.5\times10^9/L^{-1}$			
	• measurable disease			
2.	• identical to protocol 1	multi-agent chemotherapy ×2 or more	100%	3/16
		radiation	93%	
		BMT	80%	
3.	• HD histology	multi-agent		2/22
	• measurable disease	chemotherapy ×1	100%	
	• HIV negative	radiation	50%	
	• creat <1.5 mg%; bili <1.5 mg%; DLCO >50%; ejection fraction >0.50			

Administration to patients

Radioimmunoconjugates were administered IV Polyclonal AF was given in a 2- to 5-mg total protein dose by rapid bolus infusion. Cold (unlabeled) polyclonal AF was not added to radiolabeled polyclonal AF. Blood and urine samples were taken at 1, 20, 40 and 120 hours after administration of the radioimmunoconjugate for pharmacokinetic analysis. The percent of administered activity eliminated in first and second 24-hour urine after administration was calculated. Rapid and long blood half-lives (T alpha and T beta) were calculated. Alpha/beta ratios were determined. Gamma camera whole-body scans were always performed 1, 20, 40 and 120 hours after indium-labeled immunoconjugates. SPECT scans were performed at 40 hours of tumor-bearing areas only. In seven patients, whole body scans were repeated after yttrium-90–labeled AF administrations. A medium energy collimator was used, with an energy setting between 70–140 KeV for yttrium and an energy setting of 140–250 KeV for indium.[11]

RESULTS

Patient accrual

All patients signed institutional and FDA approved consent forms. Studies were performed under FDA-IND number BB 4731. The eligibility criteria, prior treatment and number of unevaluable patients are listed per protocol in Table 2. Patients in protocols 1 and 2 had more advanced disease and had failed many prior "conventional" therapies. The unevaluable patients for protocols 1 and 2 were lost to follow-up (2 of 8), died from early toxicity (5 of 8) or were in violation of the protocol (1 of 8, patient changed to BMT protocol prior to being evaluable for response). In retrospect, patients with early toxicity did not benefit from the therapy and should not have been entered on the study. They died from HD, not from the therapy. They all had bulky disease and an ECOG performance status of 2 or higher at study entry. Accrual was relatively rapid for a disease as uncommon as HD at approximately one new patient every 2 weeks. Patients were allowed to be retreated for protocol 1 (no limitation on the number of cycles) and protocol 2 (maximum of 3 cycles).

Toxicity: Administration of radioimmunoconjugates

All patients were treated as outpatients, with some of the early toxicity patients of Table 2 being admitted for disease complications prior to their last yttrium-90 RAF administration. Vital signs were recorded every 15 minutes for 1 hour after each radioimmunoconjugate administration. In over 300 administrations, 3 patients on 2 separate days showed temperature elevations and shaking chills 45 minutes after administration. This was responsive to acetaminophen and diphenhydramine within an hour. By exclusion of other sources, a batch of human albumin was considered to be contaminated with a small amount of pyrogen. This problem did not recur in the affected patients or other patients after the introduction of a new batch of human albumin. Wipe tests of the administration room were performed immediately after and were always negative, with the exception of patients' chair pads of the patients with disease-related fever. Presumably, a small amount of the administered radioactivity is eliminated in perspiration. Patients received instruction on how to collect their urine for the first 48 hours after administration. Thereafter, they were instructed to flush twice after urination. They were asked to bring in linen, clothes, etc., for radioactivity measurements if they suspected contamination. Patients rarely brought in items to be checked for radioactivity but when they did, they were not radioactive. Patients were asked to abstain from sexual activity for 1 week after administration.

Toxicity: Hematology

The only side effects after yttrium-90–labeled AF were hematological. HD patients had lymphopenia prior to initiation of treatment. Within days after

Table 3. Hematological side effects after Yttrium-90–labeled antiferritin IV

Parameter 1	*Parameter 2*	*Statistical test*	*p value*
Yttrium-90 administered in mCi/kg (0.3;0.4; 0.5)	ratio of pre and lowest post administration platelet level	Wilcoxon	<0.05
	ratio of pre and lowest post administration white blood cell level	Wilcoxon	<0.05
	1 hour blood radioactivity level μCi/g	correlation coefficient 0.61	<0.01
1 hour blood radioactivity level μCi/g	platelet level $<100\times10^9$/L in weeks	correlation coefficient 0.31	<0.05
area under the Tα curve	platelet level $<100\times10^9$/L in weeks	correlation coefficient 0.37	<0.025

yttrium-90 administration peripheral blood lymphocyte levels decreased further, presumably due to radiation-induced apoptosis of lymphocytes. Recovery took weeks. Granulocytopenia and thrombocytopenia occurred within 3–4 weeks after administration with recovery taking 8–14 weeks. The first study (escalation) demonstrated that hematological toxicity correlated with the amount of yttrium-90–labeled AF administered. The one-hour blood radioactivity level correlated with the administered dose. At later time points, (20 and 40 hours after administration) this correlation was no longer statistically significant. This appeared to be due to a larger volume of distribution for patients with B symptoms. The hypothesis is that patients with B symptoms have varying degrees of a capillary leak syndrome, which will lower blood radioactivity levels at later time points for those patients and decrease the correlation between administered activity and blood activity. New endpoints were examined for hematological toxicity, such as ratio between pretreatment and lowest post-treatment blood counts and the duration in weeks of platelet levels below 100×10^9/L. Significant correlations were found (Table 3). The advantages of these new endpoints are that they provide more quantitative endpoints than the grading system currently in use for hematological toxicity (grade 1 to 4 for different ranges of blood cell counts) and that they provide a time-factor (how long does the toxicity last?).

In the next section, the blood pharmacokinetics of the radioimmunoconjugates are described. Blood elimination was biphasic, with a rapid (T-alpha) component and a slow (T-beta) component. The area under the curve (AUC) for the T-alpha component correlated significantly with hematological toxicity. The addition of T-beta AUC was numerically insignificant and did not improve the correlation. Presumably, under a certain dose rate no further hematological damage occurred. Antiferritin did not target normal BM unless prior granulocyte colony-stimulating factor (G-CSF) treatment had been given.[3,12] Hematological damage was caused by radiation of hematopoietic stem cells in BM, not because these cells were targeted, but because they were radiated by yttrium-90 labeled AF in blood perfusing through BM. The range of yttrium-90 energy deposition is long enough to radiate the marrow spaces from its position in the circulatory system.[12]

Bone uptake of free yttrium-90 was not measured in these patients. The radioimmunoconjugate was stable in vivo (see next section). Therefore free yttrium and bone uptake were not anticipated. In addition, in animal studies the hematological damage inflicted by single intravenous administration of free low molecular weight, bone seekers is limited and much more pronounced after chronic repeated administration.[12] Protocol 1 consisted of a single intravenous administration of a stable, high molecular weight (150,000 MW) yttrium-90–labeled AF. In protocols 2 and 3, two intravenous administrations were used.

Blood and urine pharmacokinetics

In Figure 1, blood radioactivity levels are given for one of the protocol 1 patients after indium-111 and yttrium-90–labeled AF administration. Yttrium counts were collected in the window over 500 KV to exclude the radioactivity caused by the previously injected indium-111. The calculation methods for T-alpha and T-beta half-lives are indicated in the figure. The values are not corrected for radioactive decay and define the so-called effective half-lives. Biological half-lives can be derived from incorporating the known physical half-life of the radioisotope in the formula

$$T_e = \frac{T_b \times T_p}{T_b + T_p},$$

in which e = effective, b = biological and p = physical. The alpha/beta ratio is found by dividing the intercepts of the alpha and beta curves with the Y axis. Results have been reported previously.[8] Alpha/beta ratios are below 3.0, T-alpha half-life is usually around 5–7 hours, while a T-beta half-life is usually around 40 hours. The respective biological half-lives for the patient in Figure 1 are 6.5 hours and over 100 hours.

Blood pharmacokinetics are similar for indium- and yttrium-labeled AF, if the specific activity for yttrium is kept below 15 mCi/mg. The pharmacokinetics are

Table 4. Y-90 RAF in HD

Protocol	*Response rate*		*p value*	*Conclusion*
1. escalation	0.3 mCi/kg	25%		positive dose effect curve 0.5 mCi/kg too high, increased radiolysis, hematological toxicity and third spacing
	0.4 mCi/kg	62%	<0.05	
	0.5 mCi/kg	85%		
2. fractionation	0.25 mCi/kg ×2	55%	>0.05	same response rate, less radiolysis, similar or less hematological toxicity as protocol 1
3. conditioning	0.3 mCi/kg ×2	90%		G-CSF opens blood marrow barrier tumor targeting decreased by prior chemo improved survival over historical controls by CBV conditioning alone

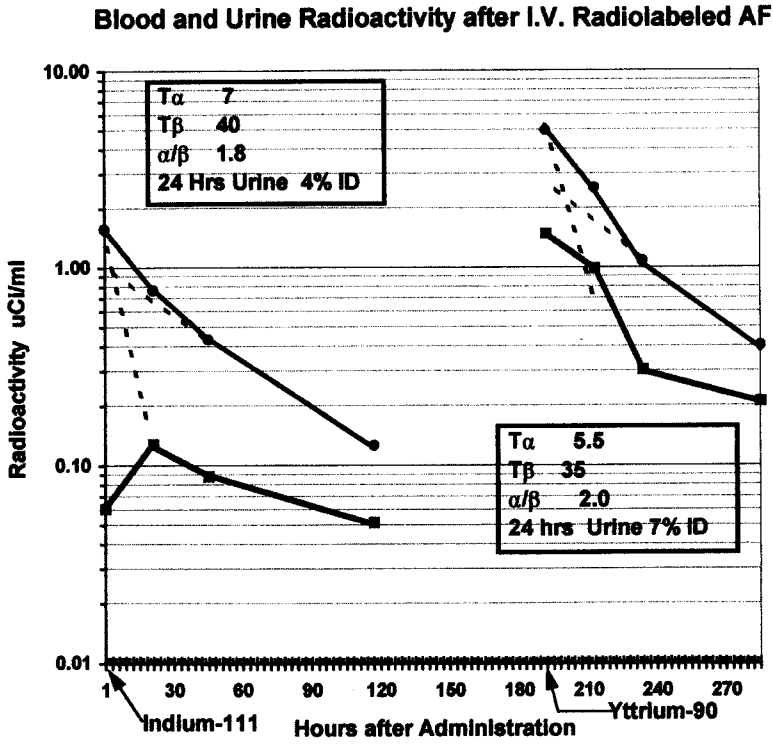

Figure 1. *The thick lower lines connect urine radioactivity levels; the thin upper lines connect blood radioactivity levels at similar timepoints. The dashed lines indicate the derived alpha and beta half-lives.*

similar between patients on all protocols and for the same patient for first and subsequent cycles, with the exception of one patient. This patient made anti-rabbit IgG antibodies after his first RIT cycle and had a high (>4) alpha/beta ratio after his second indium-111 AF administration, indicating a predominantly short half-life and rapid elimination of the radioimmunoconjugate. This patient did not receive a second yttrium-90 AF administration.

Response of Hodgkin's Disease

Table 4 summarizes response rates and preliminary conclusions for protocols 1, 2 and 3. Tumor responses are more frequent after higher administered activities of yttrium-90 ($p<0.05$). Formal tumor dosimetry studies have not been completed.[14,15] This, to our knowledge, is the first time that a positive dose-effect relationship has been demonstrated in a clinical RIT study. The protocol design called for a stable, low protein dose of 2–5 mg of rabbit AF. This increases the

Table 5. Number of Yttrium-90 AF cycles and response of HD (protocols 1 and 2)

Number of cycles	*Patients with response*	*Patients without response*
1	10	14
2	13	7
3	8	1
>3	2	0

1. Cycle length: 8–14 weeks.

2. Some patients have short-lasting response and disease progression prior to 2nd cycle.

3. Patients with stable disease (listed as without response) do not obtain objective response on second or third cycle.

4. Some patients have waning and waxing HD between cycles. Others retain response for over one year (median six months). Patients with a CR after one cycle were retreated once.

specific activity (mCi/mg) for patients receiving higher yttrium-90 activities. In patients who received yttrium-90–labeled AF with specific activities over 15 mCi/mg of protein, lower blood radioactivity, higher % ID urine elimination and shorter T-betas in blood were found. This appeared to be due to increased in vivo radiolysis and uptake of the yttrium-90–labeled AF in normal interstitial tissues due to endothelial damage ("third spacing"). This phenomenon was observed in patients treated with 0.5 mCi yttrium-90/kg. Protocol 1 included 9 patients treated at the 0.3 mCi yttrium-90/kg level and was completed at the 0.4 mCi yttrium-90/kg level after the initial seven patients each treated at the 0.4 and 0.5 mCi yttrium-90/kg level. Protocol 2 (fractionated RIT) showed a lower response rate than protocol 1, although this difference is not statistically significant ($p>0.05$). Less radiolysis and less or similar hematological toxicity were observed in comparison to protocol 1. Protocol 3 (RIT as part of conditioning prior to hematopoietic rescue) is chaired by Dr. B.S. Andersson.[16] In comparison to historic controls, patients treated with CBV only,[17] no increase in toxicity was observed. With relatively short follow-up (median 1 year) approximately 50% of protocol 3 patients appear to be free of disease in comparison to 30% long-term disease-free survival (DFS) for patients treated with CBV alone. Protocol 1 was closed July 1995, protocols 2 and 3 would still accrue more patients.

Combined modality therapy

In a single patient on protocol 1, 30 Gy was given in 2 Gy fractions to bulky abdominal HD immediately preceding indium-111/yttrium-90 AF administration. Tumor targeting was observed only in unirradiated HD. All protocol 3 patients had ifosfamide, etoposide and G-CSF immediately preceding indium-111/yttrium-90 AF (Table 1). The tumor targeting observed in protocol 3 patients was significantly

lower than the tumor targeting observed in protocol 1 and 2 patients. No targeting was observed in 3 patients; they did not receive yttrium-90 labeled AF, one recurred in approximately 6 months, two continue without evidence of disease. G-CSF appeared to open the blood/marrow barrier for indium-111 AF.[12] All protocol 3 patients showed symmetric BM uptake of AF with extension into long bones of upper and lower extremities and sparing of previously irradiated BM areas. In the setting of a hematopoietic rescue protocol (12 days after last yttrium-90 administration) BM uptake on indium-111 scans did not preclude yttrium-90 administration. All protocol 3 patients showed timely engraftment and hematopoietic regeneration.

Repeated RIT cycles

The lack of anti-antibody formation in HD patients allowed for repeat cycles of AF therapy. New cycles were always preceded by indium-111 AF administrations. This precluded the need for anti-antibody determinations in patients' serum and provided a cost effective and sensitive restaging of the patient.

The response rates after the first cycle of yttrium-90 AF are given in Table 4. Table 5 summarizes the distribution of patients with and without a response after different cycles. Some patients with a response after the first cycle were not retreated due to disease progression prior to becoming eligible for a second cycle. Patients without response or progressive disease were not retreated. Patients with stable disease (not counted as response) were retreated. Some continued to experience stable disease, none obtained a PR or CR after new cycles. Patients with a PR after one cycle received repeat cycles (unlimited in protocol 1, limited to 3 in protocol 2). Patients with a CR after one cycle were retreated once, then observed and remained eligible for retreatment if a recurrence was observed. New responses were noted in some such patients after new yttrium-90–labeled AF therapy.

Factors controlling HD response/recurrence after RIT

No responses of HD were observed in 3 patients under 20 years of age. Responses were significantly more frequent in patients over the age of 30 (80 versus 45% response rate). In 5 patients with large (>5 cm) necrotic tumors (as evidenced by "cold" areas on SPECT scans after indium-111 AF) a response after yttrium-90–labeled AF was not obtained. Responses were more frequent in patients with low tumor volumes. After a PR or a CR, recurrences of HD were observed at sites of previous bulky disease (one-third of patients), in previously involved and new areas (another one-third of patients) or only in new areas (the final one-third of responding patients). Patient survival after yttrium-90–labeled AF for protocols 1 and 2 was similar to survival of HD patients studied earlier at Johns Hopkins Oncology Center.[6] Median survival was approximately 8 months with approximately 20% of patients surviving over 2 years.

DISCUSSION

Yttrium-90–labeled AF provides a patient-friendly treatment modality with a high response rate in an extensively pretreated and essentially end-stage patient population with HD. Patients remain outpatients, experience hematological toxicity only (which is familiar to all patients from prior non-RIT therapies) and incur charges of less than $15,000 per cycle. The responses of HD to RIT are due to radiation, not to immunotherapy as ferritin is an interstitial antigen, not a cell membrane antigen. The decrease in HD targeting observed in patients recently treated with chemotherapy or external beam radiation prior to RIT might be explained by a decrease in ferritin secretion by HD cells after therapy. Ferritin deposited earlier in tumor interstitium will be removed over time by catabolism or transportation into the general circulation (patients with HD have increased blood levels of ferritin). The optimal timing of radiolabeled AF treatment appears to be prior to chemotherapy or external beam therapy.

Decreases in the size of HD lesions after yttrium-90–labeled AF appear to be radiation dose related. Higher yttrium-90 activities correlate with higher response rates, smaller tumors respond more frequently and larger tumors with a perfusion problem (necrosis) have lower response rates. Evidently, insufficient radiation is delivered to most tumors, as even in the best responding patients recurrences are noted. One of the problems noted after RIT is recurrence in previously uninvolved areas, comparable to the recurrence problem seen in the early days of external beam radiation for HD, when so-call "involved fields" were used.[18] Presumably, small volume HD present at the time of initiation of RIT is not identified by indium-111–labeled AF staging studies and does not show yttrium-90–labeled AF targeting. Possible explanations for untargeted HD are low ferritin concentrations in small volume HD or the absence of tumor neovasculature in small volume HD. Successful RIT of small volume HD appears to require a new immunoglobulin, reactive with tumor associated antigens present in small volume HD and a radioisotope with a smaller range of energy deposition than yttrium-90.[2,9] Failure in previously involved ("bulky") disease can be solved by radioimmunoconjugates with better tumor penetration (Ig fragments), monoclonal AF or new RIT fractionation regimens. The low response rates observed in large, necrotic HD masses might be improved upon by intralesional AF IgM administration.[19] IgM provides a better vehicle for such therapy than IgG.

The updated results from the clinical radiolabeled AF studies performed at M.D. Anderson indicate that important "mechanistic" information can be obtained from clinical studies of a relatively simple design. This is in strong contrast to phase I studies of chemotherapeutic agents, which are not tumor selective and cannot be imaged or quantified as easily as RIT agents. The rewards of seeing clinically significant responses after RIT in a heavily pretreated HD

patient population are enhanced by the new options available for further improvements in the already high therapeutic ratio of yttrium-90–labeled AF treatment. These options will be explored in further studies at the Arlington Cancer Center in the near future.

REFERENCES

1. Eshbar Z, Order SE, Katz DH: Ferritin, a Hodgkin's disease associated antigen. *Proc Natl Acad Sci USA* 71:3956–3960, 1974.
2. Vriesendorp HM, Quadri SM, Williams JR: Radioimmunoglobulin therapy. In: Armitage JO, Antman KH (eds) High Dose Cancer Therapy. Pharmacology, Hematopoietins, Stem Cells. Baltimore: Williams & Williams, 1992, pp 84–123.
3. Vriesendorp HM, Quadri SM, Stinson RL et al.: Selection of reagents for human radioimmunotherapy. *Int J Radiat Biol Phys* 22:37–46, 1991.
4. Lenhard RE, Order SE, Spunberg JJ et al.: Isotopic immunoglobulin. A new systemic therapy for advanced Hodgkin's disease. *J Clin Oncol* 3:1296–1300, 1985.
5. Vriesendorp HM, Herpst JM, Germack MA et al.: Phase I-II studies of yttrium labeled AF treatment for relapsed Hodgkin's disease, including RTOG 8701. *J Clin Oncol* 918–928, 1991.
6. Herpst JM, Klein JL, Leichner PK et al.: Survival of patients with resistant Hodgkin's disease after polyclonal yttrium-90 labeled AF treatment. *J Clin Oncol* 13:2394–2400, 1995.
7. Bierman PJ, Vose JM, Leichner et al.: Yttrium-90 labeled AF followed by high-dose chemotherapy and autologous bone marrow transplantation for poor prognosis Hodgkin's disease. *J Clin Oncol* 11:698–703, 1993.
8. Vriesendorp HM, Morton JD, Quadri SM: A review of five consecutive studies of radiolabeled immunoglobulin therapy in Hodgkin's disease. *Cancer Res* 55:5888s–5892s, 1995.
9. Vriesendorp HM, Quadri SM, Jaeckle KA et al.: Proposal for translational analysis and development of clinical radiolabeled immunoglobulin therapy. *Radiother Oncol* (in press, 1996)
10. Quadri SM, Vriesendorp HM, Leichner PK: Evaluation of indium-111 and yttrium-90 labeled linker immunoconjugates in nude mice and dogs. *J Nucl Med* 34:938–945, 1993.
11. Bhadkamlkr VA, Vriesendorp HM, Quadri SM et al.: Imaging of patients with Hodgkin's disease after yttrium-90 labeled AF administration. *J Immunother* 16:170, 1994 [abstr 90].
12. Vriesendorp HM, Quadri SM, Andersson BS, Dicke KA: Hematologic side effects of radiolabeled immunoglobulin therapy. *Exp Hematol* 24:1183–1190, 1996.
13. Wang S, Quadri SM, Tang XZ et al.: Liver toxicity induced by combined external beam irradiation and radioimmunoglobulin therapy. *Rad Res* 141:294–302, 1995.
14. Leichner PK, Vriesendorp HM, Hawkins WG et al.: Quantitative SPECT for indium-111 labeled antibodies in the liver of beagle dogs. *J Nucl Med* 32:1442–1444, 1992.
15. Leichner PK, Kwok CS: Tumor dosimetry in radioimmunotherapy: Methods of calcu-

lation for beta particles. *Med Phys* 29:529–534, 1993.

16. Andersson BS, Vriesendorp HM, Quadri SM et al.: The transplant conditioning therapy with yttrium-90 labeled AF (Y^{90}-AF) and high dose chemotherapy is well tolerated in patients with Hodgkin's disease-W36. In: Monoclonal Antibodies and Cancer Therapy: The Next Decade; Symposium Cancer Research Institute, New York, New York, 1995.
17. Jagannath S, Dicke KA, Armitage JO et al.: High dose cyclophosphamide, carmustine and etoposide and autologous bone marrow transplantation for relapsed Hodgkin's disease. *Ann Intern Med* 104:163–168, 1986.
18. Kaplan HS: Hodgkin's disease, Ed. 2. Cambridge, MA: Harvard University Press, 1980.
19. Quadri SM, Borchardt PE, Ali MS et al.: Intralesional administration of radiolabeled immunoglobulin for human solid tumors. In: Dicke KA, Keating A (eds) Autologous Marrow and Blood Transplantation: Proceedings of the Eighth International Symposium. Arlington, Texas, 1997.

CYTOMEGALOVIRUS

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Cytomegalovirus (CMV) infection remains a considerable cause of morbidity and mortality in marrow transplantation. CMV reactivation is expected to occur in approximately 70% of seropositive patients undergoing allogeneic marrow transplant. Approximately 30% of patients undergoing high-dose chemotherapy and autologous marrow transplant will have reactivation of CMV. Primary infection occurs in 20 to 50% of seronegative allogeneic transplant recipients. The use of CMV seronegative blood products and/or leukocyte depletion have tremendously decreased the risk of primary infection.

There are several risk factors associated with progression of CMV infection to CMV disease. CMV infection is defined as asymptomatic viral excretion detected by cultures from urine, throat, blood or bronchoalveolar lavage (BAL). CMV disease is defined as the presence of constitutional symptoms: generalized malaise, fevers, myalgias, with or without organ affectation (pneumonia, hepatitis, cystitis, retinitis and gastroenteritis). Lymphocytosis, neutropenia, thrombocytopenia or graft failure can be manifestations of hematological involvement of CMV. The degree of immunosuppression seems to be the main predictive factor of the development of serious CMV infection. CMV infection will progress into CMV disease particularly in patients receiving immunosuppressants.

The availability of the anti-viral agents ganciclovir and foscarnet has allowed major progress to be made in the treatment of CMV disease. Before the availability of these agents, CMV pneumonia had a mortality rate of approximately 80–90%. Treatment of CMV pneumonia with ganciclovir (plus intravenous gamma globulins) results in cure in approximately 50–70% of patients. Even though significant advances in the treatment of CMV disease have been accomplished with the availability of these agents, the best treatment or prevention remains to be determined.

Most of our efforts in marrow transplantation have been in the early detection of CMV infection through surveillance cultures. Throat, urine, blood and bronchoalveolar lavage fluids have been routinely performed at timely intervals, usually on a weekly basis, to detect early infections and therefore treat patients earlier before organ damage occurs. The detection of the virus in the blood (viremia) has a predictive value for progression or development of CMV disease of 60%. Predictive values for viruria and positive throat cultures for disease

Table 1. Early treatment

		Development of CMV disease		
ganciclovir	5/20	(25%)*	1/37	(3%)**
placebo	14/20	(70%)*	15/35	(43%)**

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***NEJM 325:1601, 1991.*

development are 40 and 41%, respectively. Of more clinical importance is that 40 to 50% of patients who develop CMV pneumonia may present without antecedent positive cultures, either in blood or urine. For this reason, it is clearly necessary to have a more sensitive test to detect early CMV infection. Two tests have been recently introduced into the clinical practice: CMV antigenemia and CMV DNAemia. The CMV antigenemia test is a sensitive test based on the identification of viral antigens on the surface of leukocytes even before the virus has entered into a replicative state. These viral antigens expressed on the cell surface of infected cells are amenable to detection using monoclonal antibodies in an immunofluorescence assay. The test has a high predictive value of 70 to 75% for the development of CMV disease, and enables earlier detection of viral infection (7 to 10 days earlier) compared with conventional viral cultures.

DNA from CMV can be detected by polymerase chain reaction (PCR) techniques (DNAemia test). This test will provide the earliest indication of CMV infection and becomes positive 4 to 8 days earlier than the antigenemia test, or 12 to 14 days earlier than conventional cultures. Because it is very sensitive, it will be positive in a substantial number of patients not at risk for progression to CMV disease (therefore, it has a lower predictive value). However, because of high sensitivity it may prove very useful in determining when to stop treatment in a particular patient.

For the most part, prevention of CMV infection results in improved outcome after bone marrow transplant. Seronegative recipients undergoing marrow transplantation should receive CMV negative or leukocyte-depleted blood products. Seropositive patients should receive either early treatment or early prophylaxis. Early treatment may consist of treatment when the PCR first becomes positive at the first sign of excretion of the virus from urine, blood, throat or any other fluids. The effectiveness of early treatment has been well documented in two randomized studies (Table 1).

One study performed surveillance viral cultures from urine, blood and throat on a weekly basis. In the other study, bronchoalveolar lavage was performed on day 28–35 after transplant. The patients were randomized to receive either ganciclovir or placebo at the time of positive cultures. Both studies revealed a significant reduction in CMV disease when early treatment with IV ganciclovir was given.

Table 2. Early prophylaxis

CMV infection				
ganciclovir:	1/33	(3%)*	8/40	(20%)**
placebo:	14/31	(45%)	25/45	(56%)
CMV disease				
ganciclovir:	0/33*		4/40	(10%)**
placebo:	9/31	(29%)	11/45	(24%)

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***Ann Int Med 118:179, 1993.*

However, significant neutropenia due to ganciclovir was seen in 30 and 35% of patients, respectively. In 12 and 13% of patients registered in the two studies, respectively, CMV disease occurred without antecedent excretion of the virus.

Some drawbacks of "early treatment" include:

1. High incidence of neutropenia
2. Significant occurrence of overt CMV disease without antecedent positive surveillance cultures.

Early prophylaxis is an alternative approach to early treatment. Antiviral agents are given at the time of engraftment, before any evidence of CMV infection. Two randomized trials compared the efficacy of "early prophylaxis" with intravenous ganciclovir (Table 2).

In both studies, the incidence of CMV infection and CMV disease was significantly reduced in the group receiving ganciclovir. A problem with early prophylaxis is that a substantial number of patients not at risk receive treatment. This number varies from 40 to 65%. In addition, 30 and 58% of patients developed significant neutropenia. Up to 30% of patients developed a bacterial sepsis that was directly related to ganciclovir-induced neutropenia. No difference in survival was noted in these studies, perhaps due to the high incidence of serious complications related to drug-induced neutropenia. Early treatment or early prophylaxis is effective in reducing the incidence of CMV infection and/or CMV disease, but both approaches have numerous disadvantages.

A trial has been initiated utilizing oral ganciclovir. In this trial, early prophylaxis with oral ganciclovir is compared with placebo in a double-blind randomized fashion. The oral ganciclovir will produce blood levels that are in the range of the IC50 of most of the CMV isolates. However, since blood levels achieved with the oral agent are considerably lower than the IV doses used in prior studies, a lower frequency of neutropenia is expected. In this new trial, early treatment will be initiated if the patient develops a positive CMV antigenemia test (in contrast to earlier trials in which treatment was based on results of conventional cultures). The potential benefit is that early prophylaxis may be provided without a high incidence

of neutropenia. In addition, by following patients with the CMV antigenemia test, we hope to initiate intravenous ganciclovir therapy earlier. This approach may result in a reduction in CMV infection and disease while minimizing toxicity.

Oral ganciclovir has been used in maintenance treatment for CMV retinitis in AIDS patients. No significant difference in retinitis recurrence was noted after oral versus IV maintenance treatment. At our institution, oral ganciclovir has been used in a small group of patients. Most have chronic graft-versus-host disease that requires the prolonged use of immunosuppressants and therefore are susceptible to recurrent CMV infections. While the availability of oral ganciclovir may improve the care of these patients, trials evaluating the efficacy of oral ganciclovir in this clinical setting are needed.

In summary, the treatment and prevention of CMV has improved the outcome of patients undergoing allogeneic marrow transplantation. We still need to identify the best prophylactic regimen and determine the most appropriate way of using intravenous and oral ganciclovir.

CHAPTER 10
New Avenues

AUTOLOGOUS STEM CELL THERAPY FOR TREATMENT OF AUTOIMMUNE DISEASES

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INTRODUCTION

Being honored at this meeting with the first CMOMC award gives me the privilege to dwell on certain aspects of my work that are not strictly of scientific interest. My motivation for applying bone marrow transplantation (BMT) to autoimmune diseases (AID) did not spring from my usual inclination to cross traditional borders between disciplines, but was forced upon me by my paymasters. It began about 10 years ago when the Ministry of Health, which traditionally provided substantial structural funding to the Radiobiological Institute, announced that part of this money would have to be spent on applied research of chronic diseases and, furthermore, that autoimmune diseases, in particular rheumatoid arthritis, should be a priority. The research program of the Institute was at that time devoted to radiation protection, including large projects on carcinogenesis, on radiation therapy with cancer as the outstanding issue and on hemopoiesis with stem cells and BMT as its leading motive. All of these programs had been well established during the 25 years of the Institute's existence, so that the political demand for a shift towards an area of research in which we had neither experience nor reputation was received with very mixed feelings. This reluctance was also based on the concern that competing for grants by newcomers in a field of investigation is always very difficult. Accordingly, the enthusiasm among the staff to develop this new program or even start the process of thinking about it was minimal. Moreover, the financial situation did not allow recruitment of new staff familiar with arthritis research. Thus, it was left to the director to prepare the grounds for the new enterprise with his small group of one research assistant, a technician and the occasional graduate student.

The Dutch Health Ministry made no exception to the universal strong preference of government agencies for prevention as opposed to treatment. Our survey of the literature showed that most AID in humans have genetic components that influence susceptibility to unknown environmental initiating factors. Among those factors, likely candidates are bacteria present in the intestinal microflora carrying antigens that mimic or cross-react with autoantigens present on cells of the target tissue of AID. Such a mechanism is

apparently operative in several animal models of AID. AID-prone mice from the same stock were found to exhibit a strikingly different incidence when bred in different laboratories, and germ-free mice have a different incidence compared with conventional mice kept in the same place.[1] Among the facilities of the Institute were a large colony of gnotobiotic and germ-free rodents for ongoing research on graft-versus-host disease (GVHD) and the production of disease-free animals for radiation research, as well as adequate bacteriological support for this operation. Considering our long-standing experience with this technology, it seemed most opportune to exploit these resources for starting a program on the role of microflora in the development of AID, with the ultimate objective of developing prevention via modification of the microflora. Obviously, this would have been a long-term effort requiring stable funding, but that prospect did not appeal to the authorities, who were only interested in fast results. We therefore began to compare the various animal models of arthritis in rats and mice in order to identify the best model for intervention studies.

The fascinating therapeutic results of autologous BMT (autoBMT) and the ongoing implementation of those findings in the clinic show that commissioned research can have its rewards. Following our exciting findings in the arthritis model, we made numerous attempts to get funding for similar studies in other autoimmune diseases, from disease-oriented charity foundations, since by that time I had retired from my position at the Radiobiological Institute. All these agencies (including the one for rheumatoid arthritis research) rejected our applications as being too far-fetched, except the Dutch Foundation, "Friend of MS research," which has generously funded all our work with the experimental allergic encephalitis (EAE) model.

Selection of an animal model of rheumatoid arthritis

A large variety of antigens and immunization schedules was known to induce various forms of inflammatory arthritis in susceptible experimental animals. Among the antigens are Complete Freund Adjuvant (CFA), collagens and streptococcal cell wall preparations.

The most widely studied are the Lewis rat and CFA, but in our hands, as in most other laboratories, immunization with adjuvant induced a typically acute form of the disease resulting in resistance after remission. We then studied a variety of mouse, rat and guinea pig strains using adjuvant and collagen as sensitizing agents and compared the clinical picture as well as the histopathology of the lesions. Our conclusion from this extensive exploration was that adjuvant arthritis (AA) in the Buffalo (BUF) rat strain provided the best resemblance to rheumatoid arthritis in humans. In the BUF rat, a single intracutaneous inoculation of Mycobacterium tuberculosis in Incomplete Freund Adjuvant induces within 3–4 weeks a chronic progressive type of polyarthritis

in over 80% of the animals.[2] The inflammation involves chiefly the distal extremities, as a chronic proliferative synovitis with pannus formation, destruction of cartilage and subchondral bone, vasculitis, pericapsular fibrosis and extensive reactive bone formation. In some animals the inflammation recedes after 10 weeks at the earliest, in others progressive inflammation continues for as long as 30 weeks, by which time the experiments were usually terminated. Clinically, there is swelling of the affected joints, which is measured with calipers and expressed as the arthritic score: the sum of the increase in thickness of the 4 paws compared with preimmunization values. During the progressive chronic stage the inflamed joints are reddish and painful. Following extinction of the inflammation, either spontaneously or as a result of treatment, the osseous deformities persist. Treatment of rats with fully developed arthritis with cyclosporin A causes partial regression but the disease progresses again after discontinuation of the drug.

Is AID a disease of the CMOMC?[1]

([1]Cells meeting our morphological criteria [of hemopoietic stem cells][3])

Many, if not all, of the autoimmune diseases are T cell initiated or mediated, and the presence of autoantibodies in the serum of the affected subjects is now considered to be an associated- or epiphenomenon. The activation of T lymphocytes against self-antigens may be induced by the release of an excessive amount of tissue-specific antigens to which the organism has not developed tolerance during development, as in the case of sympathetic ophthalmia. For the majority of the human AID, however, the inducing agent is unknown. Epidemiological research has not been able to identify specific triggers, suggesting perhaps that exposure to such antigens is universal and that affected individuals have an unusually high susceptibility, a predisposition that is genetically determined. Numerous reports describe the linkage of certain MHC genes of both the HLA and the D/DR regions with specific AID, but the linkages are far from absolute.[4] Also, concordance in monozygotic twins is limited: in multiple sclerosis (MS) it is 20–30%, and in RA 11%,[5] indicating that both genetic and environmental factors are involved.

The animal models of AID are of two distinct categories: the hereditary and the induced forms. In the first category the disease develops spontaneously in a large proportion, or in all of the individuals of a particular inbred strain of mice or rats. The induced AID develop following immunization with certain antigens in certain inbred strains—the susceptible ones—and not in others, the resistant ones. The best described models are AA and EAE. The latter is currently considered to be the best model for human multiple sclerosis (MS). Susceptibility and resistance are genetically determined as demonstrated by cross breeding. The induced models for AID are obviously best suited for the experimental approach to human diseases because of their similar dual etiology.

Table 1. Transfer (T) and prevention (P) of hereditary AID by alloBMT

Disease	*AI strains*	*Non-AI strains*	*T and/or P*	*Ref.*
SLE-like syndromes (mice)	NZB, (NZB×SJL/J)F1 (NZB×C57BL/6)F1	BALB/c, SJL/J, C57BL/6	T	7
	NZB	BALB/c, B10.D2,C57BL/Ks,DBA/2	T/P[1]	8,9,10
	MRL/1pr	C57BL/6J	P	11
	(NZW×BXSB)F1	C3H/HeN,C57BL/J	T	12
insulin-dependent diabetes mellitus (mice)	NOD	NON,(NODxNON)F1	T	13
	NOD	(NODxB10)F1	T	14
	NOD	BALB/c *nu/nu*	P	15
	NOD	C57BL/6, B10.BR/cd	T/P	16
(rats)	BB	BB diabetes-resistant subline	P	17
	BB	WF	P	18
"moth-eaten" syndrome (mice)	me/me	+/+	T	19
skin fibrosis (mice)	tsk	+/+	T	20

Having proposed this, we may turn our attention to the immune system, which is instrumental in causing and maintaining the pathology of AID. Failure to abstain from or limit self-destruction may be regarded as a defect either of restrictive immune mechanisms or of suppression. The next question is whether this occurs at the level of the lymphoid cell population; for example, a dysregulation resulting from an abnormal formation—too much or not enough—of subpopulations that could be brought about by an unusual sequence or strength of stimuli.[52] Examples are the diverse autoimmune syndrome that has been described in neonatally thymectomized mice[6] and the AID that occurs in rodents following treatment with cyclosporin A.

The alternative hypothesis assumes a defect in the hemopoietic stem cell (HSC), which is expressed in certain lymphoid cells. The latter hypothesis was inspired by experiments demonstrating the transfer of hereditary AID to lethally irradiated animals from normal strains by BM grafts from the affected strain and vice versa (Table 1).

Table 2. Transfer (T) and prevention (P) of induced AID by alloBMT

AID	*AI strains*	*Non-AI strains*	*T and/or P*	*Ref.*
adjuvant arthritis				
(rats)	Lewis	F344	T/P	21
(rats)	BUF (AA)	WAG	T/P	30
streptococcal cell wall arthritis				
(rats)	Lewis	F344	T/P	21
collagen-induced arthritis				
(rats)	WAG(CIA)	BUF	T	22
(rats)	DBA/1 (CIA)	SWR	conflicting results	23
encephalomyelitis (EAE)				
(rats)	Lewis	BN	T/P[3]	24
(guinea pigs)	strain 13, (2x13)F1	strain 2	T/P	25
(rats)	Lewis	Le-R	T/P	26
(mice)	SJL/J	Bl0.S	conflicting results	27,28
(mice)	SJL/J	Bl0.S	T/P	29
(rats)	BUF	WAG	T/P[3]	22
(rats)	BUF	BN.1B	T/P	31
	(BUF, Lewis , SJL/J)	C.B.-17-scid/scid	T	32

Such results led Ikehara[15] to propose that AID is due to a defect of the HSC—in other words, a stem cell disease. However, this conclusion is not wholly justified. Many of the AID in animals can also be transferred by mature lymphocytes. As long as similar transfers have not been performed with highly purified stem cells, it seems more appropriate to use the term stem cell-associated diseases in the case of the hereditary models.

In regard to the indelible models, experiments have been performed with radiation chimeras of resistant recipients and susceptible donors and vice versa. (Tables 2, 3 and 4). In the case of AA (various rat strain combinations) and EAE (rat, mouse and guinea pigs), the responses were determined by the BM donor strain, in other words, both the susceptibility and the resistance could be transferred with the BM. In the case of collagen-induced arthritis (CIA) the susceptibility was transferred from WAG rats to resistant BUF rats, but the results of an experiment with mice were conflicting, presumably because the resistant strain was C5 deficient. Interestingly, susceptibility to induction of EAE has been transferred to SCID mice with BM grafts from

Table 3. Transfer of susceptibility and resistance to EAE by T cell depleted BM[1]

Strain or BM chimera (donor→recipient)	*Incidence of clinical EAE*	*Incidence of histological lesions in CNS*	*% activated T ly of WAG haplotype in CNS*
BUF	12/12	6/6	0 (3×)
BUF→BUF 7/7		ND	ND
BUF→WAG	9/9	8/8	1,4
WAG	0/10	1/9	
WAG→BUF	2/11	4/10	82, 88, 100
WAG→WAG	0/7	0/3	

[1]*Adapted from reference 22.*

Table 4. Transfer and resistance to AA by BMT[1]

Strain or BM chimera (donor→recipient)	*incidence of arthritis*	*(%)*
BUF	130/192	(67)
BUF→BUF	7/10	(70)
BUF→WAG	3/6	(50)
WAG	0/20	
WAG→WAG	0/5	
WAG→BUF	0/14	

[1]*D.W. van Bekkum (30).*

susceptible mouse or rat strains. These results with inducible models—which have the most resemblance to clinical AID—do not argue a priori against the use of autoBMTs for treating patients with severe AID, as will be demonstrated below.

Treatment of fully developed AID with BMT

In view of the transfer data described in the previous section it appeared logical to attempt to cure full-blown AID by eradication of the lymphohemopoietic system with high-dose ablative treatment (analogous to treatment of leukemia to be designated as conditioning) followed by rescue with allogeneic BM from a resistant strain donor. Such treatment was first explored by Ikehara and Good in hereditary AI models with remarkable success (Table 5). When the disease had already developed in BXSB mice (SLE-like disease with lymphoproliferation and glomerulonephritis) complete remissions were obtained with

Table 5. Treatment of fully developed experimental AID with alloBMT

AI strain	*Normal donor strain (conditioning)*	*Effect*	*Ref.*
Hereditary AID			
B/W, BXSB	BALB/c nu/nu (TBI)	lasting reduction of glomerular damage and deposits of IgG and C, as well as reduction in circulating immune complexes	33
MRL/lpr	C57BL/6 (TBI)	complete and lasting amelioration of glomerulonephritis, arthritis and correction of immunological abnormalities	34
Induced AID			
AA (BUF)	WAG(TBI)	complete remission of severe arthritis	35
EAE(BUF)	WAG, BN.1B	remission-induction, prevention of spontaneous and induced	31
	(TBI or CY+BU)	relapse in 90% of the animals	36
CIA (DBA/1J)	BALB/c (TBI)	no remission-induction, but complete prevention of progression	37

Abbreviations: AI, autoimmune; AA, adjuvant-induced arthritis; BU, busulfan; C, complement factor; CIA, collagen-induced arthritis; CY, cyclophosphamide; EAE, experimental autoimmune encephalomyelitis; TBI, total body irradiation.

transplants of allogeneic marrow lasting for as long as 1 year after transplantation.[33] In similarly treated mice of the MLR/lpr strain (another model of SLE) the disease recurred after an initial remission and the relapses were associated with a reversal of the chimeric state.[34]

We began similar experiments in 1988 with adjuvant-induced arthritis in rats using lethal total-body irradiation (TBI) and allogeneic BM from a resistant strain and obtained complete and lasting remission in all animals. In control experiments we employed syngeneic BMT, expecting that the disease would relapse. However, this was not the case. Much to our surprise syngeneic BM was just as effective as allogeneic BM. Apparently, the complete remissions were followed by a state of tolerance to the autoimmune antigens which may or may not persist beyond the moment of treatment, 4–5 weeks after the primary induction. When the rats treated with syngeneic BM were reimmunized at 24 hours and at 28 days following the BMT, relapses did not occur in any of the animals.[35]

Table 6. Results of treatment of fully developed experimental induced AID with syngeneic or (pseudo-) autoBMT

AID (strain)	*BM origin (conditioning)*	*Effect*	*Ref.*
EAMG (Lewis)	pseudoautologous (CY + TBI)	reduction of a-AChR titer by CY, elimination of memory response by TBI; reimmunization induces a new primary response	39
AA (BUF)	autologous and pseudoautologous (TBI)	complete and lasting remission; no regression of excessive bone formation when treated at late stage of the disease	38
CIA (DBA/1)	syngeneic (TBI)	no remission-induction, but complete prevention of progression	37
EAE (SJL/J)	syngeneic (CY)	remission-induction, only 7% spontaneous relapses; 25% induced relapses (versus 100% in controls)	40
EAE (BUF)	syngeneic and pseudoautologous (TBI,CY/ALS + TBI)	remission-induction, 30% spontaneous relapses (conditioning-dose dependent); 45% induced relapses in syn BMT, 72% in psa BMT	41 42

Abbreviations: AA, adjuvant-induced arthritis; a-AChR, anti-acetyl choline receptor antibodies; CIA, collagen-induced arthritis; CY, cyclophosphamide; EAE, experimental autoimmune encephalomyelitis; EAMG, experimental autoimmune myasthenia gravis; TBI, total body irradiation; syn, syngeneic; psa, pseudoautologous.

These findings made us investigate the effects of autoBMT following the same conditioning regimen.[38] The BM was harvested from the femurs of arthritic rats by a surgical procedure, followed by TBI and intravenous return of their BM after the TBI. This resulted in complete and lasting remission, similar to the results obtained with allogeneic or syngeneic BM.

For subsequent studies on the curative effects of autologous marrow transplants, both in the arthritis model and in EA, we have always used the pooled marrow harvested from syngeneic rats that suffered from the same fully developed AID as the animals to be treated. This procedure was adopted to avoid unnecessary suffering of the sick animals (polyarticular inflammation or in the case of EAE paresis and paralysis) from the surgical procedure of BM harvesting. For each experiment about 100 rats were immunized and—4 or 5

weeks later in the case of AA, 3 weeks later in the case of EAE—each animal was scored using a grading scale for the clinical symptoms and the animals were distributed over the various experimental groups and the donor group of rats, assuring that the average score of all groups was similar. Animals without symptoms (10–20%) were always excluded. The donor marrow obtained in this way is designated as pseudoautologous; its composition is identical to that of autologous BM.

The EAE rat model

Similar experiments and procedures to those used in the AA model were subsequently performed with M. van Gelder in the EAE model, which was also developed in BUF rats. In contrast to the widely employed Lewis rat, which in our hands developed only an acute and time-limited encephalitis, the BUF rat reacts to immunization with spinal cord tissue in Complete Adjuvant with relapsing neurological symptoms, accompanied by characteristic inflammatory lesions throughout the CNS. These can be found even before the appearance of clinical symptoms. With the immunization mixture of 6–12 mg of spinal cord in Complete Adjuvant supplemented with 0.14–0.28 mg of M. tuberculosis strain H37 RA, clinical paralysis and paresis appear from day 11 onwards. By day 20, when treatment was given, between 70 and 100% of the rats had-full blown EAE. If untreated, the neurological defects continue in some or gradually recede in others, to be followed by one or more spontaneous relapses in 70% after the first attack. About 10% of the animals die during this period. Between day 40 and 90 most of the rats have entered a stable remission and when reimmunized during that period, 85% respond with a relapse, a rate similar to the incidence of EAE following primary immunization. The onset of these induced relapses is between 3 and 11 days after immunization, compared with day 11 onwards following the primary immunization.

Therefore, the induced relapses are probably similar to secondary immune responses following sensitization, which are ascribed to activation of memory cells. The effects of treatment with BMT include the registration of spontaneous and induced relapses, although we cannot interpret their relevance for relapses in MS, because the immunological mechanism underlying the latter is unknown.

We shall discuss the results of autoBMT in AA and EAE in BUF rats together since the treatment methods were basically similar, so that differences in responses between the models may well be specific for each disease.

RESULTS OF AUTOBMT

Few results have been published so far on the treatment of full-blown AID with syngeneic BMT. Our group and that of Pestronk[39] are the only ones having

explored the use of autoBMT (Table 6). The latter results will be reviewed here, as autoBMT will be in most cases the best option for clinical application of this treatment modality.

In both diseases, high-dose TBI (8–10 Gy) followed by pseudoautologous (PSA) BMT causes rapid and complete remission in all animals. Spontaneous relapses were not seen in AA, but occurred in 30% on average in EAE (Table 7). Following treatment with allogeneic BMT (alloBMT) from a resistant strain, the incidence of spontaneous relapses was only 5%.[36] The higher incidence after PSA BMT is not due to T lymphocytes in the grafted marrow because a similar spontaneous relapse rate is observed with syngeneic BMT derived from normal rats of the same strain. This conclusion is borne out by the observation that T cell depletion of both PSA and syngeneic grafts does not prevent or diminish the spontaneous relapse rate. Spontaneous relapses following PSA BMT are therefore attributed to residual T lymphocytes that have survived the conditioning. Analogous to leukemia, we use the term "minimal residual autoimmune disease." Such cells capable of initiating a spontaneous relapse are probably activated T cells. The notion that residual T cells are operative in spontaneous relapses is supported by the increased relapse rate following less intensive conditioning and a decreased relapse rate following conditioning with high-dose TBI and antilymphocytic antibody. The near absence of spontaneous relapses following alloBMT can at this time only be explained by assuming a reduction of residual host lymphocytes by a subclinical graft-verus-host reaction (analogous to the term graft-versus-leukemia reaction, this putative phenomenon was named graft-versus-autoimmunity by A.M. Marmont).

In AA rats, less intensive conditioning—such as high-dose cyclophosphamide—does not result in spontaneous relapses but in less complete remission, which reflects the chronic progressive nature of AA. This also points to the necessity of extensive eradication of the lymphocyte population for obtaining complete remission, which is supported further by the recurrences that occur in patients with AID who have been treated in the past years with repeated medium high doses of cyclophosphamide.[43,44]

The absence of a contribution to spontaneous relapse incidence by autologous grafts in our experiments does not infer a similar situation in humans. Rat BM contains about ten times fewer T lymphocytes than human BM. Furthermore, the number of activated T cells that may cause relapses may be fewer in the human autoimmune diseases; after all, animal models are not complete images of human disease. Recently, relapses were reported in 5 patients suffering from diverse connective tissue autoimmune disorders who were treated with unmanipulated autologous BM or mobilized peripheral blood cells,[45] loosely called "blood stem cells" or "peripheral blood progenitor cells," and the one relapse out of 15 patients with the progressive form of MS treated

by Fassas et al.[46] with autologous blood cells, who had not received antilymphocyte antibodies (ALS) after the transplant (which is effective in depleting the graft of T cells in vivo).

T cell depletion of the graft is essential in autoBMT for autoimmune disease, as is tumor cell depletion in autoBMT for malignant disease. A patient with active AID is to be compared with a leukemic patient in relapse. In the latter case nobody will seriously consider transplanting autologous BM without an effective leukemic cell depletion. We showed that addition of PSA spleen cells to the BMT increases the spontaneous relapses in EAE from 30–40% in rats treated with BM only, to 93%.[42] In view of the uncertainties mentioned above, our recommendation for the clinic is to go for at least 3 log of T cell depletion for autologous BM grafts and for 4 log when using autologous mobilized peripheral blood progenitor cells. An even better guideline is to set a maximum at 10^5 T cells/kg body weight for both types of autologous grafts in accordance with the recommendations formulated at the International Meeting on Hemopoietic Stem Cell Therapy in Autoimmune Diseases at Basel, September 1996.

As regards the induced relapses, the responses after reimmunization differ in AA and EAE. In the nontreated AA controls, the reimmunization was performed 15 weeks after the primary immunization. At that time a varying proportion of the animals still suffer from severe stable or progressive inflammatory arthritis; in others the inflammation has remitted, but the osseous malformation does not regress. Reimmunization does not influence the course of the disease. We did not investigate the effects of reimmunization in AA rats treated with alloBMT because alloBMT is not a realistic option for the treatment of patients suffering from severe connective tissue autoimmune diseases. This stems from the fact that a proportion of the patients may develop chronic GVHD, the symptoms of which are hard to distinguish from the primary diseases; also, the transplantation associated risks of alloBMT are still higher than those associated with autoBMT.

Reimmunization of the treated animals was performed at 11 weeks after PSA BMT, that is 15 weeks after the primary immunization. When optimal conditioning schedules had been employed, a few (11%) mild relapses were recorded (Table 7). This low incidence precluded the design of experiments which can distinguish between minimal residual autoimmune disease and grafted T cells as the effector mechanism of these relapses. Following suboptimal conditioning, which resulted in partial remissions, reimmunization caused accelerated disease progression in 50% of the animals. Here, the obvious cause is inadequate elimination of host lymphocytes, but a certain contribution by grafted T lymphocytes cannot be excluded.

Reimmunization of rats with relapsing EAE was performed when most of the non-treated animals had recovered from their last spontaneous relapse (day 40

onwards). As described earlier, it induces a high incidence of new relapses; in most cases, probably by activation of memory T lymphocytes. Interestingly, we observed 11% induced relapses following alloBMT from resistant rats. The cause of these relapses is likely to reside in a small number of residual host type lymphocytes, the presence of which cannot be excluded even in so-called complete chimeras. EAE rats treated with PSA BMT developed a high incidence (72%) of induced relapses, that is significantly more than following syngeneic BMT (44%), suggesting that both residual T cells of the host and grafted T cells contribute to induced relapses after PSA BMT.

In both models we investigated various conditioning schedules. High-dose TBI-9G in AA, 10 Gy in EAE was the most effective conditioning regimen, equaled by a combination of cyclophosphamide (2×60 mg/kg) and a sublethal dose of TBI: 4 Gy or 7 Gy in AA and 7 Gy in EAE. High-dose cyclophosphamide as the sole conditioning agent (2×60, 2×80 or a single dose of 120 mg/kg) was tested only in AA. These regimens resulted in partial remissions only. In AA, fractionated TBI (total dose adjusted for the fractionation effect) was as effective as a single TBI. For reasons explained below, fractionated TBI was not investigated in EAE rats.

In EAE we found a combination of 2×60 mg/kg cyclophosphamide followed by a single dose of 30 mg/kg of busulfan only marginally less effective than high-dose TBI in terms of incidence of spontaneous relapses.[31] As mentioned before, it is essential to employ agents for conditioning that effectively eradicate both activated T lymphocytes and memory T lymphocytes in the lymphatic and hematopoietic tissues as well as those in the sites of inflammation. In both AA and EAE, irradiating the affected tissues (legs or CNS respectively) only, or shielding those parts of the body while irradiating the rest proved to be ineffective.[35,38,41]

In rats with EAE, irradiation of the CNS only or with TBI induces an acute exacerbation of the clinical symptoms within a few hours after irradiation, with a maximum at 24 hours, after which the condition of the rats returns to pre-irradiation levels within a few days. This complication occurs even after a dose as low as 1.5 Gy. We have seen an occasional fatal outcome of such exacerbations. Conditioning with cyclophosphamide + busulfan did not induce this side effect.

The aggravation is probably related to the release of cytokines[47] or enhanced edema due to increased blood brain barrier permeability.[48] This adverse effect of irradiation did not occur in mice,[49] nor in MS patients following irradiation of the brain and spinal cord with 8–12 daily fractions 1.5 Gy.[50] However, these patients had not deteriorated in the previous six months, which may imply a less active disease compared with those with EAE. In the clinical study, radiation-induced damage to the blood–brain barrier was demonstrated, which was

Table 7. Incidence of relapses after treatment of experimental AID with BMT in rats

	Remission induction (%)	*Spontaneous relapses (%)*	*Relapses after reimmunization (%)*	*Ref.*
AA (TBI 9 Gy)				
autologous	100	0	11	30
alloBMT	100	0	not done	
EAE (TBI 10 Gy)				
pseudoautoBMT	100	30	72	42
alloBMT	100	5	11	36
EAMG (Cyclo + TBI 6 Gy)				
pseudoautoBMT	100	NA	11	39

Abbreviations: AA, adjuvant arthritis; EAE, experimental allergic encephalitis; EAMG, experimental autoimmune myasthenia gravis.

completely prevented by ACTH and corticosteroid therapy. Unfortunately, we could not determine whether corticosteroids protect against the exacerbations, because paretic BUF rats also responded adversely to this medication. Support for a risk of TBI in MS patients is in a recent case report of two patients in whom quiescent inflammatory demyelinating polyneuropathy exacerbated during conditioning with TBI, which contributed to their death 48 and 175 days after BMT.[51] Although the pathogenesis of this disease may be completely different from that of MS, it argues against the use of irradiation in the treatment of demyelinating diseases like MS. Until data to the contrary become available, the use of TBI for conditioning of MS patients should be discouraged.

CONCLUSIONS AND RECOMMENDATIONS FOR CLINICAL APPLICATION

Treatment with BMT following myelo-lympho ablative conditioning proved to be highly effective in both AA and EAE, justifying prudent exploration of this modality for treating selected patients with severe progressive AID. This new approach finds encouragement in a few case reports of long lasting remissions of various AID in patients undergoing BMT for malignancy or aplastic anemia.[4] Candidates should be in the inflammatory phase, as our models showed that extensive scar lesions do not respond. Because of its universal availability and lower morbidity and mortality, the use of autologous BM is to be preferred over HLA-identical sibling marrow. An exception might perhaps be made for MS patients in view of the much lower occurrence of relapses with alloBMT in the EAE model. Conditioning should be as intensive as safely tolerated to minimize residual T lymphocytes in the inflammatory lesions as well as elsewhere in the

body. For connective tissue AID, a combination of cyclophosphamide and a sublethal dose of TBI (4 or 5 Gy) seems to fulfill that condition, perhaps to be supplemented by ALS or another specific lymphocytotoxic agent. As radiation had been found to be a potential hazard in the EAE model, it should not be used for conditioning of MS patients. In this disease, busulfan and cyclophosphamide, or high-dose cyclophosphamide, or the BEAM regimen employed with encouraging results by Fassas et al.[46] are recommended with an addition of pretransplant ALS. In designing a conditioning regimen it should be kept in mind that Pestronk et al.[39] observed in his model of myasthenia gravis that cyclophosphamide as the sole conditioning agent was inadequate; it had to be supplemented with sublethal TBI for eradication of memory T cells.

In all cases, extensive T cell depletion of the autologous BM graft is mandatory. The grafting of autologous mobilized peripheral blood precursors (MPBP) is not recommended because the larger numbers of T cells are more difficult to remove down to the low level required. The advantage of autologous MPBP over BM for the more rapid recovery of peripheral blood cells does not outweigh the chances of reinfusing larger numbers of T cells with an increased risk of relapse.

At the time of writing, "stem cell treatment" of AID is entering the clinic.[44–46,53] We are about to witness a highly inspiring collaborative effort of hematologists with rheumatologists and neurologists. The attempts to extend the applications of BMT to the treatment of this important group of nonmalignant diseases will profit greatly from the pioneering work of the initiator of these meetings and the "father" of autoBMT: Dr. Karel A. Dicke. May the Healing Power of CMOMC[54] once more be revealed!

REFERENCES

1. Leiter EH: The role of environmental factors in modulating insulin-dependent diabetes. In: De Vries RRP, Cohen IR, Van Rood JJ (eds) The role of micro-organisms in non-infectious diseases. 1990, pp 39–51.
2. van Bekkum DW et al.: Regression of adjuvant-induced arthritis in rats following bone marrow transplantation. *Proc Natl Acad Sci USA*, 86:10090–10094, 1989.
3. van Bekkum DW et al.: Attempts at identification of hemopoietic stem cell in mouse. *Blood* 38:547–558, 1971.
4. van Bekkum DW: Review: BMT in experimental autoimmune diseases. *Bone Marrow Transplant* 11:183-187, 1993.
5. Silman AJ et al.: Twin concordance rates for rheumatoid arthritis results from a nationwide study. *Br J Rheumatol* 32:903–907, 1993.
6. De Vries MJ et al.: Signs of autoimmune reactivity in neonatally thymectomized mice. La greffe des cellules hematopoietiques allogeniques. (Ed. du Centre National de la Recherche Scientifique), 1965, CNRS, Paris, 7–9 September 1964, 115–117.

7. Morton JL et al.: Transplantation of autoimmune potential. I. Development of antinuclear antibodies in 1 1-2 histocompatible recipients of bone marrow from New Zealand Black mice. *Proc Natl Acad Sci USA* 71:2162–2165, 1974.
8. Sardina EE et al.: Transplantation of wheat germ agglutinin-positive hematopoietic cells to prevent or induce systemic autoimmune disease. *Proc Natl Acad Sci USA* 83:3218–3222, 1991.
9. De Heer DH et al.: Evidence for a B lymphocyte defect underlying the anti-X antierythrocyte autoantibody response of NZB mice. *J Immunol* 5:1858–1863, 1977.
10. Morton IL et al.: Transplantation of autoimmune potential. *Transplant* 2:133–134, 1979.
11. Ikehara S et al.: Rationale for transplantation of both allogeneic bone marrow and stromal cells in the treatment of autoimmune diseases. In: Champlin RE, Gale RP (eds) New Strategies in Bone Marrow Transplantation, UCLA symposia on Molecular and Cellular Biology New Series 137:251, 1991.
12. Panitch HS: Influence of infection on exacerbations of multiple sclerosis. *Ann Neurol* 36:S25–S28, 1994.
13. Serreze DV et al.: NOD marrow stem cells adoptively transfer diabetes to resistant (NOD × NON)F1 mice. *Diabetes* 37:252–255, 1988.
14. Wicker LS et al.: Expression of genetically determined diabetes and insulitis in the nonobese diabetic (NOD) mouse at the level of bone marrow-derived cells: Transfer of diabetes and insulitis to nondiabetic (NOD × B 10) F1 mice with bone marrow cells from NOD mice. *J Exp Med* 167:1801–1810, 1988.
15. Ikehara S et al.: Prevention of type I diabetes in nonobese diabetic mice by allogeneic bone marrow transplantation. *Proc Natl Acad Sci USA* 82:7743–7747, 1985.
16. LaFace DM et al.: Reciprocal allogeneic bone marrow transplantation between NOD mice and diabetes nonsusceptible mice associated with transfer and pretension of autoimmune diabetes. *Diabetes* 38:894–901, 1989.
17. Naji A et al.: Influence of islet and bone marrow transplantation on the diabetes and immunodeficiency of BB rats. *Metabolism* 32:62–68, 1983.
18. Scott J et al.: Bone marrow irradiation chimeras in the BB rat: Evidence suggesting two defects leading to diabetes and lymphopenia. *Diabetologia* 30:774–781, 1987.
19. Kuntz L et al.: Adoptive transfer of viable motheaten pathology in sublethally irradiated beige recipient mice. *Immunol* 73:356–362, 1991.
20. Walker MA et al.: Adoptive transfer of tsk skin fibrosis to +/+ recipients by tsk bone marrow and spleen cells. *PSEBM* 192:196–200, 1989.
21. Van Bruggen MCJ et al.: Streptococcal cell wall-induced arthritis and adjvant arthritis in F344 → Lewis and in Lewis → F344 bone marrow chimeras. *Cell Immunol* 136:278–290, 1991.
22. Van Gelder et al.: Both bone marrow and non-bone marrow associated factors determine susceptibility to experimental autoimmune encephalomyelitis of BUF and WAG rats. *Cellular Immunology* 168:39–48, 1996.
23. Fujita M et al.: A study on type II collagen-induced arthritis in allogeneic bone marrow chimeras. *Immunol* 66:422–427, 1989.
24. Singer DE et al.: EAE in rat bone marrow chimeras: Analysis of the cellular mecha-

nism of BN resistance. *J Immunol* 126:1553–1557, 1981.
25. Ben-Nun A et al.: Genetic control of autoimmune encephalomyelitis and recognition of the critical nonapeptide moiety of myelin basic protein in guinea pigs are extended through interaction of lymphocytes and macrophages. *Eur J Immunol* 11:311–316, 1981.
26. Pelfrey CM et al.: Genetic resistance in experimental autoimmune encephalomyelitis. I. Analysis of the mechanism of LeR resistance using radiation chimeras. *Cell Immunol* 122:504–516, 1989.
27. Korngold R et al.: Acute experimental allergic encephalomyelitis in radiation bone marrow chimeras between high and low susceptible strains of mice. *Immunogenetics* 24:309–315, 1986.
28. Lublin FD et al.: Relapsing experimental allergic encephalomyelitis in radiation bone marrow chimeras between high and low susceptible strains of mice. *Clin Exp Immunol* 66:491–496, 1986.
29. Binder TA et al.: Relative susceptibility of SJL/J and B 10.S mice to experimental allergic encephalomyelitis (EAE) is determined by the ability of prethymic cells in bone marrow to develop into EAE effector T cells. *J Neuroimmunol* 42:23–32, 1993.
30. van Bekkum DW: unpublished observations.
31. Van Gelder M, et al.: Treatment of relapsing experimental autoimmune encephalomyelitis with largely MHC matched allogeneic bone marrow transplantation. *Transplantation,* in press.
32. Jones RE et al.: Induction of experimental autoimmune encephalomyelitis in severe combined immunodeficient mice reconstituted with allogeneic or xenogeneic hemapoietic cells. *J Immunol* 150:4620–4629, 1993.
33. Ikehara S et al.: Long-term observations of autoimmune-prone mice treated for autoimmune disease by allogeneic bone marrow transplantation. *Proc Natl Acad Sci USA* 86:3306–3310, 1989.
34. Ishida T et al.: Requirement of donor-derived stromal cells in the bone marrow for successful allogeneic bone marrow transplantation. Complete prevention of recurrence of autoimmune diseases in MRL/MP-lpr/lpr mice by transplantation of bone marrow plus bones (stromal cells) from the same donor. *J Immunol* 152:3119–3127, 1994.
35. van Bekkum DW et al.: Regression of adjuvant-induced arthritis in rats following bone marrow transplantation. *Proc Natl Acad Sci USA* 86:10090–10094, 1989.
36. Van Gelder M et al.: Treatment of relapsing experimental autoimmune encephalomyelitis in rats with allogeneic bone marrow transplantation from a resistant strain. *Bone Marrow Transplant* 16:343–351, 1995.
37. Kamiya M et al.: Effective treatment of mice with type II collagen induced arthritis with lethal irradiation and bone marrow transplantation. *J Rheumatol* 20:225–230, 1993.
38. Knaan-Shanzer S et al.: Remission induction of adjuvant arthritis in rats by total body irradiation and autologous bone marrow transplantation. *Bone Marrow Transplant* 8:333–338, 1991.
39. Pestronk A et al.: Combined short-term immunotherapy for experimental autoimmune myasthenia gravis. *Ann Neurol* 14:235–241, 1983.

40. Karussis DM et al.: Chronic-relapsing experimental autoimmune encephalomyelitis (CR-EAE): Treatment and induction of tolerance, with high dose cyclophosphamide followed by syngeneic bone marrow transplantation. *J Neuroimmunol* 39:201–210, 1992.
41. Van Gelder M et al.: Treatment of experimental allergic encephalomyelitis in rats with total body irradiation and syngeneic bone marrow transplantation. *Bone Marrow Transplant* 11:233–241, 1993.
42. Van Gelder M et al.: Effective treatment of relapsing experimental autoimmune encephalomyelitis with pseudoautologous bone marrow transplantation. *Bone Marrow Transplant*, in press.
43. Marmont AM.: Immune ablation followed by allogeneic or autologous bone marrow transplantation: A new treatment for severe autoimmune diseases? *Stem Cells* 12:125–135, 1994.
44. van Bekkum DW et al.: Severe autoimmune disease: A new target for bone marrow transplantation. Sandoz, Keystone Symposium Abstracts. *Stem Cells* 14:460–472, 1996.
45. Dreger P et al.: Recurrence of persistence of autoimmune diseases following autologous and unmanipulated stem cell transplantation in five patients. The International Meeting 'Haemopoietic stem cell therapy in autoimmune diseases.' *Basel* 1996 [abstr].
46. Fassas A et al.: Peripheral blood stem-cell transplantation for treatment of the progressive form of multiple sclerosis. The International Meeting 'Haemopoietic stem cell therapy in autoimmune diseases' *Basel* 1996 [abstr].
47. Xun CQ et al.: Effect of total body irradiation, busulfan-cyclophosfamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H2 incompatible transplanted SCID mice. *Blood* 83:2360–2367, 1994.
48. Oldendorf WH et al.: A comparison of total body and local spinal cord irradiation in experimental allergic encephalomyelitis. *J Neuropathol Exp Neurol* 36:50–61, 1977.
49. Van Gelder M.: Bone marrow transplantation for treatment of experimental autoimmune encephalomyelitis in rats. Prospects for therapy of severe multiple sclerosis. Thesis 1995, Leiden.
50. Tourtelotte WW et al.: Multiple sclerosis de novo CNS IgG synthesis: Effect of CNS irradiation. *Arch Neurol* 37:620–624, 1980.
51. Openshaw H et al.: Exacerbation of inflammatory demyelinating polyneuropathy after bone marrow transplantation. *Bone Marrow Transplant* 7:411–414, 1991.
52. Bucy RP et al.: Cyclosporin A-induced autoimmune disease in mice. *J Immunol* 151:1039-1050, 1993.
53. Tyndall A et al.: Haemopoietic stem and progenitor cells in the treatment of severe autoimmune diseases. *Ann Rheum Dis* 55:149–151, 1996.
54. From "The Ballad of CMOMC." van Bekkum DW. Bone marrow transplantation: A story of stem cells. In: Terasaki PI (ed) History of Transplantation: Thirty-five Recollections. Los Angeles: UCLA Tissue Typing Lab, 1991, pp 395–433.

A part of the fourth verse of the Ballad, describing the first clinical success with T cell depleted bone marrow in 1968 at Leyden, is printed here:

So, the concept of stem cells pluripotential
For the treatment of SCID became rather essential
DeVries killed the dogma defended by Good
That a thymic defect was its pathological root,
And in view of no T cells nor B cells detected
It must be the stem cell that is badly affected.
It followed that SCID patients should receive
Bone marrow stem cells for complete relief.
Well, that's how it happened that stem cell concentrations
Were used for the first time in clinical transplantations.
With T cell removed there was no need for postponing
This treatment at Leyden said Leo Jan Dooren and DeKoning.

INTRALESIONAL ADMINISTRATION OF RADIOLABELED IMMUNOGLOBULIN FOR HUMAN SOLID TUMORS

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ABSTRACT

The use of radiolabeled monoclonal antibodies for the selective radiation of tumors has been explored intensively for the last two decades. Clinical radioimmunotherapy (RIT) studies with intravenously (i.v.) administered radiolabeled antibodies have indicated two major limitations,[1] i.e., low deposition of radioactivity in solid tumors and[2] the formation of anti-antibodies. The low tumor uptake of IV RIT can be rectified by depositing the RIT reagent directly into large tumor masses (intralesional [i.l.] administration). The problem of anti-antibody formation can be minimized by using human monoclonal antibodies. The high radiation doses required for the cure of a large tumor cannot be delivered with an external beam due to the lack of selectivity and the high incidence of serious damage to normal tissue. Intralesional RIT can overcome some of the limitations of external beam radiation for large tumor masses. We have evaluated IgM and IgG radioimmunoconjugates injected i.l. in nude mouse models bearing tumor xenografts of two human head and neck squamous cell carcinomas cell lines. Cell line 886 reacts with human monoclonal IgM (CR4E8) and cell line 1483 reacts with mouse antiferritin antibodies (IgM-101 and IgG-103). Normal mouse IgG and IgM were used as specificity controls. Tumor nodules were grown s.c. on the hind leg of nude mice. Antibodies were conjugated to a 2B3M-DTPA chelator and radiolabeled with ^{111}In or ^{90}Y. Biodistribution was performed at 4 hours, 1, 2, 4 and 6 days after injection. The distribution of radioactivity was also analyzed by immunoscintigraphy studies at 1, 2, 4 and 6 days post injection. Eighty to ninety percent of ^{111}In or ^{90}Y labeled CR4E8 (tumor specific human IgM) was retained in the tumor (886) for up to four days. Blood activity was negligible (0.13–0.01% ID/g). Liver (0.4–2.27% ID/g), kidney (1.06–2.87% ID/g), and spleen (0.05–1.14% ID/g) showed very low uptake of radioactivity. The serial static gamma camera images of mice confirmed that most of the radioactivity resided in the tumor and gradually moved to the tumor periphery. In the 1483 tumor model (ferritin positive), biodistribution data showed that approximately 50% of radiolabeled IgG (antiferritin) perfused out of the tumor into the circulation and deposited into normal tissues, whereas most of IgM (antiferritin) remained bound to the tumor mass. More tumor specific IgM and IgG

were retained in tumor than nonspecific IgM or IgG. Specific IgM appears to be a better vehicle for i.l. RIT than specific IgG. It is anticipated that much higher radiation doses can be obtained in large tumors by i.l. RIT than is possible by external beam irradiation. Dose-limiting levels for normal tissue still need to be defined for this new approach.

INTRODUCTION

Clinical radioimmunotherapy (RIT) with intravenously (iv) administered radiolabeled antibodies suffers from a low deposition of radioactivity in solid tumors.[1–3] This could be rectified by depositing the RIT reagent intracompartmentally, as, for example, in the peritoneal cavity for peritoneal carcinomatosis[4,5] or intralesionally, i.e., directly into large tumor masses.[6] Such locally deposited radioimmunoconjugates might slowly diffuse over the whole tumor volume, including the thin, long tentacles infiltrating into normal tissues. The radioimmunoconjugates would be retained in the tumor by specific antigen-antibody binding. This approach could overcome some of the difficulties experienced in the delivery of external beam radiation to large or compartmentally dispersed tumor masses. External beam radiation cannot deliver the high doses needed for cure, owing to its lack of selectivity and unacceptable damage to surrounding normal organs in large radiation fields.[7] This problem can be illustrated by the experience obtained in patients with large (>6 cm) head and neck cancers in whom local tumor recurrence and/or severe normal tissue side effects are common after external beam irradiation with curative intent.[8]

We postulate that the efficacy of direct deposition of radioimmunoconjugates in compartments or tumors is influenced by tumor antigen location (interstitial, membrane, intracellular), number of antibody binding sites per radioimmunoconjugate and size of the radioimmunoconjugate. We anticipate that significantly higher radiation doses can be delivered to tumors with these new approaches than was previously possible. We report on i.l. (intralesional) RIT in two different nude mouse models, both bearing subcutaneous human head and neck squamous cell carcinoma xenografts. Intralesional RIT, particularly when using IgM, can deliver selective, high-dose radiation to large tumors. Dose-limiting toxicity to normal organs remains to be defined, and will require toxicology studies in large animals such as beagle dogs.

METHODS AND MATERIALS

Purification of antibodies

Two monoclonal IgM antibodies (CR4E8, AF-101) were isolated from cell culture supernatant and purified by column chromatography using Sephacryl S-300 gel filtration media. The IgG (AF-103) was also isolated from cell culture

supernatant and purified by ion-exchange/affinity chromatography. CR4E8 is a human Monoclonal IgMλ[9] that reacts with a human squamous cell carcinoma of head and neck (886) cell line. AF-101 is a mouse monoclonal antiferritin (IgM) and AF-103 is also a mouse monoclonal antiferritin (IgGi). Both antibodies react with 1483 cell line. Tumor nodules of the 1483 cell line contain ferritin.

Cell lines and tumor xenograft

The human head and neck squamous cell carcinoma cell line (886 or 1483) was cultured in vitro. Single cell suspensions (8×10^6 cells) were used to inoculate subcutaneous (s.c.) tumor xenografts in nude mice.

Preparation of immunoconjugates

The antibodies were conjugated to 2-(p-isothiocyanato) benzyl-3-methyl-diethylene triamine pentaacetic acid (ITC-2B3M-DTPA) by reacting them at a 1:14 molar ratio in 0.05 M Hepes buffer, pH 8.5, at 4°C for 12 hours. The conjugated antibodies were purified from unconjugated chelate molecules with an Amicon Centricon-50 unit. Purity of the antibody-chelate conjugates were determined by size-exclusion HPLC.

Radiolabeling of immunoconjugates

Indium-111 was obtained from DuPont NEN Products (Wilmington, DE). A 20 μL aliquot of $^{111}InCl_3$ (3.7 mCi) was equilibrated with 250 μL of 0.6 M sodium acetate buffer, pH 5.3, and 250 μL of 0.06 M sodium citrate buffer, pH 5.5. Two hundred and fifty microliters of immunoconjugate (9 mg/mL) in 0.1 M 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 radiolabeled immunoconjugates were separated from low molecular weight compounds by gel filtration chromatography with a 1.5×20 cm Sephadex G50 gel column using 0.1 M PBS as eluent.

^{90}Y was obtained from Westinghouse (Richland, WA). A 5 μL aliquot of $^{90}YCl_3$ (6.5 mCi) in 0.1 M HCl was equilibrated with 200 μL of 2.0 M acetate buffer, pH 6.0. An aliquot of 200 μL of immunoconjugate solution (9 mg/mL) in 0.1 M PBS was added to buffered ^{90}Y, mixed well and incubated at room temperature for 90 minutes. DTPA challenge and gel-filtration chromatography purification followed similarly to ^{111}In labeling, except that a 1.5×30 cm Sephadex G100 column was used.

Incorporation of radiometals to immunoconjugates was monitored by ITLC and TLC analyses after the incubation period (radiochemical yield), after DTPA challenge and after column elution (final product). Radiolabeling efficiency, radiochemical yield and specific activity of all the radioimmunoconjugates were determined. Before the final product was administered to mice, the labeled

Table 1. Quality control analysis of immunoconjugates

Analysis	*^{111}In-IgM (CR4E8)*	*^{90}Y-IgM (CR4E8)*	*^{111}In-IgM (AF-101)*	*^{111}In-IgG (AF-103)*
DTPA/Ig	4.0	4.0	4.0	1.5
purity of immunoconjugates				
size-exclusion HPLC	98%	98%	96%	96%
protein-bound radioactivity	98.3%	95.9%	96.5%	96.5%
specific activity	2.48 mCi/mg	3.25 mCi/mg	2.4 mCi/mg	2.6 mCi/mg
immunoreactivity	61%	61 %	85%	74%
serum stability[a]	90.2%	94.2 %	88.7%	92.8%

[a]*Protein bound fraction after 48 hours incubation at 37°C in human serum.*

immunoconjugate solution was filtered through a sterile 0.2 mm Acrodisc syringe filter (Gelman Sciences, Inc., Ann Arbor, MI).

Radioimmunoglobulin administration and biodistribution

The nude mice bearing s.c. 886 tumor xenografts (0.5–1 cm diameter) were injected with 50 μL of either ^{111}In or ^{90}Y labeled CR4E8 conjugates (15 μCi) directly into tumor. For biodistribution studies, 4–6 mice were sacrificed at each time point (3, 24, 48, 96 and 144 hours post-injection).

The nude mice bearing s.c. 1483 tumor xenografts (0.5 cm diameter) were injected with 50 μL of ^{111}In-labeled IgG or IgM conjugates 10 μCi) directly into the tumor. Three mice were sacrificed at each time point (1, 2, 4 and 6 days post-injection).

For biodistribution studies, the mice were euthanized, and approximately 0.5 mL of blood was removed by cardiac puncture, weighed and prepared for radioactivity assay. Tumor and normal-tissues samples (whole organs in every case, except for the liver and small intestines) were removed, cleaned in saline, weighed and assayed for radioactivity. A Cobra II gamma counter (Packard Instrument Co., Downers Grove, IL) was used to determine counts per minute per gram of sample. A standard of the injectate was counted simultaneously in order to correct data for the physical decay of the isotopes. Percent injected dose/gram was calculated for all samples.

Imaging studies

Athymic nude mice bearing human tumor xenografts were injected with 25 μCi of ^{111}In-labeled immunoconjugate directly into tumor for immunoscintigraphic

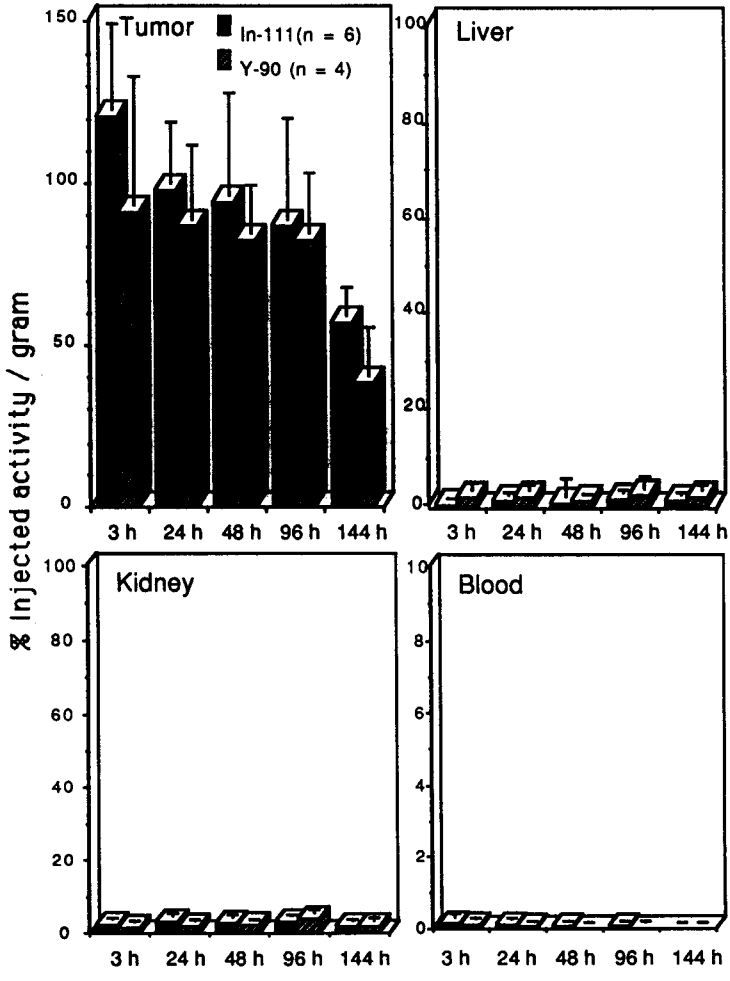

Figure 1. *Comparative biodistribution of intralesionally administered ^{111}In- and ^{90}Y-labeled CR4E8 in nude mice with 886 subcutaneous xenografts.*

analysis. Static gamma camera images of nude mice were acquired in the dual-energy mode, with 10% windows centered on the 172- and 247-keV photopeaks of ^{111}In, and stored in 128×128 matrices. Mice were placed on a parallel-hole collimator, protected by absorbent padding, and serial images were acquired at 1, 2, 4 and 6 days postinjection to visualize the localization of IgM and IgG immunoconjugates in tumor and normal tissue compartments.

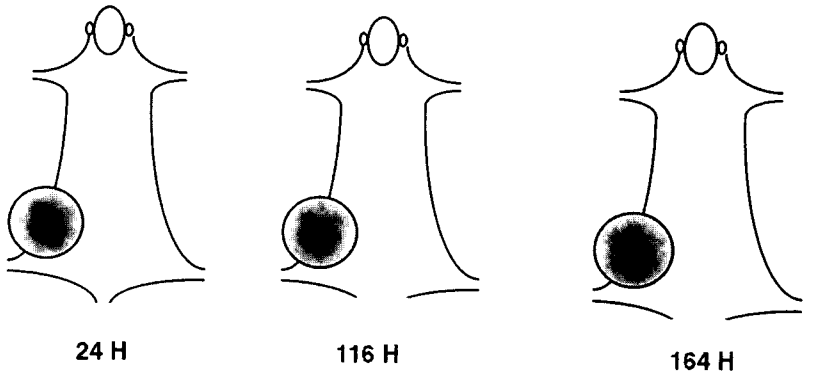

Figure 2. *Immunoscintigraphy of a nude mouse that had a 886 s.c. xenograft injected intralesionally with ^{111}In-labeled CR4E8. The mouse was imaged at 24, 116 and 164 hours post injection. The radioactivity in the tumor remained high, with sparse activity apparent in the abdomen.*

RESULTS

Table 1 summarizes the data for in vitro quality control analyses of radioimmunoconjugates used in this study. On average, four chelators were conjugated per IgM molecule and 1.5 chelators were conjugated per IgG molecule. All immunoconjugates have purity greater than 95%. Radiolabeled products carried at least 95% of radioactivity bound to immunoconjugates. A moderate to high level of immunoreactivity was observed as determined by affinity chromatography or direct cell binding assay. Serum incubation test indicated a high chelation stability of ^{111}In or ^{90}Y with immunoconjugates under physiological conditions.

Figure 1 shows the comparative biodistribution of ^{111}In- and ^{90}Y- labeled CR4E8 injected directly into s.c. 886 tumor nodules of nude mice. Both In and Y-labeled CR4E8 demonstrated similar pharmacokinetics. Eighty to ninety percent of ^{111}In or ^{90}Y labeled CR4E8 was retained in the tumor nodules up to four days. Blood activity was negligible (0.13–0.1% ID/g), while liver, kidney and spleen showed very low uptake of radioactivity. The serial static images of nude mice after i.l. injection of ^{111}In-labeled IgM conjugates indicated that most of the radioactivity resided in the tumor and gradually moved to the tumor periphery (Figure 2).

Figure 3 compares the i.l. biodistribution between radiolabeled IgM (antiferritin) and IgG (antiferritin) in nude mice with 1483 tumor xenograft. Biodistribution data showed that approximately 50% of radiolabeled IgG perfused out of the tumor into the circulation and was deposited into normal tissues, whereas most of the IgM conjugates remained bound to the tumor mass. The IgM conjugate showed five-fold higher tumor retention than that of IgG at 48 to 144 hours. Much higher normal tissue uptake was observed with IgG compared with IgM.

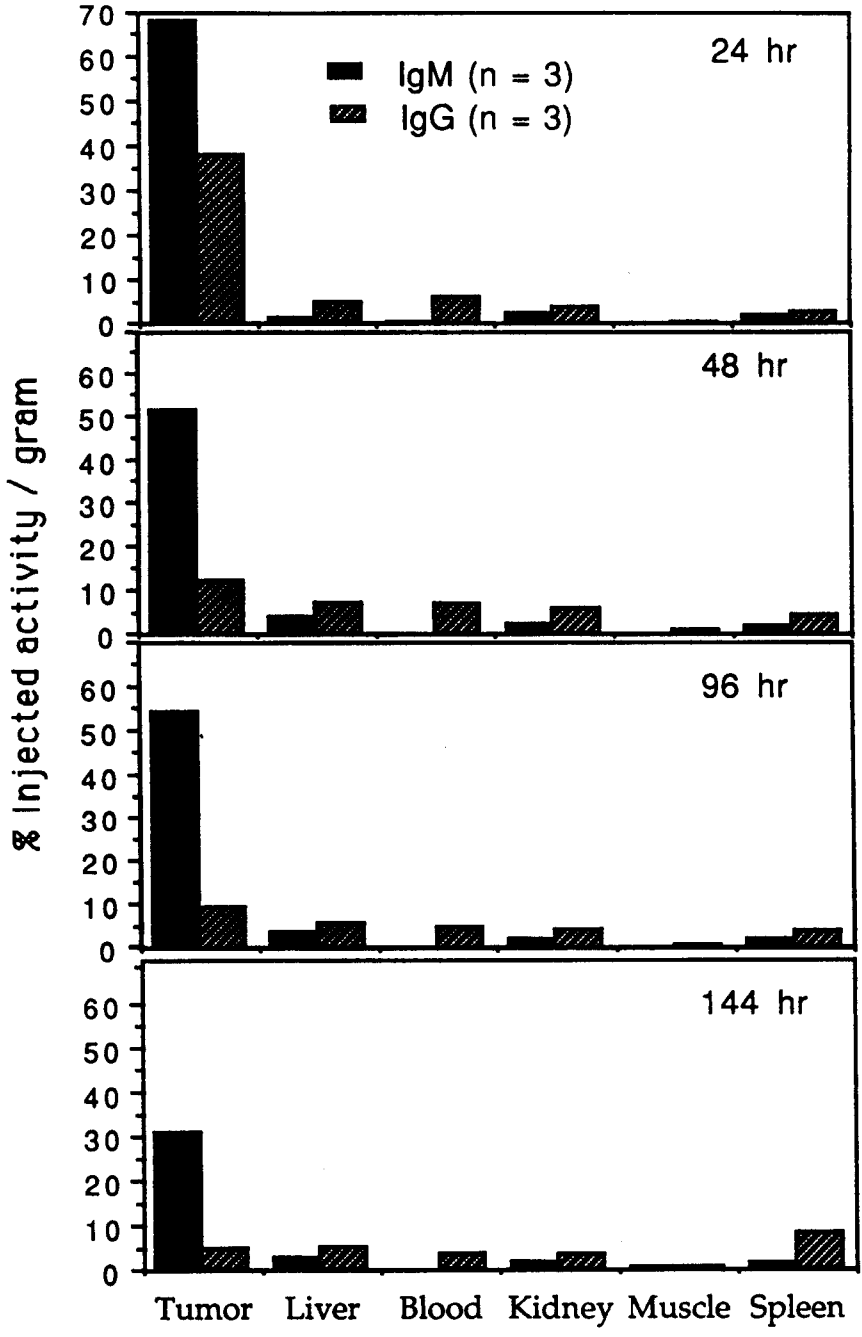

Figure 3. *Biodistribution of intralesionally injected ^{111}In-labeled IgM (AF-101) and IgG (AF-103) in nude mice bearing s.c. head and neck tumor xenografts (1483).*

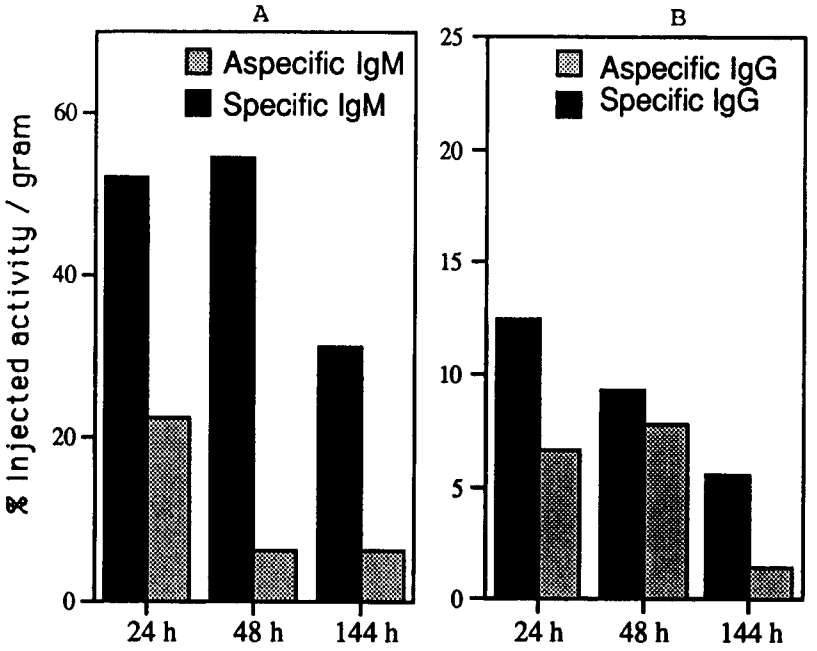

Figure 4. **(A)** *Tumor retention of specific IgM (AF-101) and aspecific IgM (normal mouse) after intralesional injection.* **(B)** *Tumor retention of specific IgG(AF-103) and aspecific IgG (normal mouse) after intralesional injection.*

Specificity control experiments indicated higher tumor uptake and longer retention with tumor-reactive (specific) antibody than non-tumor-reactive (aspecific) antibody in tumor nodules. Figure 4A illustrates the difference in tumor retention between specific IgM and aspecific IgM. The ratio of specific-to-aspecific tumor uptake was 2 at 24 hours but increased to at least five-fold at later time points. Figure 4B compares the tumor retention of specific IgG and aspecific IgG. The difference in the tumor retention between specific and aspecific IgG was less prominent, however, the specific IgG showed two-fold higher tumor uptake than aspecific IgG, except at 48 hours.

DISCUSSION

The objective of this report is to investigate whether i.l. RIT can deliver selective, antibody-mediated radiation therapy to head and neck squamous cell carcinoma (HNSCC). Most HNSCC are locally invasive and metastasize first to regional lymph nodes rather than distant sites.[10] We chose to study i.l. RIT in a HNSCC model since large regional nodal metastases are readily accessible for i.l. injection

and are not easily controlled with surgery and/or external beam radiation. Both indium-111 and yttrium-90 labeled IgM (CR4E8) conjugates showed high in vivo stability and similar biodistribution patterns when injected directly into the tumor. Our experience in nude mouse models indicates that i.l. radiolabeled IgM provides high tumor doses without causing significant normal tissue radiation. Liver, spleen and kidney show low uptake of radioactivity while the rest of the normal organs receive little radioactivity. Tumor-to-normal tissue ratios are approximately 50 or higher. Previously, we showed that intravenous radiolabeled tumor specific IgM does not lead to significant tumor targeting.[4] More than 60% of injected activity is taken up by liver and spleen. Intralesionally administered IgM has a longer tumor retention time than intralesionally administered IgG. Two properties of the IgM molecule may mediate its longer residence within a tumor mass following i.l. injection as compared to IgG antibody. The large size of IgM (over 900 kDa versus 150 kDa of IgG) may slow its diffusion. In addition, IgM, with its pentameric configuration and ten antigenic binding sites, may bind more strongly and be retained in the tumor longer than the monomeric IgG with two antigenic binding sites. In this study IgM appears to be a better vehicle for i.l. RIT than IgG. The tumor retention of radioimmunoconjugates also depends upon the specificity of antibody. A 5-fold higher tumor retention of specific IgM indicates that the tumor reactive (specific) IgM is superior to tumor nonreactive (aspecific) IgM. The differences between tumor reactive IgG and tumor nonreactive IgG are smaller.

Intralesional RIT has several advantages over the intravenous delivery of radioimmunoconjugates. The radioisotope is concentrated in the target immediately upon delivery before significant radioactive decay has occurred. Levels of circulating radioactivity remain low with lower normal tissue exposure. The accumulation of injected antibody within the tumor is not dependent upon adequate vascularization of the tumor or migration of the antibody from the intravascular to the extravascular compartment, as it is with the intravenous route. A disadvantage of i.l. approach is the requirement of an accessible tumor target for injection, and the lack of systemic effects for micrometastatic disease as is possible with IV RIT.

Intralesional RIT with ^{90}Y-labeled IgM will have a very high therapeutic ratio. Initial dose calculations indicate that in a 1 cm diameter tumor a dose of 100 Gy in one week can be obtained from 1 mCi ^{90}Y labeled i.l. IgM. Dose-limiting toxicity to normal tissues with i.l. RIT remain to be defined. This might involve vascular, nervous or interstitial structures in the vicinity of the tumor. The nude mouse anatomical dimensions are so small in comparison to equivalent structures in human patients that a proper preclinical toxicology analysis requires the administration of i.l. IgM to normal tissues in larger experimental animals (such as dogs).

The advantages of selective cancer treatment over nonselective cancer treatment are so important that i.l. RIT as a prime example of the former deserves to be

evaluated expeditiously. Patients with accessible tumor may benefit from i.l. RIT as the radiation will localize within the tumor margin without affecting the neighboring normal tissue. Intralesional administrations of IgM to human cancer patients can be single visit, outpatient, low toxicity studies. This would provide a patient-friendly and low cost treatment modality. If i.l. RIT can be developed successfully for patients with HNSCC, it can be easily adapted to the treatment of patients with other solid tumors such as glioblastoma multiforme, prostate cancer or breast cancer.

REFERENCES

1. Epenetos AA, Snook D, Durbin H et al.: Limitations of radiolabeled monoclonal antibodies for localization of human neoplasms. *Cancer Res* 46:3183–3191, 1986.
2. Goldenberg DM: Future role of radiolabeled monoclonal antibodies in oncological diagnosis and therapy. *Semin Nucl Med* 19:332–339, 1989.
3. Mach JP, Chatal JF, Lumbroso JD et al.: Tumor localization in patients by radiolabeled monoclonal antibodies against colon carcinoma. *Cancer Res* 43:5593–5600, 1983.
4. Quadri SM, Malik AB, Tang X-Z et al.: Preclinical analysis of intraperitoneal administration of indium-111 labeled human tumor reactive monoclonal IgM AC6C3-2B12. *Cancer Res* 55:5736–5742, 1995.
5. Quadri SM, Malik AB, Chu HB et al.: Intraperitoneal ^{111}In and ^{90}Y-labeled administration of indium-111 labeled human IgM (AC6C3-2B12) in nude mice bearing peritoneal carcinomatosis. *J Nucl Med* 37:1545–1551, 1996.
6. Quadri SM, Borchardt PE, Ali MS et al.: Administration of radiolabeled immunoglobulin directly into human malignancies. *Tumor Targeting* 2:170, 1996.
7. Tupchoug L, Scott CB, Blitzer PH et al.: Randomized study of preoperative versus postoperative radiation therapy in advanced head and neck carcinoma: Long term follow-up of RTOG study 73-03. *Int J Radiat Onco Biol Phys* 20:21–28, 1991.
8. Haraf DJ, Weichselbaum RR, and Vokes EE: Timing and sequence of chemotherapy. In: Hong WK, Weber RS (eds) Head and Neck Cancer: Basic and Clinical Aspects. Kluwer MA 1994, pp 173–198.
9. Chen P-F, Freedman RS, Chernajovsky Y, Platsouscas CD: Amplification of immunoglobulin transcripts by the non-palindromic adaptor polymerase chain reaction (NPA-PCR). Nucleotide sequence analysis of two human monoclonal antibodies recognizing two cell surface antigens expressed in ovarian, cervix, breast, colon and other carcinoma. *Hum Antibodies Hybridomas* 5:131–142, 1994.
10. Shah JP, Lydiatt W: Treatment of cancer of the head and neck. *CA Cancer J Clin* 45:352–368, 1995.

IMPROVED AMPHOTROPIC RETROVIRUS-MEDIATED GENE TRANSFER INTO HEMATOPOIETIC STEM CELLS

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ABSTRACT

The efficiency of amphotropic retrovirus-mediated gene transfer into human hematopoietic stem cells (HSC) is less than 1%. This has impeded gene therapy for hematopoietic diseases.[1-3] In this study we demonstrate that populations of mouse and human HSC contain low to undetectable levels of the amphotropic virus receptor messenger RNA (ampho R mRNA), and are resistant to transduction with amphotropic retroviral vectors. In a subpopulation of mouse HSC expressing 3-fold higher levels of ampho R mRNA, transduction with amphotropic retrovirus vectors was 30-fold higher. We conclude that retrovirus transduction of HSC correlates with ampho R mRNA levels. Our results predict that alternative sources of HSC or retroviruses will be required for human gene therapy of hematopoietic diseases. One alternative source of stem cells is from individuals treated with cytokines. We have previously shown that mice treated with granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) have an immediate increase in peripheral blood HSC immediately after treatment, followed by a 10-fold increase in bone marrow (BM) HSC 14 days after treatment.[4] In this report we show that when rhesus monkey BM cells collected 14 days after G-CSF and SCF treatment were transduced with amphotropic retroviruses, gene transfer levels were approximately 10% and easily detected by Southern blot analysis. We conclude that the increased gene transfer may be the result of increased expression of the amphotropic retrovirus receptor, increased numbers of cycling HSC or both.

INTRODUCTION

Hematopoietic stem cells (HSC) are among the most attractive targets for retrovirus-mediated gene transfer.[5-6] Since HSC can completely and

permanently repopulate the hematopoietic system following bone marrow transplantation (BMT),[7] integration of proviral DNA into the genome[8] of HSC would result in continuous production of hematopoietic cells with the transferred gene. The concept has been demonstrated in murine models using mouse specific ecotropic retrovirus vectors.[9–14] As examples, retrovirus-mediated gene transfer of the β-glucuronidase gene has cured murine β-glucuronidase deficiency,[15] and retrovirus-mediated gene transfer of the Multiple Drug Resistance gene has protected the hematopoietic system from taxol treatment.[16] In these studies, gene transfer was observed into 20% or more of hematopoietic cells for over 1 year.[9–16]

Amphotropic murine retroviruses have been used as gene transfer vectors for human gene therapy.[5,6] Studies using different transduction protocols have reported gene transfer efficiencies of less than 0.1 to 1.0% using amphotropic retroviral vectors. A fundamental obstacle to gene therapy of human hematopoietic diseases has been the low level of gene transfer observed in human[1–3] or primate[17–18] studies using amphotropic retroviral vectors.

Ecotropic and amphotropic retroviruses differ in the gp70 proteins in the retrovirus envelope.[8] The gp70 protein of ecotropic viruses uses an amino acid transport protein on the surface of target cells as its receptor.[19,20] The gp70 binding site on this protein is not conserved among mammals. This restricts ecotropic retrovirus transduction to mouse cells.[20] The gp70 protein of amphotropic virus uses as its receptor a phosphate channel protein in which the gp70 binding site is conserved, allowing amphotropic viruses to transduce most mammalian cells.[21–23] We hypothesized that the relatively poor transduction of HSC with amphotropic viruses may be due to low levels of the amphotropic retrovirus receptor.

Recent studies have shown that murine HSC can be enriched over 1000-fold so that as few as 10–50 cells can repopulate irradiated or W/W^{v} mice. A variety of HSC enrichment strategies have been developed,[24] including removal of cells expressing lineage markers (Lin^{-}), and positive selection for cells expressing Sca-1, c-*kit* or other markers.[25–27] We have previously shown that a population of murine Lin^{-} cells expressing high levels of c-*kit* (c-*kit*^{HI}) was highly enriched for HSC,[27] and we have used these cells to study mRNA expression in HSC.[28,29] Studies with human BM have shown that the most primitive hematopoietic cells express the CD34 antigen but not the CD38 antigen, while less primitive hematopoietic progenitor cells expressed both the CD34 and CD38 antigens.[30,31]

In all of these studies, only 3% or less of the enriched HSC have been demonstrated to be progressing through the cell cycle.[24,25] The number of cycling HSC may be higher in populations of HSC other than BM. Cytokine treatment of both mice and patients can mobilize HSC into the peripheral blood,[32] with an accompanying decrease in the repopulating ability of the BM.[33,34] We have

previously shown that the number of HSC in the BM recovers to 10-fold greater than normal levels after treatment with granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF).[4] Because retroviruses preferentially integrate into cycling cells,[8] we hypothesized that conditions that would cause the stem cell number to increase would improve gene transfer efficiency.

In this study, we assayed amphotropic retrovirus receptor mRNA (ampho R mRNA) expression in both mouse and human HSC. The level of ampho R mRNA was nearly undetectable in enriched populations of HSC. In contrast, ecotropic retrovirus receptor mRNA (eco R mRNA) was easily detected in the same cells. We isolated a subpopulation of murine HSC comprising approximately 15% of the total number that express higher levels of ampho R mRNA. We compared the efficiency of amphotropic and ecotropic retrovirus transduction of highly enriched murine HSC. We found that in most HSC populations, transduction with ecotropic retrovirus vectors was more than 10-fold more efficient than with amphotropic retrovirus vectors. The exception was the subpopulation expressing higher levels of ampho R mRNA where transduction with amphotropic retrovirus vectors was comparable to ecotropic retrovirus vectors. We also examined gene transfer into mouse and rhesus monkey peripheral blood and BM cells collected after treatment with G-CSF and SCF. The frequency of gene transfer into mouse HSC was comparable to gene transfer into HSC from 5-FU treated BM. Amphotropic retrovirus-mediated gene transfer into G-CSF and SCF primed rhesus monkey was estimated at up to 10%, a level 10-fold higher than previous attempts, and easily detectable by Southern blot analysis. We conclude that the low levels of expression of ampho R mRNA in HSC are responsible for the inefficient gene transfer observed in human and primate gene transfer experiments. In addition, we feel that the increased gene transfer we observed in cytokine pretreated rhesus monkeys may be the result of increased expression of the amphotropic retrovirus receptor, increased numbers of cycling HSC or both.

METHODS

Mice and cells

All mice were purchased from the Jackson laboratory, Bar Harbor, ME. Young adult C57BL/6J female mice, 3–5 weeks old, were used for hematopoietic stem cell enrichment. $WBB6F_1$-*W/W*^{v} mice were used as recipients of transduced hematopoietic stem cells. The ψ-CRE MFG-lacZ and ψ-CRIP MFG-NLSlacZ cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% newborn calf serum (NCS; both from Gibco, Gaithersburg, MD). The LNL6 and G1Na retroviral vectors carry an identical bacterial phosphotransferase gene.[35,36] Supernatants from all 4 cell lines were harvested from producer cell lines grown to confluence in Dulbecco's modified

Table 1. Primers and PCR conditions

Gene	*Primer sequence*	*Conditions*
mouse ecotropic R	sense: 5′CTG CCT CAA CAC CTA TGA CC3′	94°C-1 minute
	anti-sense: 5′TGC TGA CGT GAG AAC TCT CC3′	58°C-1 minute
	fragment size: 308 bp	72°C-2 minutes
mouse amphotropic R	sense: 5′CGG GCG GAA GAC GAG AAG GA3′	94°C-1 minute
	anti-sense :5′GAA GCC ACT GGA CGG TGT GA3′	65°C-1 minute
	fragment size: 309 bp	72°C-2 minutes
mouse β-2 micro globulin	sense: 5′TGC TAT CCA GAA AAC CCC TC3′	94°C-1 minute
	anti-sense: 5′GTC ATG CTT AAC TCT GCA GG3′	55°C-1 minute
	fragment size: 258 bp	72°C-2 minutes
human ecotropic R	sense: 5′CTG CCT GAA CAC TTT TGA TCT GGT GGC3′	94°C-1 minute
	anti-sense: 5′GAG GTC ATG TGT GTC CGT GAG AAC TCC3′	58°C-1 minute
	fragment size: 371 bp	72°C-2 minutes
human amphotropic R	sense: 5′CGG AAC ATC TTC GTG GCC TG3′	94°C-1 minute
	anti-sense :5′GCT GGT CAT GAG AGA GCC GTG3′	62°C-1 minute
	fragment size: 220 bp	72°C-2 minutes
human β-2 micro globulin	sense: 5′CTC GCG CTA CTC TCT CTT TC3′	94°C-1 minute
	anti-sense: 5′CAT GTC TCG ATC CCA CTT AAC3′	55°C-1 minute
	fragment size: 330 bp	72°C-2 minutes
lac Z	sense: 5′GCC GAC ACC AGA CTA AGA AC3′	94°C-1 minute
	anti-sense: 5′CCT CTT CGC TAT TAC GCC AG3′	58°C-1 minute
	fragment size: 289 or 310 bp	72°C-2 minutes
neo	sense:5′CGG ATC GCT CAC AAC CAG TC3′	94°C-1 minute
	antisense:5′ AGC CGA ATA GCC TCT CCA CC3′	60°C-1.5 minutes
	fragment size: 483 or 467	72°C-2 minutes

Cycle number for all primer pairs=35.

Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco/BRL, Gaithersburg, MD).

Enrichment of HSC

Mouse BM HSC were isolated as previously described.[27] Briefly, cells were fractionated by counterflow centrifugal elutriation and collected at flow rates of 25 mL/min, 30 mL/min (discarded) and 35 mL/min (FR25, FR35). FR25 or FR35 cells were incubated in a cocktail of rat anti-mouse monoclonal antibodies directed against lineage and cells expressing lineage markers were removed using antibody coated immunomagnetic beads. Lin^- cells were incubated with biotinylated anti-c-*kit* antibody (ACK-4; a gift of Dr. S.I. Nishikawa), and high levels of c-*kit* (c-kit^{HI}) were collected. We have previously shown that all hematopoietic stem cells (HSC) reside in the c-kit^{HI} population.[27]

Human BM cells were obtained with informed consent from volunteer donors. Mononuclear cells were enriched population for CD34 positive cells and stained with antibodies to FITC labeled human lineage markers, APC labeled CD34, and PE labeled CD38. Lin^- $CD34^+$ $CD38^-$ cells (90% pure) were collected by cell sorting.

Isolation and analysis of RNA

Total cellular RNA was isolated according to the manufacturer's instructions using RNAzol B. First strand cDNA synthesis was performed on an aliquot of RNA according to the manufacturer's instructions (Perkin Elmer Cetus, Norwalk, CT). An estimate of the amount of cDNA in each sample was obtained by limiting dilution RT-PCR (Perkin Elmer Cetus, Norwalk, CT), using the β-2 microglobulin (β-2 M) (primers and conditions shown below), with 0.1 μL of α^{32}PdCTP added per reaction. Phosphorimager analysis was used to identify the linear range of the β-2 M amplification curve. These values were used to determine equivalent quantities of cDNA for amplification with primers specific for the retrovirus receptor mRNAs shown in Table 1.[28]

Rhesus stem and progenitor cell harvesting

Young adult (age 3–5 years) rhesus macaques (Macaca mulatta) were housed and handled in accordance with the guidelines set by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS Publication No. NIH 85-23). Recombinant human stem cell factor (200 μg/kg/day) and recombinant human granulocyte colony-stimulating factor (10 μg/kg/day) were given as subcutaneous injections for 5 days. Peripheral blood apheresis of 2.5 times the animal's blood volume was performed as described on day 5.[37] Bone marrow was harvested from the femurs, iliac crests and ischial tuberosities under

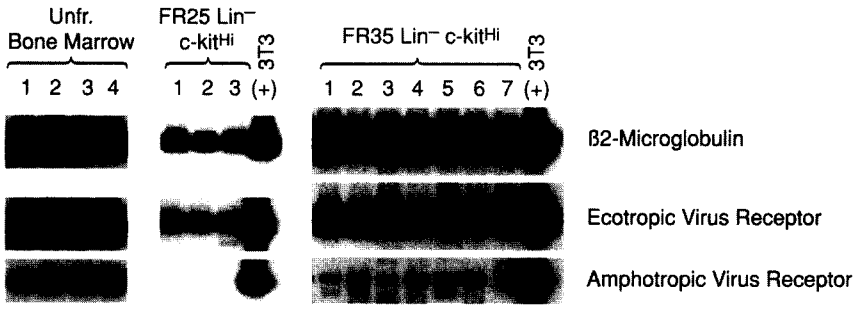

Figure 1. *Retrovirus receptor mRNA expression in enriched populations of mouse hematopoietic stem cells. The level of expression of the mRNAs encoding the mouse ecotropic and amphotropic retrovirus receptors were compared by reverse transcriptase PCR analysis using primers specific for each mRNA. The level of mouse β-2 microglobulin mRNA was measured to quantify the amount of c-DNA analyzed in each reaction. RNA isolated from NIH 3T3 cells (which are efficiently transduced by both amphotropic and ecotropic retroviruses) served as a positive control. Top Panel: Mouse β-2 microglobulin mRNA expression in unfractionated bone marrow cells (4 independent RNA isolates; left of panel); FR25 Lin⁻ c-kit^{HI} cells (3 independent RNA isolates; center of panel); and 3T3 cells (right of panel). Center Panel: Mouse ecotropic retrovirus receptor mRNA expression in the same RNA populations. Bottom Panel: Mouse amphotropic retrovirus receptor mRNA expression in the same RNA populations.*

general anesthesia, either prior to G-CSF and SCF treatment, or 14 days after discontinuation of cytokine treatment.

Retrovirus transduction

For transduction of mouse hematopoietic stem cells, 70% confluent plates of the producer cell lines were grown overnight in DMEM supplemented with 15% Hyclone FCS. The medium was aspirated from the plates, cytokines (f.c. mouse IL-3 10 ng/mL; rat SCF 100 ng/mL; human IL-6 100 ng/mL) and polybrene (f.c.: 6 μg/mL) were added, and the mixture was filtered (0.45 μm) and added to the target cells. The cells were cultured for 96 hours at 37°C (4 changes of medium) before being returned to recipient W/W^v mice. Proviral integration was analyzed by PCR of DNA extracted from peripheral blood cells using the following MFG-lacZ primers shown below. For rhesus monkey transduction, $CD34^+$ cells were transduced in undiluted LNL6 or G1Na supernatants supplemented with 4 μg/mL protamine sulfate, 20 ng/mL human IL-3, 100 ng/mL

human SCF, 50 ng/mL human IL-6. Cells were cultured 96 hours at 37°C (4 changes of medium). After transduction the cells were cryopreserved. Seven to 10 days after BM harvesting the animals received 650 rads total-body irradiation on each of two days. The next day, the transduced CD34-enriched cells were thawed and infused. Standard supportive care and transfusion support for rhesus transplantation recipients were given.

After reconstitution, peripheral blood and BM cells were collected at 1–3 month intervals. PCR was performed using the neo primers shown. For Southern blotting, 10 μg of DNA were digested with either Sac I or Bgl II, electrophoresed on a 1% agarose gel, transferred to a nylon membrane and before hybridized with a Neo probe.

RESULTS

Analysis of retrovirus receptor mRNA expression in mouse hematopoietic stem cells

We compared ampho R and eco R mRNA levels in unfractionated BM RNA and RNA isolated from a variety of cell lines by both Northern blot and RT-PCR analysis. We were able to demonstrate that our RT-PCR primers and conditions gave identical results to the Northern analysis (data not shown). Because of the scarcity of HSC in the BM we compared ampho R and eco R mRNA levels by RT-PCR in two populations of enriched murine HSC. Lin^- c-kit^{HI} cells elutriated at a flow rate of 25 mL/min. (FR25) represent approximately 25% of the HSC in C57BL/6 mice, and Lin^- c-kit^{HI} cells elutriated at a flow rate of 35 mL/min. FR35 cells represent approximately 15% of the HSC in C57BL/6 mice.[27,38] Eco R mRNA was present in similar levels in 3T3 cells, four independent isolates of unfractionated BM cells and 7 independent isolates of FR35 Lin^- c-kit^{HI} cells, and was approximately 50% of the 3T3 cell level in FR25 Lin^- c-kit^{HI} cells (Figure 1). The level of ampho R mRNA in unfractionated BM cells was approximately 50% the level in 3T3. Ampho R mRNA was present at low but detectable levels in FR35 Lin^- c-kit^{HI} cells, but was nearly undetectable in FR25 Lin^- c-kit^{HI} cells ($p=0.035$; Figure 1).

Analysis of retrovirus receptor mRNA expression in human hematopoietic stem cells

Expression of retrovirus receptor mRNAs was analyzed in two populations of human primitive hematopoietic cells. Cells co-expressing the CD34 and CD38 antigens are enriched for progenitor cells, while $CD34^+/CD38^-$ cells are enriched for more primitive cells.[30,31] The human homologue of the ecotropic virus receptor does not serve as a retrovirus receptor on human cells due to changes in the virus binding site. This mRNA was expressed in $CD34^+/CD38^+$ cells, and at lower

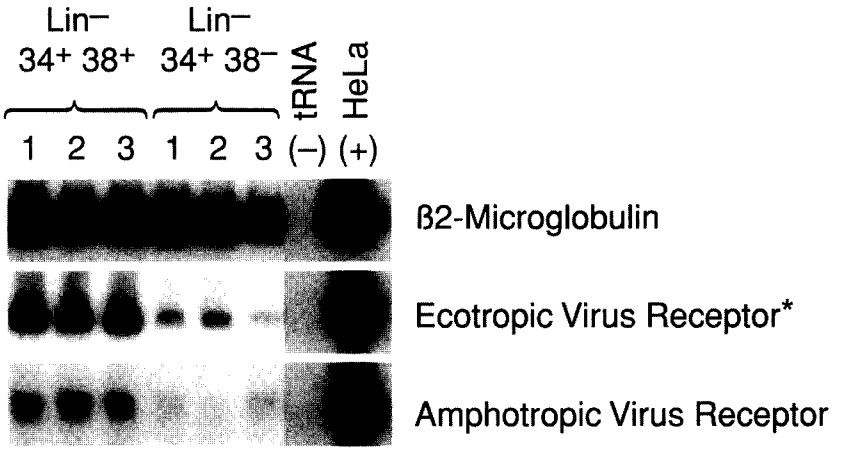

Figure 2. *Retrovirus receptor mRNA expression in enriched populations of human primitive hematopoietic cells. The level of expression of the mRNAs encoding the human equivalent of the mouse ecotropic retrovirus receptor (this molecule does not function as a retrovirus receptor in human cells) and the human amphotropic retrovirus receptor were compared with reverse transcriptase PCR using primers specific for each mRNA. The level of β-2 microglobulin mRNA was measured to quantify the amount of c-DNA analyzed in each reaction. RNA isolated from HeLa cells (which are efficiently transduced by amphotropic retroviruses) served as a positive control. Top Panel: Human β-2 microglobulin mRNA expression in Lin⁻ CD34⁺ CD38⁺ cells (3 independent RNA isolates; left of panel), Lin⁻CD34⁺CD38⁻ cells (3 independent RNA isolates; center of panel), and negative control (tRNA) and positive control (HeLa cell) RNA samples. Center Panel: Human ecotropic retrovirus receptor equivalent mRNA expression in the same RNA populations. Bottom Panel: Human amphotropic retrovirus receptor mRNA expression in the same RNA populations.*

levels in CD34⁺/CD38⁻ cells (Figure 2). Human ampho R mRNA was expressed in CD34⁺/CD38⁺ cells, but was nearly undetectable in CD34⁺/CD38⁻ cells. These results were similar to the pattern of expression seen in mouse HSC.

Transduction of mouse HSC with ecotropic and amphotropic retroviruses

To determine the relationship between eco R and ampho R mRNA expression and gene transfer efficiency, a co-transduction assay was developed. Two cell lines producing ecotropic and amphotropic lacZ retroviruses (ψ-Cre MFG-lacZ and ψ-Crip MFG-NLSlacZ, respectively) were selected. These retroviruses differ by a 21 base pair nuclear localization signal (NLS) and could be distinguished by

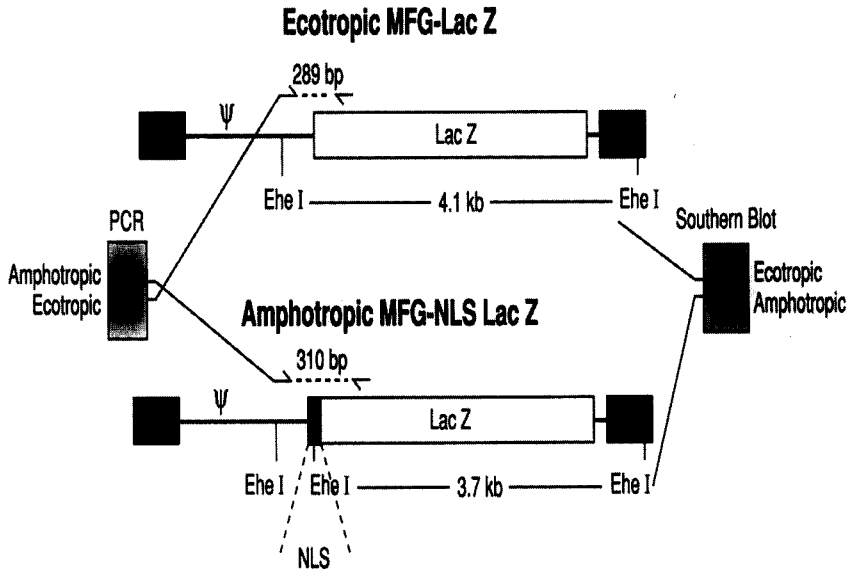

Figure 3. *Co-transduction assay using the MFG-lacZ and MFG-NLS lacZ virus vectors. The two proviruses are depicted in the center of the figure. The provirus from the amphotropic producer cell line can be distinguished from the provirus from the ecotropic producer cell line by either Southern blot analysis using Ehe I for digestion (right of figure) or PCR analysis (left of figure) based on the presence of a 21 base pair Nuclear Localization Signal (NLS) in the NLS lacZ retrovirus.*

either PCR or Southern blot analysis (Figure 3). Equal volumes of supernatant from the two producer cell lines were combined and used to transduce 3T3 cells, FR25 Lin$^-$ c-*kit*^{HI} cells and FR35 Lin$^-$ c-*kit*^{HI} cells. Southern blot analysis of DNA extracted from transduced 3T3 cells demonstrated that the producer cell lines generated equivalent titers of each retrovirus (Figure 3). Ecotropic MFG-lacZ proviral sequences were detected in DNA extracted from peripheral blood cells of 10 of 13 recipients of transduced FR25 Lin$^-$ c-*kit*^{HI} cells. No proviral sequences were detected in 3 recipients. Consistent with the low level of expression of amphotropic retrovirus receptor mRNA detected in FR25 Lin$^-$ c-*kit*^{HI} cells, amphotropic MFG-NLSlacZ proviral sequences were detected in only one animal, at a low level. The ratio of amphotropic to ecotropic retrovirus transduction of FR25 Lin$^-$ c-*kit*^{HI} cells was 0.022 ± 0.07 (Figure 4). Ecotropic MFG-lacZ proviral sequences were detected in DNA extracted from peripheral blood cells of all 11 recipients of transduced FR35 Lin$^-$ c-*kit*^{HI} cells. Consistent with the higher level

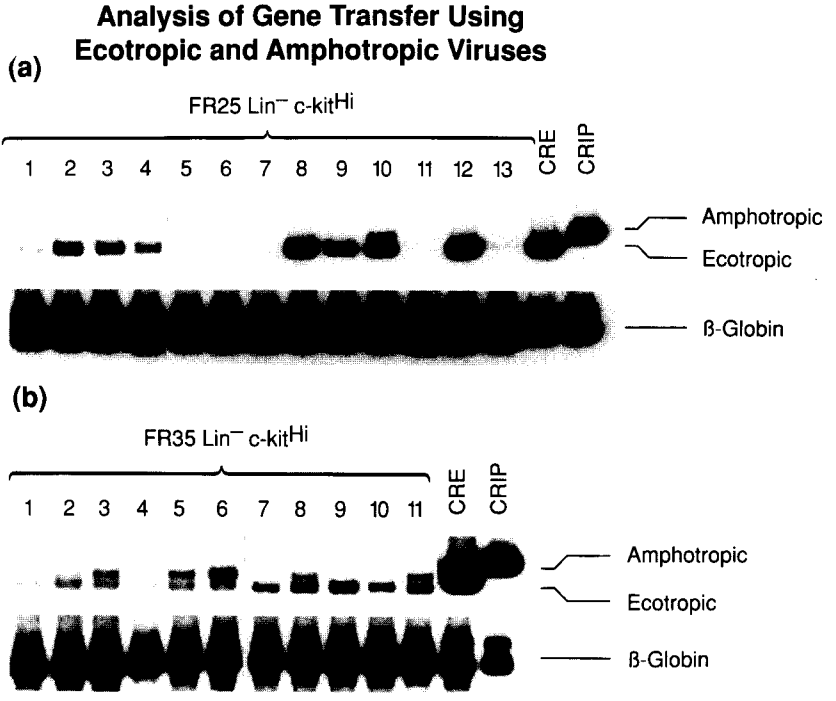

Figure 4. *Co-transduction of mouse FR25 Lin⁻ c-kit^{HI} and FR35 Lin⁻ c-kit^{HI} stem cells. Equal volumes of supernatant from the MFG-lacZ and MFG-NLS lacZ producer cell lines were mixed and used to transduce enriched stem cell populations (96 hours; 4 changes) or 3T3 cells (overnight exposure). Top panel: Analysis of DNA extracted from the peripheral blood of W/W^{v} mice 16 weeks after transplantation with transduced FR25 lin⁻ c-kit^{HI} cells. DNA was amplified using the PCR primers shown in Figure 3. The sizes of the fragments corresponding to the amphotropic and ecotropic retroviruses are 311 and 290 bp respectively. These mice represent two independent experiments. The mouse adult β-globin genes (4 genes per cell) were co-amplified as an internal control (bottom). Lower panel: Analysis of DNA extracted from the peripheral blood of W/W^{v} mice 16 weeks after transplantation with transduced FR35 lin⁻ c-kit^{HI} cells. These mice represent two independent experiments. The mouse β-globin gene was co-amplified as an internal control (bottom).*

of expression of amphotropic retrovirus receptor mRNA detected in FR35 Lin⁻ c-*kit*^{HI} cells, amphotropic MFG-NLSlacZ proviral sequences were detected in 6 of the 11 animals. In three mice, the level of amphotropic proviral sequences was greater than or equal to the ecotropic proviral sequences in the same animal. The ratio of amphotropic to ecotropic retrovirus transduction into FR35 Lin⁻ c-*kit*^{HI} cells was 0.625 ± 0.7 (p=0.018; Figure 4). To exclude the possibility that

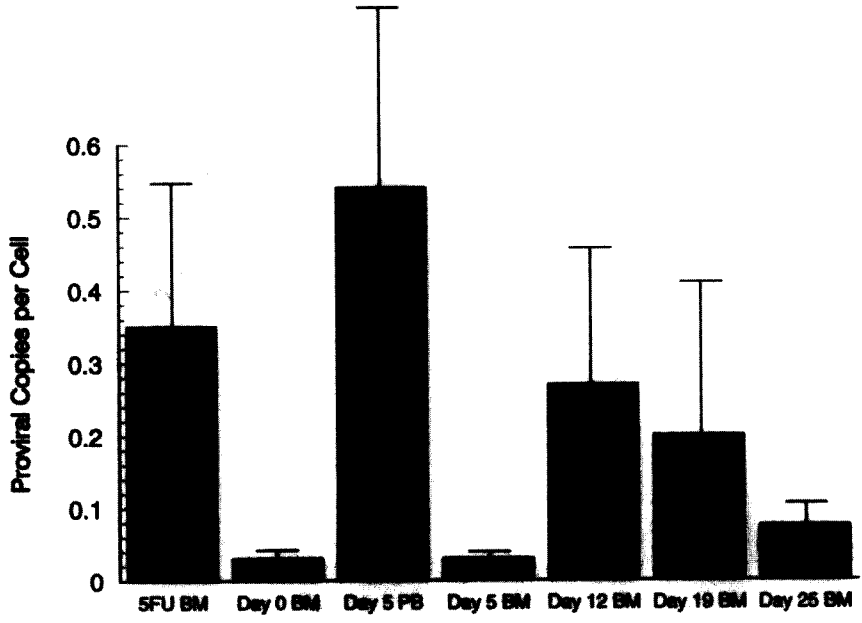

Figure 5. *Proviral copy number in the peripheral blood of mice 16 weeks after transplantation. The bars represent the mean provirus copy number (± standard deviation) in the peripheral blood of mice transplanted with transduced cells collected at the times indicated below the figure. 5-FU: Positive control animals transplanted with transduced 5-FU treated marrow. Day 0: Pretreated bone marrow cells. Day 5 PB: Peripheral blood cells collected after 5 days of treatment with G-CSF and SCF. Day 5 (12,19,25) BM: Bone marrow cells collected 5 (12,19,25) days after treatment with G-CSF and SCF was initiated (G-CSF/SCF treatment was terminated on day 5). Each bar represents the pooled data from a minimum of 20 mice in 4 independent experiments.*

transduction of progenitors among the FR35 Lin^- c-kit^{HI} cells was responsible for the positive signals, animals were analyzed 16 weeks post transplantation, and complete repopulation with donor cells was demonstrated using polymorphisms in the mouse β-globin gene.[10,16]

Retroviral transduction efficiency of G-CSF/SCF primed BM and PB in the rhesus autologous transplantation model

We have previously shown that following a 5-day course of treatment with G-CSF and SCF, mice have an immediate increase in the number of peripheral blood HSC, followed by a 10-fold increase in BM HSC activity that peaks at 14 days after cytokine treatment.[4] We hypothesized that the increase in HSC activity might

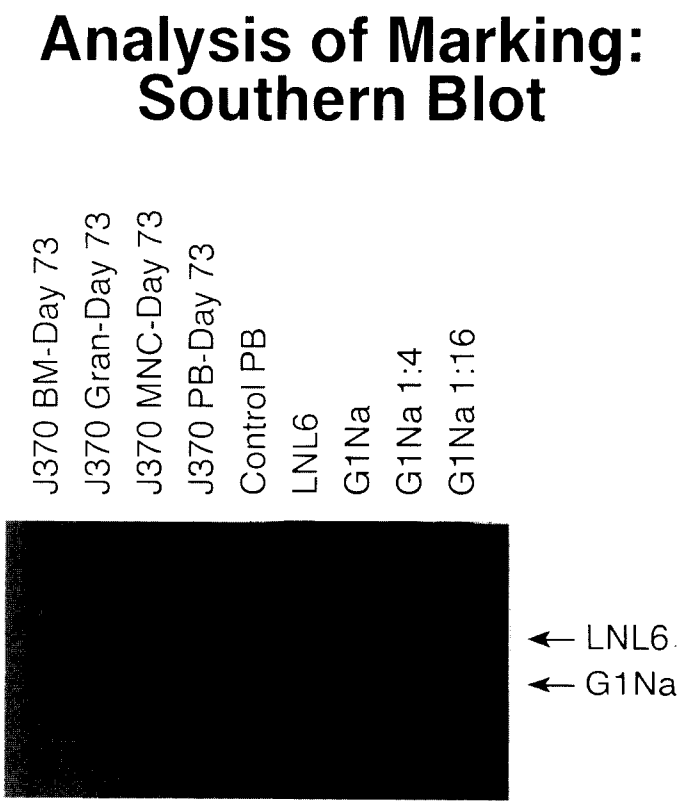

Figure 6. *Southern blotting for the presence of the Neo gene in samples from monkey J370 post-transplantation. DNA samples from nucleated bone marrow cells (BM), purified granulocytes (GRAN) and mononuclear cells (MNC), and unfractionated peripheral blood nucleated cells (PB) from J370, negative control PB from a nontransplanted monkey, and positive control standards of dilutions of (single copy) producer cell line DNA into normal BM DNA were cut with SacI, an enzyme which flanks the Neo transgene in both LNL6 and G1Na, and probed with a neo gene probe.*

be accompanied by an increase in cycling HSC, which would facilitate retrovirus-mediated gene transfer. To test this hypothesis, we compared ecotropic retrovirus transduction of HSC in populations of untreated BM cells, G-CSF and SCF mobilized peripheral blood mononuclear cells, and G-CSF and SCF primed BM cells collected at various times after G-CSF and SCF treatment to our standard

transduction of BM from 5-FU treated donors. A total of 4 experiments were performed using 2 different retrovirus vectors, and the pooled results for all 4 experiment are shown in Figure 5. Compared with 5-FU treated marrow, minimal levels of transduction HSC in untreated BM and in BM collected immediately after G-CSF and SCF treatment were detected. In contrast, transduction of G-CSF and SCF mobilized peripheral blood HSC was higher than that of 5-FU treated BM HSC, and the transduction of BM collected 7–14 days after G-CSF and SCF treatment was equivalent to that of 5-FU treated BM. We concluded from these results that G-CSF and SCF priming of either the BM or peripheral blood promoted gene transfer with the same or greater efficiency as 5-FU treatment.

We then compared amphotropic retrovirus transduction into rhesus monkey peripheral blood and BM collected after G-CSF/SCF treatment. Two animals were treated with SCF and G-CSF for five days and peripheral blood cells were collected via apheresis on the fifth day. Previous studies in rhesus monkeys and baboons by us and others have shown maximal CFU-GM mobilization on days 4–6 after initiation of SCF and G-CSF.[39] CD34-enriched peripheral blood stem and progenitor cells were transduced for 96 hours in suspension culture with either LNL6 or G1Na Neo vectors (titer: $1–5\times10^5$) using a protocol that is similar to that used in human clinical trials.[2] Fourteen days after discontinuation of the G-CSF/SCF treatment, BM was harvested, enriched for cells expressing CD34, and transduced under identical conditions to the peripheral blood, using whichever vector was *not* used to transduce the peripheral blood cells.

All animals received over 10 million CD34-enriched and cultured cells per kilogram. Both peripheral blood and BM cells were reinfused after total-body irradiation. The two animals recovered granulocyte counts to greater than 500/μL on days 10 and 14, respectively, faster than rhesus monkeys in our previous studies transplanted with CD34-enriched steady-state BM that engrafted on days 18–24.[17] The animals were followed for the presence and the origin of the Neo gene after engraftment. Similar to the strategy described in Figure 3, the LNL6 and G1Na vectors can be distinguished by the presence of additional polylinker sequences in G1Na. Both proviruses can be amplified in the same reaction with one set of primers and separated by denaturing gel electrophoresis. Semiquantitative PCR analysis demonstrated high levels of the vectors exposed to the post cytokine treatment marrow and lower levels of the vector exposed to the peripheral blood cells. Mononuclear cells, granulocytes and BM cells had Neo gene signals corresponding to a copy number of 0.05/cell (5%). FACS-sorted populations of T cells in both animals and B cells in one animal were also positive for the marker gene, at levels of 3–5%.

The relatively high level of vector-containing blood cells we observed using semiquantitative PCR was confirmed by Southern blot analysis. A band hybridizing to a Neo probe could be detected after digestion of DNA from

animal J370 (Figure 6). The intensity was estimated to be between 5–10%. The band size indicates origin from the G1Na vector, which was used to mark the primed BM. When the samples were cut with a restriction enzyme cutting once within the provirus in order to study the number of insertion sites, no bands were detectable, suggesting that more than one clone was contributing to the marking (data not shown).

DISCUSSION

Retrovirus binding to target cells and cell cycle status are two factors recognized to be important for retrovirus transduction.[6] Our results indicate that amphotropic retroviruses transduce mouse HSC at lower frequencies than ecotropic retroviruses and at ratios consistent with the level of ampho R mRNA. In a previous study, Osborne et al.[40] showed that amphotropic retrovirus-mediated gene transfer into mouse HSC was inefficient, requiring preselection for transduced BM cells to achieve detectable levels of transduction. This study did not compare the efficiency of transduction of ecotropic and amphotropic retroviral vectors. In another study, Richardson et al.[41] were unable to detect amphotropic retrovirus-mediated gene transfer to unfractionated fetal liver cells, while transduction with ecotropic retroviruses was detected. Gene transfer into fetal liver HSC was not analyzed.

Our results are consistent with the hypothesis that the low level of retrovirus-mediated gene transfer into murine and primate HSC is a consequence of a low level of expression of ampho R mRNA. Human hematopoietic progenitor cells ($CD34^+$ $CD38^+$) exhibit higher levels of ampho R mRNA and are efficiently transduced by amphotropic retroviruses. In the mouse, the levels of ecotropic receptor mRNA are similar in HSC and progenitor cells (c-kit^{LO}), both of which can be transduced relatively efficiently. The similarity of gene transfer efficiencies into mouse and primate HSC using amphotropic vectors[17–18] indicates that the mouse may be a suitable model system to develop improved amphotropic retrovirus-mediated gene transfer protocols.

There are potential solutions to the problems presented by the low level of expression of ampho R mRNA. We have shown that we can isolate a subpopulation of mouse HSC expressing higher levels of ampho R mRNA, which are more efficiently transduced by amphotropic retrovirus vectors. We predict that if a population of HSC expressing higher levels of ampho R receptor mRNA can be isolated, gene transfer efficiency can be improved. Our results in rhesus monkeys indicate that amphotropic retrovirus-mediated gene transfer efficiency is greater in G-CSF- and SCF-primed BM and peripheral blood. We hypothesize that this may be due to an increase in the number of HSC expressing higher levels of the mRNA encoding the amphotropic retrovirus receptor mRNA. This hypothesis is being

tested. Additional cell populations to examine would include cord blood stem cells, which have been identified as excellent targets for gene transfer using amphotropic retroviral vectors based on a greater efficiency of gene transfer into progenitor cells.[42,43]

In both murine and human experiments the percentage of cells with the HSC phenotype (Lin^- $Sca\text{-}1^+$ $Thy\text{-}1.1^-$; Lin^- c-kit^{HI}; Lin^- $CD34^+$ $CD38^-$) that are in the G_2 or M stage of the cell cycle is less than 3%.[24] This low level of cycling among HSC remains a problem for retrovirus-mediated gene transfer into HSC since retroviral integration requires cell division.[8] We have shown that treatment of normal mice with G-CSF and SCF causes a transient increase in HSC number, first in the peripheral blood and later, in the BM.[4] Cytokine priming may cause the treated HSC to engraft more efficiently or become "activated"—or more likely—cytokine priming causes an increase in the absolute number of HSC. This hypothesis is supported by the demonstration of increased numbers of Lin^- c-kit^{HI} cells in murine peripheral blood and BM and increased numbers of $CD34^+$ $CD38^{LO/-}$ cells in the BM of the rhesus monkey following G-CSF and SCF treatment (Dunbar et al., in press). A critical experiment will be to determine the cell cycle status and the level of ampho R mRNA in these cells. The results of these experiments should help to determine whether the increase in amphotropic retrovirus-mediated gene transfer is due to increased levels of ampho R mRNA, increased HSC cycling or a combination of the two.

REFERENCES

1. Brenner MK et al.: Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. *Lancet* 342:1134–1137, 1993.
2. Dunbar CE et al.: Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. *Blood* 85:3048–3057, 1995.
3. Kohn DB et al.: Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. *Nature Med* 1:1017–1023, 1995.
4. Bodine DM, Seidel NE, Orlic D: Bone marrow collected 14 days after in vivo administration of granulocyte colony-stimulating factor and stem cell factor to mice has 10-fold more repopulating ability than untreated bone marrow. *Blood* 88:89–97, 1996.
5. Anderson WF: Prospects for human gene therapy. *Science* 226:401–409, 1984.
6. Mulligan RC: The basic science of gene therapy. *Science* 260:926–932, 1993.
7. Thomas ED et al.: Bone marrow transplantation (two parts). *N Engl J Med* 292:832–902, 1975.
8. Varmus H: Retroviruses. *Science* 240:1427–1435, 1988.
9. Dzierzak EA, Papayannopoulou TH, Mulligan RC: Lineage specific expression of a human β-globin gene in murine bone marrow transplant recipients reconstituted with retrovirus-transduced stem cells. *Nature* 331:35–41, 1988.

10. Bodine DM, Karlsson S, Nienhuis AW: Combination of interleukins 3 and 6 preserves stem cell function in culture and enhances retrovirus-mediated gene transfer into hematopoietic stem cells. *Proc Nat Acad Sci USA* 86:8897–8901, 1989.
11. Lim B, Apperly JF, Orkin SH, Williams DA: Long-term expression of human adenosine deaminase in mice transplanted with retrovirus infected hematopoietic stem cells. *Proc Nat Acad Sci USA* 86:8892–8896, 1989.
12. Belmont JW et al.: Expression of adenosine deaminase in murine hematopoietic cells. *Mol Cell Biol* 8:5116–5125, 1988.
13. Wilson JM, Danos O, Grossman M, Raulet DH, Mulligan RC: Expression of human adenosine deaminase in mice reconstituted with retrovirus-transduced hematopoietic stem cells. *Proc Nat Acad Sci USA* 87:439–443, 1990.
14. Jordan CT, Lemischka IR: Clonal and systemic analysis of long-term hematopoiesis in the mouse. *Genes Dev* 4:220–232, 1990.
15. Wolf JH et al.: Reversal of pathology in murine mucopolysaccharidosis type VII by somatic gene transfer. *Nature* 360:749–753, 1993.
16. Sorrentino BP et al.: Selection of drug-resistant bone marrow cells in vivo after retroviral transfer of human MDR1. *Science* 257:99–103, 1992.
17. Bodine DM et al.: Long-term in vivo expression of a murine adenosine deaminase gene in rhesus monkey hematopoietic cells of multiple lineages after retroviral mediated gene transfer into $CD34^+$ bone marrow cells. *Blood* 82:1975–1980, 1993.
18. van Beusechem VW, Kakler A, Meidt PJ, Valerio D: Long-term expression of human adenosine deaminase in rhesus monkeys transplanted with retrovirus infected bone marrow cells. *Proc Nat Acad Sci USA* 89:7640–7644, 1992.
19. Albritton LM, Tseng L, Scadden D, Cunningham JM: A putative murine ecotropic retrovirus receptor encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. *Cell* 57:659–666, 1989.
20. Albritton LM, Kim JW, Tesng L, Cunningham JM: Envelope-binding domain in the cationic amino acid transporter determines the host range of ecotropic murine viruses. *J Virol* 67:2091–2096, 1993.
21. Miller DG, Edwards RH, Miller AD: Cloning of the cellular receptor for amphotropic murine retroviruses reveals homology to that for gibbon ape leukemia virus. *Proc Nat Acad Sci USA* 91:78–82, 1994.
22. van Zeijl M, Johann SV, Closs E, Cunningham J, Eddy R, Shows TB, O'Hara B: A human amphotropic retrovirus receptor is a second member of the gibbon ape leukemia virus receptor family. *Proc Nat Acad Sci USA* 91:1168–1172, 1994.
23. Kavanaugh MP, Miller DG, Zhang W, Law W, Kozak SL, Kabat D, Miller AD: Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters. *Proc Nat Acad Sci USA* 91:7071–7075, 1994.
24. Spangrude GJ, Smith L, Uchida N, Ikuda K, Hiemfeld S, Friedman J, Weissman IL, Mouse hematopoietic stem cells. *Blood* 78:7835–7840, 1991.
25. Spangrude GJ, Heimfeld S, Weissman IL: Purification and characterization of mouse hematopoietic stem cells. *Science* 241:58–62, 1988.
26. Visser JWM, Bauman JGJ, Mulder AH, Eliason JF, de Leeuw AM: Isolation of murine

pluripotent hematopoietic stem cells. *J Exp Med* 59:1576–1590, 1984.
27. Orlic D, Fischer R, Nishikawa SI, Neinhuis AW, Bodine DM: Purification and characterization of heterogeneous pluripotent hematopoietic stem cell populations expressing high levels of c-*kit* receptor. *Blood* 82:762–770, 1993.
28. Orlic D, Anderson S, Biesecker LG, Sorrentino BP, Bodine DM: Pluripotent hematopoietic stem cells contain high levels of mRNA for c-*kit*, GATA-2, p45 NF-E2, and c-myb and low levels or no mRNA for c-fms and the receptors for granulocyte colony-stimulating factor and interleukins 5 and 7. *Proc Nat Acad Sci USA* 92:4601–4605, 1995.
29. Sorrentino BP, McDonagh KT, Woods D, Orlic D: Expression of retroviral vectors containing the human multidrug resistance 1 cDNA in hematopoietic cells of transplanted mice. *Blood* 86:491–501, 1995.
30. Terstappen LWMM, Huang S, Safford M, Lansdorp Loken MR: Sequential generations of hematopoietic colonies derived from single nonlineage-committed $CD34^+CD38^-$ progenitor cells. *Blood* 77:1218–1225, 1991.
31. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, Peault B: Isolation of a candidate human hematopoietic stem-cell population. *Proc Nat Acad Sci USA* 89:2804–2808, 1992.
32. Eaves CJ: Peripheral blood stem cells reach new heights. *Blood* 82:1957–58, 1993.
33. Bodine DM, Seidel NE, Zsebo KM, Orlic D: In vivo administration of stem cell factor to mice increases the absolute number of pluripotent hematopoietic stem cells. *Blood* 82:445–55, 1993.
34. Bodine DM, Seidel NE, Gale MS, Nienhuis AW, Orlic D: Efficient retrovirus transduction of mouse pluripotent hematopoietic stem cells mobilized into the peripheral blood by treatment with granulocyte colony-stimulation factor and stem cell factor. *Blood* 84:1482–91, 1994.
35. Cassel A. Cottler-Fox M, Doren S, Dunbar CE: Retroviral-mediated gene transfer into CD-34 enriched human peripheral blood stem cells. *Exp Hematol* 21:585–591, 1993.
36. Miller AD, Buttimore C: Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol Cell Biol* 6:2895–2902, 1986.
37. Donahue RE, Kirby MR, Metzger ME, Agricola BA, Sellers SE, Sullis HM: Peripheral blood $CD34^+$ cells differ from bone marrow $CD34^+$ cells in Thy-1 expression and cell cycle status in nonhuman primates mobilized or not mobilized with granulocyte colony-stimulating factor and/or stem cell factor. *Blood* 87:1644–1653, 1996.
38. Orlic D, Bodine DM: Pluripotent hematopoietic stem cells of low and high density can repopulate W/W^v mice. *Exp Hematol* 20:1291–1295, 1992.
39. Andrews RG, Briddell RA, Knitter GH, Opie T, Bronsden M, Myerson D, Appelbaum FR, McNiece IK: In vivo synergy between recombinant human stem cell factor and recombinant human granulocyte colony-stimulating factor in baboons: Enhanced circulation of progenitor cells. *Blood* 84:800–810, 1994.
40. Osborne WRA, Hock RA, Kaleko M, Miller AD: Long-term expression of human adenosine deaminase in mice after transplantation of bone marrow infected with amphotropic retroviral vectors. *Hum Gene Ther* 1:31–41, 1990.
41. Richardson C, Ward M, Podda S, Bank A: Mouse fetal liver cells lack amphotropic retroviral receptors. *Blood* 1994:433–439, 1994.

42. Lu L, Xiao M, Clapp DW, Li ZH, Broxmeyer HE: High efficiency retroviral mediated gene transduction into single isolated immature and replatable ($CD34^{+++}$) hematopoietic stem/progenitor cells from human umbilical cord blood. *J Exp Med* 178:2089–2096, 1993.
43. Hanley ME, Nolta JA, Parkman R, Kohn DB: Umbilical cord blood cell transduction by retroviral vectors: Pre-clinical studies to optimize gene transfer. *Blood Cells* 20:539–546, 1994.

ACCURATE QUANTITATION OF CD34+ CELLS IN AN AUTOTRANSPLANT SETTING: THE ISHAGE GUIDELINES

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ABSTRACT

The increased use of peripheral blood stem cells (PBSC) to reconstitute hematopoiesis in both auto- and more recently, allo-transplant settings has not been associated with a consensus means to assess the engraftment potential of the PBSC product. Since reinfusion of the small population of cells that bear the CD34 antigen can lead to multilineage engraftment in intensively treated patients, graft assessment by flow cytometric quantitation of CD34+ cells should provide a rapid, reliable and reproducible assay. Unfortunately, the lack of a standardized method has led to the generation of widely divergent data. To address this problem, the International Society of Hematotherapy and Graft Engineering (ISHAGE) established a Stem Cell Enumeration Committee, the mandate of which was to validate a previously described flow cytometric method (FCM) to quantitate CD34+ cells in peripheral blood and apheresis products. It also sought to establish this method's applicability on a variety of flow cytometers in clinical laboratories and its reproducibility between transplant centers. Here, we describe the basic four-parameter flow protocol adopted by ISHAGE and show how it can be adapted by incorporating fluorescent beads in a three-color analysis, to generate an absolute CD34+ cell count per apheresis pack. Additionally, by incorporating a third antibody to the Thy-1 antigen in the analysis, candidate hematopoietic stem cells (HSC) exhibiting the CD34+/Thy-1+ phenotype can be enumerated. The ability to detect and quantitate this most primitive CD34+ subset at an early phase of the mobilization protocol may have implications for heavily pretreated patients from whom adequate total CD34+ cell numbers may be difficult to obtain based on an increase in total white blood cell count or on timed collections. Finally, the basic ISHAGE protocol can be adapted by incorporation of an anti-CD3 conjugate to assess residual T lymphocyte content of immuno-selected, CD34+ cell fractions of peripheral blood and bone marrow (BM) harvests in an allo-transplant setting.

INTRODUCTION

A variety of studies performed over the past several years have established that the 1–3% of cells in the bone marrow (BM) that express the cell-surface antigen CD34 are capable of reconstituting long-term, multilineage hematopoiesis after intensive, potentially myeloablative therapy.[1,2] $CD34^+$ cells can also be found in the peripheral blood of normal individuals but are extremely rare (consensus range 0.01–0.1%). However, $CD34^+$ cells can also be mobilized from the marrow to the peripheral circulation in far greater numbers by chemotherapy and/or hematopoietic cytokines.[3]

In recent years, the availability of a variety of recombinant cytokines to mobilize $CD34^+$ cells into the peripheral circulation has greatly facilitated the increased use of peripheral blood stem cells (PBSC) versus marrow for both auto-[4] and more recently, allo-transplantation.[5] With this increased use of PBSCs, it has become apparent that standardized methods are required for the accurate, sensitive and reproducible assessment of the engraftment potential of such PBSC collections. Until recently, the most widely used approach to assess the quality of PBSC grafts relied on colony forming cell (CFC) assays for lineage-committed hematopoietic progenitors such as CFU-GM. Due to variations in the way CFC assays are performed it has been difficult to compare data from different transplant centers and the minimum number of CFU-GM/Kg body weight has not been determined. The most serious limitation of CFC assays remains the 10–14 day interval required for assay readout. This characteristic alone precludes the assay any role in analysis of PBSC content to optimize timing of apheresis, as well as any role in the "on–line" quantitation of PBSC yield during apheresis. Furthermore, the CFC assays do not provide a means to assess more primitive precursors and true hematopoietic stem cells (HSC) that mediate long-term engraftment.

GRAFT ASSESSMENT BY FLOW CYTOMETRY

Since reinfusion of purified CD34 antigen-positive cells results in hematopoietic recovery following myeloablative therapy,[1,2] quantitation of cells bearing this cell surface molecule provides a rapid means to measure autograft potential. The $CD34^+$ population is heterogeneous, encompassing the earliest quiescent HSCs as well as maturing, committed progenitors of all lineages. By using multiparameter flow cytometric methodologies (FCM), it may be possible to address not only quantitative aspects of graft assessment but also qualitative composition (i.e., the candidate HSC content of the PBSC product). An FCM-based approach, since it can be performed in less than 1 hour, would also be suitable for the determination of optimal timing for apheresis collections and even the "on-line" evaluation of the apheresis product. Such a procedure, if

standardized, should be capable of generating data that is more directly comparable among different transplant centers. Finally, when "positive selection" techniques are employed to purify $CD34^+$ cells, the ability to accurately enumerate the $CD34^+$ cells as a percentage of a well-defined denominator such as total nucleated white blood cells is of critical importance in calculating the efficiency and yield of the $CD34^+$ cell purification technique employed.

Unfortunately, a consensus means to perform "rare event" analysis for $CD34^+$ cells in the PBSC transplant setting has not emerged. The traditional practice of using isotype matched control antibodies to set the "positive" analysis region for $CD34^+$ cells can result in highly erroneous enumeration of $CD34^+$ cells. Furthermore, sample variability is a significant problem with respect to numbers of red cells, platelets, platelet aggregates, nonspecifically stained adherent cells and cellular debris, all of which may be faithfully recorded by flow cytometers. Thus, the choice of different denominators, different gating strategies and methods of calculating absolute $CD34^+$ cell numbers have generated divergent data.[6] Finally, different investigators have employed a variety of CD34 antibodies conjugated to different fluorochromes. Not all CD34 antibodies and CD34 antibody conjugates efficiently detect all $CD34^+$ cells in every clinical sample.

In addressing the latter issue, there are a variety of CD34 antibodies that detect distinct epitopes on CD34. It has long been known that some of these antibodies depend on the presence of carbohydrate moieties, particularly terminal sialic acid residues, for their efficient binding. The CD34 antigen is a highly glycosylated structure containing both complex-type N-linked glycans as well as numerous highly sialylated O-linked glycans that cluster in an extended mucin-like amino-terminal domain.[7] The sensitivity of some CD34 epitopes to sialic acid removal, and the sensitivity of these and other epitopes to cleavage with a proteolytic enzyme from *Pasteurella haemolytica* that uniquely among proteases only cleaves glycoproteins rich in O-linked structures, allowed us to classify CD34 epitopes into three broad categories.[8] Of the seven CD34 antibodies designated at the IVth Leukocyte Differentiation Antigens Workshop, those whose epitopes were partially or totally sensitive to neuraminidase and also cleaved by the glycoprotease, were designated class I antibodies. Antibodies to epitopes that were resistant to sialidase, but sensitive to the glycoprotease were termed class II. Those insensitive to both enzymes were called class III antibodies. This scheme was subsequently adopted by the Vth International Leukocyte Differentiation Antigens Workshop as a standard for the classification of CD34 epitopes and their corresponding antibodies.[9]

Due to their dependence on terminal sialic acids, which are only found on the most fully glycosylated/processed forms of CD34, class I antibodies generate the most aberrant data in clinical samples, whereas class II and class III reagents detect similar, if not identical, numbers of $CD34^+$ cells in a wide variety of normal and

abnormal samples. Thus, for accurate enumeration of rare $CD34^+$ cells in PBSC collections, it is important to use a CD34 antibody that detects all glycosylation variants of the molecule, i.e., class II or class III antibodies. However, as indicated below, the choice of conjugate is also important in some instances.

$CD34^+$ CELL ENUMERATION BY FCM USING CD45/CD34 AND LIGHT SCATTER

In early attempts to circumvent problems with flow cytometric analysis of $CD34^+$ cells on highly heterogeneous clinical samples, we simultaneously stained mobilized PB stem cells with a pan-CD45 fluorescein isothiocyanate (FITC) and CD34 PE. CD45 FITC was chosen as a counter stain because it only stains nucleated white blood cells. By including only $CD45^+$ events in the analysis, red blood cells, their nucleated precursors, platelets and cellular debris, that are variably present in PB and apheresis preparations, and that are recorded by flow cytometers, are excluded from subsequent analysis. CD45 staining thus establishes a much more stable denominator.[10] Additionally, Steltzer et al.[11] and, more recently, Borowitz et al.[12] have shown that primitive normal and leukemic blast cells that exhibit similar light scatter properties of lymphocytes express lower levels of CD45 on their surfaces, thus providing a means of delineating non-specifically stained lymphocytes from normal or leukemic blast cells using this surface marker.

Although the ISHAGE protocol has recently been published in detail,[13] what follows is a brief description of how we use this simple two-color, two light-scatter parameter protocol to identify and enumerate $CD34^+$ cells in normal hematopoietic samples, cord blood and apheresis collections.

On FCM analysis of CD45 staining versus side scatter (granularity), three major populations of $CD45^+$ cells can be identified (Figure 1, plot 1): lymphocytes (low granularity and bright CD45 staining), monocytes (slightly lower CD45 staining and intermediate side scatter) and neutrophils (low CD45 staining and high side scatter). $CD45^-$ events, which by light scatter analysis and microscopy appear to consist mainly of unlysed red blood cells and platelet debris and whose numbers can vary enormously from sample to sample, are thus excluded. When the $CD45^+$ cells in region R1 are analyzed for CD34 PE staining (plot 2), a population of $CD34^+$ events can be detected in region R2. The cells contained in the $CD34^+$ fraction R2 are then displayed on another CD45 versus side scatter dot-plot (plot 3), and true $CD34^+$ events gated from region R2 form a discrete cluster. This cluster is characterized by uniform low side scatter and weak CD45 staining that can then be gated by region R3. Nonspecifically stained events from R2 scatter elsewhere in the dot plot and are excluded from region R3. In keeping with observations by microscopy, region R3 cells exhibited a relatively restricted range of (medium) cell

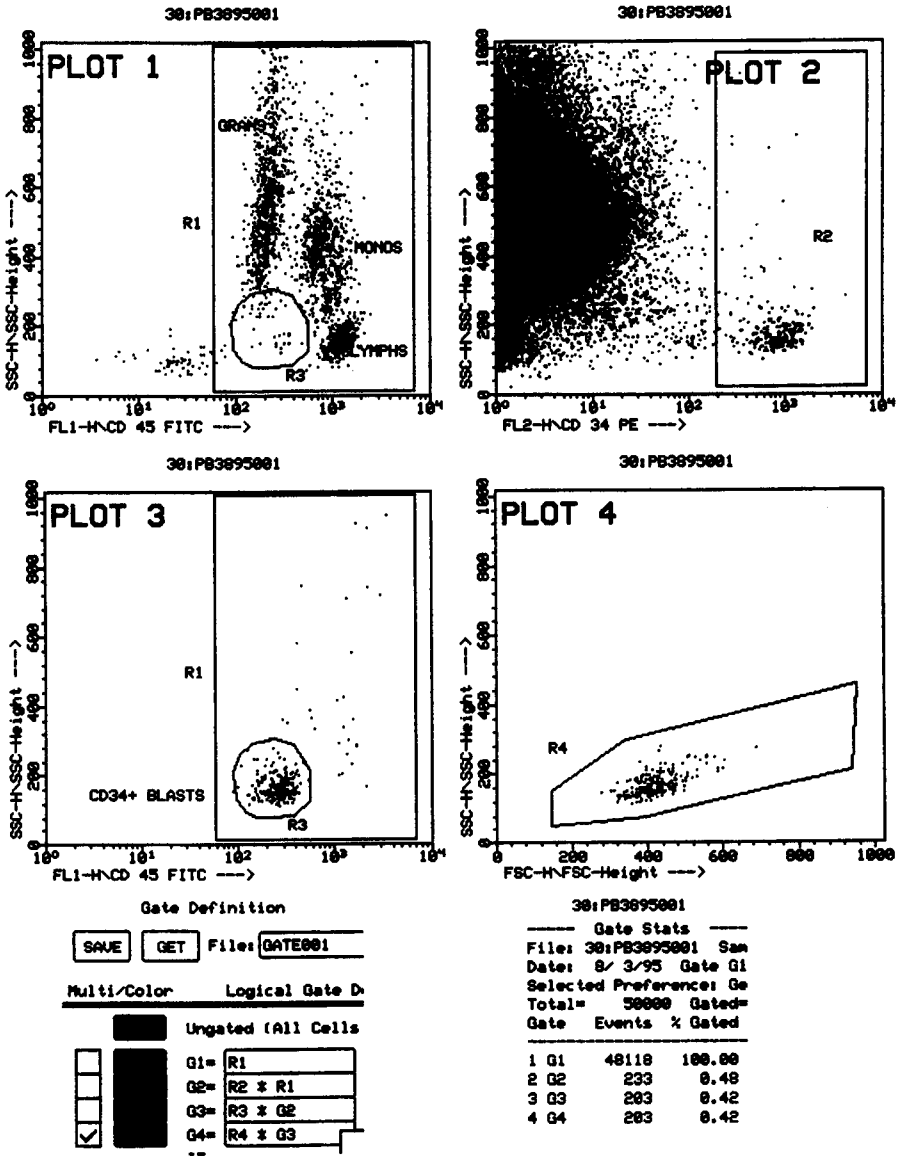

Figure 1. *Enumeration of CD34⁺ cells in an apheresis sample using CD45-FITC/CD34-PE. Precise details of gating strategy are described in reference 13. Sample analyzed on BD FACScan running Lysis II software.*

size and are found within the generic blast/lymphocyte region R4 when analyzed by side scatter and forward scatter parameters (plot 4). Statistical analysis of the gated populations in figure 1 showed the number of CD34⁺ cells to represent 0.42%

of the total nucleated white blood cell fraction ($CD45^+$) identified by region R1. $CD34^+$ events were not detected in the $CD45^-$ fraction of this sample nor in that of many other similar analyses (data not shown), confirming that if an appropriate pan-CD45 conjugate is used, all subsets of peripheral blood $CD34^+$ cells can be demonstrated to express CD45 molecules, albeit at relatively low levels.

This FCM gating strategy works well on cord blood and BM samples.[10,13] Indeed, the impetus to develop an accurate and reliable $CD34^+$ cell enumeration methodology initially arose from an inability to quantitate $CD34^+$ cells in BM aspirates, from which we were purifying $CD34^+$ cells using the glycoprotease technique.[14] To calculate meaningful estimates for the recovery of $CD34^+$ cells, starting numbers in the unseparated marrow sample had to be quantitated. In BM , however, some extra difficulties have to be dealt with. Marrow $CD34^+$ cells, unlike their peripheral blood counterparts, come in a variety of sizes—from that of small lymphocytes to intermediate size blast cells to quite large megakaryoblast/granulomonocyte progenitors. Additionally, they exhibit a wider range of granularity or side scatter characteristics as they mature into lineage-committed precursors. Finally, they exhibit a much greater range of CD34 staining intensities in comparison with mobilized peripheral blood $CD34^+$ cells. However, this gating strategy outlined above also works well for BM and, in contrast to other less sophisticated FCM procedures, generates data that are similar to that observed by fluorescence microscopy. Thus, it has greatly facilitated some of our research goals.

THE ISHAGE GUIDELINES

This $CD34^+$ cell detection procedure incorporating the sequential gating strategy outlined above was published in 1994[10] and we were subsequently invited by the International Society of Hematotherapy and Graft Engineering (ISHAGE) to establish a Stem Cell Enumeration Committee to validate this procedure in a multi-center study. The mandate of this committee was to construct clinical guidelines for the quantitation of $CD34^+$ cells in peripheral blood based upon our methodology and to design a multi-center study to assess whether reproducible data could be generated from prestained/fixed samples distributed to the participating transplant centers. We also sought to establish its utility and reproducibility on the various types of flow cytometer currently used in clinical laboratories. Looking to the future, we sought, using the data generated from the first phase of this study, to develop clinical guidelines to optimize timing of apheresis collections and establish recommendations for a threshold $CD34^+$ cell dose for rapid and sustained engraftment. We also sought, by incorporation of a third antibody conjugate, to measure the qualitative aspects of PBSC $CD34^+$ cells. For example, by incorporating an antibody to the Thy-1 antigen, one can enumerate candidate HSCs with the $CD34^+Thy\text{-}1^+$ phenotype.[15]

By April 1995, the first draft of the Clinical Guidelines was produced (Chin-Yee et al.: Enumeration of Hematopoietic Stem Cells in Peripheral Blood: Guidelines for the Performance of $CD34^+$ Cell Determination). Although this 35-page document was originally produced for the use of participants in the validation study and was not initially intended for formal publication, it was widely circulated both directly and indirectly to a large number of clinical and research laboratories in North America, Europe and the rest of the world. The basic protocol has also been included in other studies in Canada (Chin-Yee et al.: unpublished observations) and Australia (Chan et al.: J Hematother 5:605, 1996) that had started prior to the availability of the ISHAGE Guidelines. The results generated with our protocol showed greater concordance over multiple sites compared with data generated using other procedures in these studies.

The ISHAGE validation study has yet to be completed and the data from it analyzed, but the original procedure has been significantly upgraded due in part to the feedback received from a large number of transplant centers throughout the world who have adopted the protocol. Thus, an updated version of the earlier document was recently published.[13]

ANTIBODY SELECTION

CD45 antibodies

The following pan-CD45 antibodies can be safely recommended: HLE-1 (Becton Dickinson), KC56 (Coulter Corp.) and J33 (Immunotech). Although other CD45 antibodies can be used, it is important to know that the selected antibody is a pan CD45 reagent and as outlined in detail in the Guidelines document, that this epitope is sialidase and glycoprotease-resistant.

CD34 antibodies

CD34 antibodies that detect all glycoforms of this mucin-like structure (i.e., class II or class III reagents) are required for $CD34^+$ cell enumeration. The PE conjugates of the Class II clone QBEnd10 (Immunotech), and the PE conjugates of the class III antibodies HPCA2 (Becton Dickinson) and 581 (Immunotech) work interchangeably in the ISHAGE method. Although generally preferable to use PE conjugates in two-color analyses it is sometimes necessary to employ FITC conjugates of the CD34 reagent when three antibodies are used. In such cases, FITC conjugates of class III reagents detect similar numbers of $CD34^+$ cells using the ISHAGE protocol as their PE conjugated versions and can thus be utilized. However, the FITC conjugates of class II antibodies such as QBEnd10 do not identify $CD34^+$ cells in clinical samples as efficiently as their PE-labeled counterparts due in major part to the extra negative charge conferred on such conjugates by this fluorochrome. Additionally, FITC conjugates of CD34 antibodies

can increase the levels of nonspecific staining of dead cells present in some clinical samples and the FITC conjugate of QBEnd10 can be particularly problematic in this regard.[16,17] A number of studies using FITC-conjugated class II antibodies have been published without regard to this problem. The inappropriate choice of FITC conjugated class II reagents may explain the divergent results reported in some of these studies.[18] Thus, one has to be careful not only in selecting an appropriate CD34 antibody clone, but the choice of fluorochrome can also affect the results.[13]

Isotype controls

As every monoclonal antibody has slightly different "nonspecific" binding characteristics from the next, it is our view that "appropriate isotype controls" for rare event analysis do not exist. Use of an isotype control to set a positive analysis region for rare event analysis can thus result in either the inclusion of "nonspecifically" stained events in the $CD34^+$ cell analysis region, or it can mask the staining of rare, bona fide $CD34^+$ cells. However, given the sequential gating strategy at the heart of our FCM method, we believe that the use of isotype control antibodies for the purpose of establishing the positive analysis region is not necessary. Just as the appropriate titration of monoclonal reagents is critical in distinguishing the boundary between true positive and negative cells, the isotype control should also be titrated. At the appropriate concentration, the isotype control should give the same level of fluorescence staining on a CD34-negative leukocyte population (e.g., lymphocytes) as exhibited by the CD34-PE reagent. At that concentration, staining histograms of non-$CD34^+$ events generated by the two reagents should be very similar. However, even at the optimum concentration, it is our experience that some isotype control antibodies stain more leukocytes (nonspecifically) than are specifically stained by the CD34-PE antibody in use. Even in these circumstances, these events are "gated out" by the sequential gating strategy at the heart of this method since it is rare for any of them to exhibit the other characteristics of true $CD34^+$ cells. If any such isotype-positive events appear in region 4, they are simply subtracted from the $CD34^+$ cell numbers enumerated in the CD45/CD34 tube(s). Thus in the ISHAGE protocol, gating regions are established on the "positive analysis" tube (CD45/CD34) as shown in Figure 1. The nonspecifically stained events are enumerated for the CD45/isotype control tube using exactly the same gating regions.[13]

SENSITIVITY

We have analyzed a large number of PBSC samples as well as a number of normal peripheral blood samples. If 100,000 events are collected in listmode on the flow cytometer, we can readily detect 10–20 events as a cluster in regions 3 and 4 of the analysis, translating into a sensitivity of 0.01–0.02%. In our ISHAGE

validation studies, the first stained/fixed sample sent out for multicenter analysis contained only 0.04% $CD34^+$ cells. However, those centers that followed the Guidelines were able to accurately detect these cells with a high degree of reliability. Using the same gating regions to analyze non-specifically stained events in the CD45/isotype control tube, events were not detected in region 4. Notwithstanding the sensitivity of this FCM strategy, it is recommended that a minimum of 100 $CD34^+$ events be collected to maintain precision and reliability.

ABNORMAL CLINICAL SAMPLES

Working in clinical research laboratories, our method has also been able to detect abnormalities of the $CD34^+$ cell compartment in some interesting clinical samples. In one case, a BM sample from a supposedly normal donor contained approximately 14% $CD34^+$ cells as assessed by microscopy and about half of the $CD34^+$ cells contained granular cytoplasm. FACScan analysis using the CD45/CD34 technique confirmed these findings. When the $CD34^+$ cells from region R2 were analyzed for CD45 expression versus side scatter, they exhibited a wide range of granularity. When the events gated into this elongated region R3 were assessed by forward versus side scatter, only about half of the $CD34^+$ cells were resolved in the generic blast/lymphocyte gate R4. In this case, the most accurate estimate of $CD34^+$ cell numbers is represented by the events gated into region R3 (representing about 13% of the white blood cells), rather than R4, due to large numbers of $CD34^+$ cells exhibiting increased side scatter that fall outside of the blast/lymphocyte gate. Interestingly, the subsequent clinical diagnosis of myelodysplastic syndrome/refractory anemia with excess blasts provides an explanation for these observations since refractory anemia may predate the onset of overt acute myeloblastic leukemia. The most prevalent type of myeloid leukemia in these patients is FAB M2, the brief clinical definition of which is "blasts with differentiation." Significantly, the FCM analysis of this sample performed in a routine flow cytometry laboratory where the technique outlined here was not in use, produced a $CD34^+$ cell number of only 2.8%, a value well within the range for $CD34^+$ cells in normal marrow.

In other studies, perturbations in the CD34 compartment of atopic individuals were also detectable and the increased numbers of $CD34^+$ cells detected in the marrow and blood of such samples correlated with a specific increase in the basophil/eosinophil colony forming cell numbers.[19]

USING THE ISHAGE PROTOCOL IN A CLINICAL SETTING

The four-parameter procedure that has been adopted for validation by ISHAGE can be used to quantitate $CD34^+$ cells in a variety of normal hematopoietic tissues as well as abnormal clinical samples from a variety of disease states. The former is

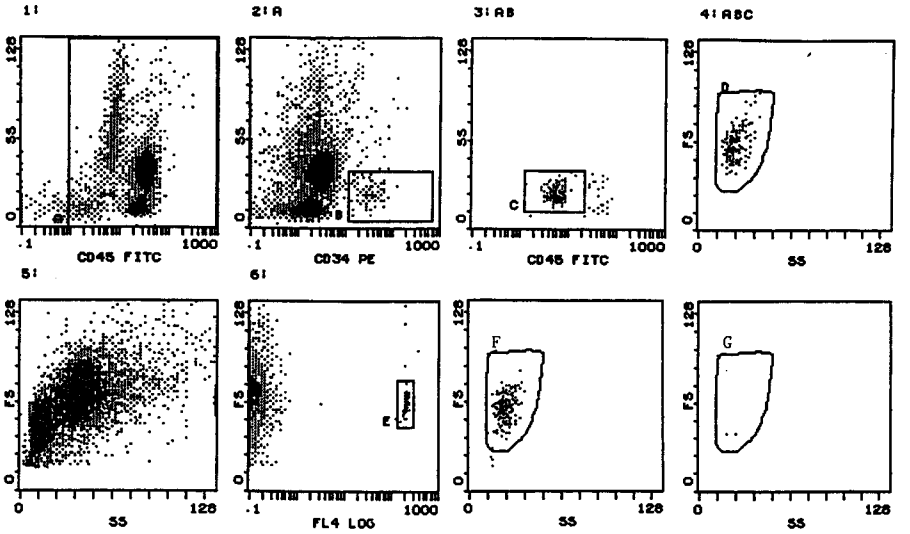

Figure 2. *Generating an absolute CD34⁺ cell count from an apheresis pack using Flow-Count beads (Coulter, Miami FL), and the ISHAGE protocol. Sample analyzed on Coulter EPICS XL. Gating regions A–D are equivalent to regions 1–4 in Figure 1. Gate F shows the analysis of the duplicate CD34/CD45 tube. Gate G enumerates the events stained using the IgG1/CD45 tube. The number of beads analyzed is gated in region E.*

important because BM transplants are still performed in some centers and cord blood transplantation is increasingly used in specific circumstances. Furthermore, CD34⁺ cell selection from both marrow and peripheral/cord blood is now being performed routinely. For researchers interested not just in graft assessment in a PBSC transplant setting, it is important to have available a reliable procedure that can generate accurate data from a variety of hematopoietic sources. It is highly sensitive, detecting 1 CD34⁺ cell in 10,000, and reproducible. It is highly specific using appropriate CD34 reagents (i.e., antibodies like QBEnd10 and 8G12 and 581) which in PE conjugated form, appear to detect all glycoforms of CD34. However, as outlined above, only class III reagents should be used where CD34 FITC conjugates are required, as in some three-color analyses.

The analysis is simple and can be performed on most single laser instruments as it requires only routine instrument settings and only basic software for data analysis, making it suitable for routine analysis in flow cytometry laboratories. Sample variability with respect to the number of red cells, dead cells, platelets, platelet aggregates and other cellular debris is minimized by the sequential gating strategy.

The interpretation is simple and one can express the results as a percentage of $CD45^+$ cells (i.e., nucleated WBC) or by incorporating a leukocyte count determined by an automated hematology analyzer in the calculation, as an absolute $CD34^+$ cell count. In the example shown below, the number of non-specific events in R4 from the isotype control tube is subtracted from the average total number of events in the duplicate CD34 stained sample tubes. This corrected number of $CD34^+$ cells is the numerator and the average number of $CD45^+$ events from the CD45-FITC/CD34-PE sample represents the denominator. This value is multiplied by the absolute LKC as determined by the automated hematology analyzer to calculate the absolute CD34 stem/progenitor cell numbers in the sample.

$$\text{Absolute LKC } (\times 10^9/\text{L}) \times \frac{(\text{CD34}^+ \text{ events})}{(\text{CD45}^+ \text{ events})} \times 1000^* = \text{Absolute CD34}^+ \text{ cells} \times 10^6/\text{L}$$

*The final value is multiplied by a factor of 1000 to convert from 10^9/L to 10^6/L.

EXAMPLE:

421 ($CD34^+$ events); 75,000 ($CD45^+$ events); 5.2×10^9/L (absolute LKC);

$$5.2\ (\times 10^9/\text{L}) \times \frac{421}{75{,}000} \times 1000 = 29.1 \times 10^6/\text{L}$$

To determine the absolute $CD34^+$ cell number per apheresis pack, the above number is multiplied by the pack volume in liters.

ABSOLUTE CD34+ CELL COUNTING USING FLUORESCENT BEADS

In a recent development of our protocol, we have used fluorescent microspheres to generate an absolute $CD34^+$ cell count on a single instrument platform. In this modification, fluorescent beads (at a known concentration) are added to the stained sample following red cell lysis. The number of $CD34^+$ cells are identified using the basic ISHAGE Guidelines and compared with the total number of beads counted in the third fluorescence channel of the cytometer. This simple modification eliminates the need to perform an absolute leukocyte count on an automated hematology analyzer. As different types of analyzers can generate different numbers from the same sample, eliminating the use of such an instrument reduces a significant source of error in the calculation of "absolute $CD34^+$ cell count." Indeed, it is the accurate determination of the latter number that clinicians rely upon when assessing graft adequacy of PBSC collections. Another benefit of including the fluorescent beads in a three-color analysis is that the CD45 positivity is no longer used as a denominator in the calculation of absolute $CD34^+$ cells. Instead, the CD45 expression is used solely as part of the sequential gating strategy to accurately identify bona fide $CD34^+$ cells. This modification also eliminates the potential introduction of errors in calculating the absolute $CD34^+$ cell count. The operator does not need to be concerned about the presence of nucleated red blood

cells which can express low levels of CD34 and little or no CD45 and are counted as LKCs by some automated hematology analyzers currently in use. While the presence of significant numbers of nucleated red cells (that are irrelevant to long-term engraftment) in apheresis samples is thought to be quite rare, excluding them reduces the possibility that their presence can affect the accurate determination of the absolute $CD34^+$ cell number. An example of the use of such beads in the ISHAGE protocol is shown in Figure 2. The calculation involved in generating an absolute $CD34^+$ cell count per apheresis pack is:

$$\frac{\#CD34^+ \text{ events} \times \text{bead concentration} \times \text{cell dilution factor} \times \text{pack volume (liters)} \times 10^6}{\#\text{beads counted}}$$

ASSESSING $CD34^+$ CANDIDATE HEMATOPOIETIC STEM CELLS

As outlined in detail elsewhere,[15] more sophisticated analysis is possible using our basic method. For example, it is possible using the third fluorescence channel of the cytometer to assess other surface antigens such as Thy-1, or CD109, the co-expression of which is associated with the most primitive stem cell subsets of $CD34^+$ cells in marrow. It was recently shown that the Thy-1^+ subset of $CD34^+$ cells in PBSC samples was also enriched for the most primitive colony-forming cells currently detectable in long-term cultures. Furthermore, the Thy-1^+ subset was capable of multi-lineage engraftment in immune deficient mice.[20] Some mobilizing regimens may prove more efficient than others at mobilizing the candidate HSC subset of $CD34^+$ cells, particularly in heavily pretreated patients. Thus, though there may be fewer *total* $CD34^+$ cells than would be desirable, there may be a sufficient number of these primitive cells to effect (albeit delayed) engraftment. Given the increasing numbers of growth factors that can be used to stimulate hematopoiesis in vivo, it may be possible to obtain an adequate autograft from fewer $CD34^+$ cells than the minimum dose currently advocated (about 2×10^6 cells/kg body weight).

In three-color analysis, cells are stained with CD45 PE:Cy-5, CD34 FITC (a class III reagent must be used) and Thy-1 PE (clone 5E10, Pharmingen Inc., San Diego CA). The $CD34^+$ cells are gated as before using the four parameter gating strategy. Duplicate samples contain either IgG1 PE control or Thy-1 PE antibodies. In this instance, the isotype control determines the "positive analysis region" for the Thy-1^+ $CD34^+$ cells.

We used this modified approach in 12 myeloma patients in a recent study in which peripheral blood samples were taken daily after chemotherapy and during mobilization with GM-CSF. Our data indicated that the level of $CD34^+$/Thy-1^+ cells was highest before collection would normally be scheduled to take place, if collection timing criteria were determined by either an increase in white cell count or by predetermined timing. Thus, the most primitive subsets of $CD34^+$ cells enter the peripheral circulation first and are most numerous before an increase in general white

cell numbers is detectable. Consequently, a window of opportunity for "optimal" collection may exist and the application of serial measurements of the $CD34^+/Thy\text{-}1^+$ cell concentration in the circulation to define early collection days may have merit. This approach may be especially useful for those heavily pretreated myeloma patients with a low likelihood of successful collection, especially given the recently reported increase in malignant cell contamination of apheresis products noted on later collection days.[21] If this window of opportunity is missed while awaiting an increase in the WBC before beginning the apheresis collection, subsequent attempts to mobilize these patients have a low likelihood of success.[22] Analysis of the results[15] also indicated that the frequency of circulating $CD34^+$ cells not only best predicted the apheresis $CD34^+$ and CFU-GM but was the only predictor of the apheresis $CD34^+/Thy\text{-}1^+$ cell count. Thus, daily peripheral blood CD34 enumeration most accurately predicts the progenitor/stem cell content of the apheresis collection, and this assay could also allow for optimal collection of candidate stem cell subsets of $CD34^+$ cells expressing the $CD34^+/Thy\text{-}1^+$ immunophenotype.

In summary, the experiments outlined above, and in rather more detail where indicated, show that the basic two-color sequential gating strategy of the ISHAGE protocol is extremely flexible and can be used to analyze $CD34^+$ cells in a variety of hematologic tissues and in different clinical situations. Using three- and four-color flow cytometers, the third and/or fourth fluorescence channel can be used to assess, in an autologous transplant setting, the qualitative composition of the $CD34^+$ cell fraction. In this regard, the quantitation of candidate HSC subsets of the $CD34^+$ population is potentially of great importance. Furthermore, the addition of a known concentration of internal standard beads converts the FCM $CD34^+$ cell assay into a single instrument platform for the determination of absolute $CD34^+$ cell count determination.

REFERENCES

1. Berenson RJ, Bensinger WI, Hill RS et al.: Engraftment after infusion of $CD34^+$ marrow cells in patients with breast cancer or neuroblastoma. *Blood* 77:1717–1722, 1991.
2. Andrews RG, Bryant EM, Bartelmez SH et al.: $CD34^+$ marrow cells, devoid of T and B lymphocytes, reconstitute stable lymphopoiesis and myelopoiesis in lethally irradiated baboons. *Blood* 80:1693–1701, 1992.
3. Siena S, Bregni M, Brando B et al.: Circulation of $CD34^+$ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide-treated patients: Enhancement by intravenous recombinant human granulocyte-macrophage colony-stimulating factor. *Blood* 74:1905–1914, 1989.
4. Kessinger A, Armitage JO: The evolving role of autologous peripheral stem cell transplantation following high-dose therapy for malignancies. *Blood* 77:211–213, 1991.
5. Goldman JM: Peripheral blood stem cells for allografting. *Blood* 85:1413–1415, 1995.
6. Brecher ME, Sims L, Schmitz et al.: North American multicenter study on flow cytometric

enumeration of CD34+ hematopoietic stem cells. *J Hematother* 5:227–236, 1996.
7. Sutherland DR, Keating A: The CD34 antigen: Structure, biology and potential clinical applications. *J Hematother* 1:115–129, 1992.
8. Sutherland DR, Marsh JCW, Davidson J et al.: Differential sensitivity of CD34 epitopes to cleavage by *Pasteurella haemolytica* glycoprotease: Implications for purification of CD34-positive progenitor cells. *Exp Hematol* 20:590–599, 1992.
9. Greaves MF, Titley I, Colman SM et al.: Report on the CD34 cluster workshop. In: Schlossman S et al. (eds) Leukocyte Typing V, 1995, pp 840–849.
10. Sutherland DR, Keating A, Nayar R et al.: Sensitive detection and enumeration of CD34+ cells in peripheral and cord blood by flow cytometry. *Exp Hematol* 22:1003–1010, 1994.
11. Stelzer GT, Shults KE, Loken MR. CD45 gating for routine flow cytometric analysis of human bone marrow specimens. *Ann NY Acad Sci* 677:265–280, 1993.
12. Borowitz MJ, Guenther KL, Schults KE et al.: Immuno-phenotyping of acute leukemia by flow cytometry: Use of CD45 and right angle light scatter to gate on leukemic blasts in three color analysis. *Am J Clin Pathol* 100:534–540, 1993.
13. Sutherland DR, Anderson L, Keeney et al.: The ISHAGE guidelines for CD34+ cell determination by flow cytometry. *J Hematother* 3: 213–226, 1996.
14. Marsh JCW, Sutherland DR, Davidson J et al.: Retention of progenitor cell function in CD34+ cells purified using a novel O-sialo-glycoprotease. *Leukemia* 6:926–934, 1992.
15. Stewart AK, Imrie K, Keating A et al.: Optimizing the CD34+Thy-1+ stem cell content of peripheral blood collections. *Exp Hematol* 23:1619–1627, 1995.
16. Sienna S, Bregni M, Brando B, Belli N, Lansdorp PM, Bonadonna G, Gianni M:. Flow cytometry to estimate circulating hematopoietic progenitors for autologous transplantation: Comparative analysis of different CD34 monoclonal antibodies. *Haematologica* 76:330–333, 1991.
17. Hughes K, Bell DN: Nonspecific binding of anti-CD34 antibody QBEnd10 to nonviable cells. *Exp Hematol.* 23:968–969, 1995.
18. Steen R, Tjonnfjord G, Gaudernack G et al.: Differences in the distribution of CD34 epitopes on normal progenitor cells and leukemic blast cells. *Br J Haematol* 94:597–605, 1996.
19. Sehmi R, Howie K, Sutherland DR et al.: Increased levels of CD34+ hematopoietic progenitors in atopic subjects. *Am J Respir Cell Mol Biol*, in press, 1996.
20. Murray L, Chen B, Galy A et al.: Enrichment of hematopoietic stem cell activity in the CD34+Thy-1+Lin- subpopulation from mobilized peripheral blood. *Blood* 85:368–378, 1995.
21. Gazitt Y, Tian E, Barlogie B, Reading C, Vesole DH, Jagannath S, Schnell J, Hoffman R, Tricot G: Differential mobilization of myeloma cells and normal hematopoietic stem cells in multiple myeloma following treatment with cyclophosphamide and GM-CSF. *Blood* 86:381–389, 1995.
22. Prince HM, Imrie K, Sutherland DR et al.: Peripheral blood progenitor cell collections in multiple myeloma: Predictors and management of inadequate collections. *Br J Haematol* 93:142–145, 1996.

ENGRAFTMENT INTO THE NONMYELOABLATED AND MYELOABLATED HOST: STEM CELL PHENOTYPE

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Studies characterizing stem cell populations have generally been carried out in lethally irradiated myeloablated mice with an underlying assumption that space needed to be created in order for donor stem cells to home and engraft. Micklem et al.[1] in 1968 showed engraftment in normal hosts, but interpreted the level of engraftment as relatively insignificant. Takada et al.[2,3] also detected low numbers of donor marrow cells following injection into normal CBA mice. Brecher, Saxe and colleagues[4,5] established that engraftment occurred and this work was extended by Stewart et al.[6] who demonstrated long-term stable chimerism when 40 million unseparated male BALB/c marrow cells were engrafted daily for 5 days (total 200 million cells) into untreated female BALB/c hosts and the percentage of male DNA determined in female host marrow, spleen and thymus at 1–25 months past transplantation. This basic observation has now been extended to different cell levels and different time intervals.[7,8] Furthermore, engraftment in the normal host approached and at times exceeded theoretical maximums of engraftment based on 2 different models.[8] In 7 individual mice the theoretical observed values were 1.1 to 3.4 times the theoretical maximums (Table 1). These data indicated that engraftment in the normal host was close to quantitative and suggested that the final engraftment phenotype is determined by competition with host marrow cells, not by "niche" availability. The differences in the final differentiated cell readout when irradiated or normal hosts are infused with the same number of stem cells is illustrated in Figure 1.

The ten highest engraftment percentages achieved in individual animals given different levels or the same level of cells were compared with their calculated theoretical levels. Theoretical levels were separated according to equal homing to bone marrow, spleen and thymus or homing of all cells only to bone marrow. These theoretical maximums assume no loss or homing to other organs and replacement rather than augmentation of host cells. Therefore, they represent a theoretical maximal value and the assumption that cells may only home to marrow is the ultimate maximal theoretical engraftment. The level to which observed values exceeded theoretical values is shown for both comparisons.

Work by Stewart et al.[6] and Ramshaw et al.[9] demonstrated that marrow harvested 6 days after the administration of 5 fluorouracil (5FU) (150 mg/kg) to male BALB/c mice, although relatively enriched in cycling progenitor/stem cells, was defective in

Table 1. Comparison of highest individual engraftments to theoretical levels

	Observed engraftment BM (%)	*Maximal theoretical engraftment (BM, SP, THY) (%)*	*Level above 3-organ theoretical*	*Maximal theoretical engraftment (BM only) (%)*	*Level above 1-organ theoretical*
1.	79	29	2.7×	51	1.5×
2.	75	24	2.6×	44	1.7×
3.	72	29	2.6×	51	1.4×
4.	71	16	4.4×	29	2.4×
5.	56	29	1.9×	51	1.1×
6.	54	9	6.0×	16	3.4×
7.	47	29	1.6×	51	—
8.	46	9	5.1×	16	2.9×
9.	44	29	1.5×	51	—
10.	40	29	1.4×	51	—

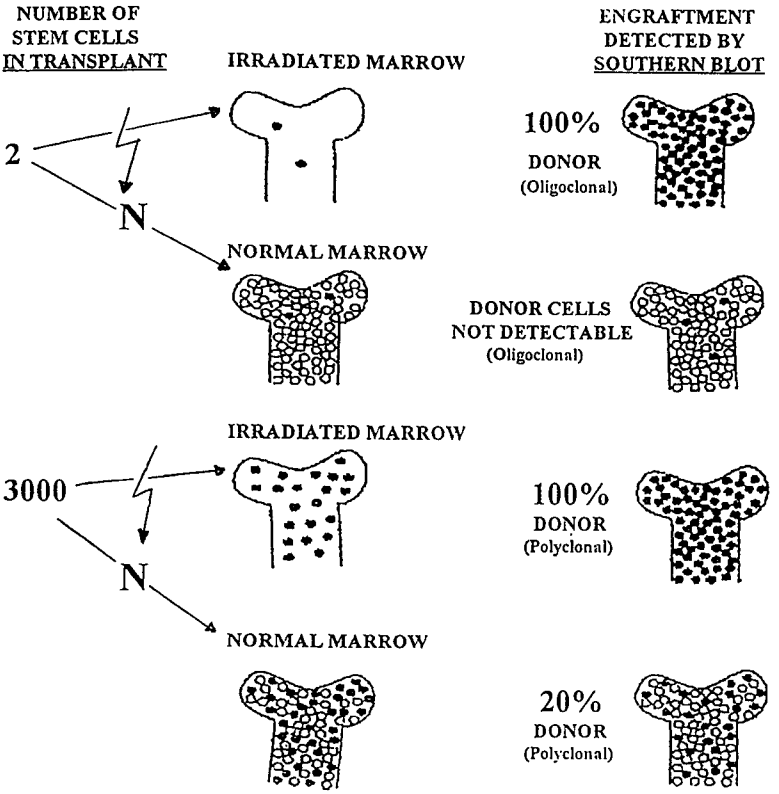

Figure 1. *Theoretical model of stem cell engraftment.*

Table 2. Cycling and stem cell engraftment

Stem cell condition	*HPP-CFC # and cycle status*	*Long-term engraftment into normal host*
normal donor	normal & dormant	high levels
post-5FU donor	preserved & cycling	defective
donor cells exposed to IL-3, IL-6, IL-11 and SCF for 48 hours in in vitro liquid culture	expanded & cycling	defective

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long-term engraftment when quantitatively compared with normal marrow in either normal or irradiated female BALB/c hosts. If marrow was harvested 35 days after 5FU it engrafted normally. Studies utilizing in vivo hydroxyurea suicide indicated that the long-term repopulating stem cells detected in normal hosts were not cycling. Similarly, those cells that did engraft from post-5FU marrow were also dormant.

The engraftment potential, HPP-CFC number and cell cycle status of male BALB/c marrow cells exposed in vitro to the early acting cytokines, IL-3, IL-6, IL-11 and steel factor were also evaluated using two separate engraftment models, male BALB/c marrow into normal female BALB/c host or an irradiated host model in which either male-female or Ly5.1/Ly5.2 congenic cells were competed.[10,11] It was demonstrated that the cytokine treated cells were markedly defective for engraftment at 6–22 weeks post-transplantation. This was in spite of the fact that HPP-CFC were expanded. The HPP-CFC were also induced into active cell cycle, as demonstrated by in vitro ^{3}HtdR suicide. Thus, in two models cycling cells were found to have preserved or expanded HPP-CFC with very defective engraftment. These studies are summarized in Table 2.

Further work has indicated that this cytokine defect may not be apparent when early times of engraftment are analyzed, suggesting the existence of a separate population of stem cells affected differentially by cytokines.[12]

Purified lineage negative rhodamine low and Hoechst low cells have also been shown to engraft, although at a lower relative frequency than whole marrow, suggesting the existence of facilitator cells in syngeneic transplants.[13]

The concept that in vivo engraftment in normal hosts is determined by competition with host cells, rather than by "opening" of occupied spaces, is further supported by studies showing that relatively low levels of whole body irradiation can result in high levels of chimerism when relatively high levels of marrow cells are infused.[14]

Overall, the present data indicate that stem cell engraftment is determined by the functional state of the engrafting stem cell, possibly related to cell cycle status, and the number of competing host stem cells. Cells engrafting early (first 1–3 weeks) do not

appear to be affected by exposure to cytokines, while those responsible for long-term engraftment appear to acquire an engraftment defect after exposure to cytokines.

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REFERENCES

1. Micklem HS, Clarke CM, Evans EP et al.: Fate of chromosome-marked mouse bone marrow cells transfused into normal syngeneic recipients. *Transplantation* 6:299, 1968.
2. Takada A, Takada Y, Ambrus JL: Proliferation of donor spleen and marrow cells in the spleens and bone marrows of unirradiated and irradiated adult mice. *Proc Soc Exp Biol Med* 136:222, 1970.
3. Takada Y, Takada A: Proliferation of donor hematopoietic cells in irradiated and unirradiated host mice. *Transplantation* 12:334, 1971.
4. Brecher G, Ansell JD, Micklem HS et al.: Special proliferative sites are not needed for seeding and proliferation of transfused bone marrow cells in normal syngeneic mice. *PNAS* 79:5085, 1982.
5. Saxe DF, Boggs SS, Boggs DR: Transplantation of chromosomally marked syngeneic marrow cells into mice not subjected to hematopoietic stem cell depletion. *Exp Hematol* 12:277, 1984.
6. Stewart FM, Crittenden RB, Lowry PA et al.: Long-term engraftment of normal and post-5-fluorouracil murine marrow into normal nonmyeloablated mice. *Blood* 81:3566, 1993.
7. Ramshaw H, Crittenden RB, Dooner M et al.: High levels of engraftment with a single infusion of bone marrow cells into normal unprepared mice. *Biol Blood Marrow Transplant* 1:74–80, 1995.
8. Rao SS, Peters SO, Crittenden RB et al.: Stem cell transplantation in the normal nonmyeloablated host: Relationship between cell dose, schedule and engraftment. *Exp Hematol* 25:114–121, 1997.
9. Ramshaw HS, Rao SS, Crittenden RB et al.: Engraftment of bone marrow cells into normal unprepared hosts: Effects of 5-fluorouracil and cell cycle status. *Blood* 86:924–929, 1995.
10. Peters SO, Kittler EL, Ramshaw HS et al.: Murine marrow cells expanded in culture with IL-3, IL-6 and SCF acquire an engraftment defect in normal hosts. *Exp Hematol* 23:461–469, 1995.
11. Peters SO, Kittler ELW, Ramshaw HS et al.: Ex vivo expansion of murine marrow cells with interleukin-3, interleukin-6, interleukin-11, and stem cell factor leads to impaired engraftment in irradiated hosts. *Blood* 87:30–37, 1996.
12. Peters SO, Vergilis KL, Habibian HK et al.: Culture of murine hematopoietic progenitor cells cultured with IL-3, IL-6, IL-11 and SCF for 47 hours leads to expansion of short term repopulating cells but impairs long-term engraftment. *Blood* 88:595a, 1996 [abstr].
13. Nilsson SK, Dooner MS, Tiarks CY et al.: Early events in the homing of murine hematopoietic stem cells. *Blood* 88:593a, 1996 [abstr].
14. Stewart FM, Zhong S, Quesenberry PJ: Donor cells dominating a competitive marrow transplant model where host receives minimal myeloablation: A potential delivery system for human gene therapy. *Blood* 88:591a, 1996.

CHAPTER 11
Summaries

AUTOLOGOUS STEM CELL TRANSPLANTATION IN LEUKEMIA: SYNTHESIS AND SUMMARY

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ACUTE MYELOCYTIC LEUKEMIA (AML)

Several multicenter collaborative trials have compared autologous bone marrow transplantation (autoBMT) with intensive combination chemotherapy in first-remission AML and have shown strikingly similar results. Most of these studies also evaluated the outcome of allogeneic BMT (alloBMT) in those patients who had histocompatible related donors. Most (but not all) of these trials demonstrated the superiority of alloBMT over either autoBMT or chemotherapy, with event-free survival (EFS) of 50–55% after alloBMT, 40–55% after autoBMT, and 30–40% after intensive chemotherapy. It is also evident that the outcome from the chemotherapy arm is helped by several patients who have been salvaged and continue to survive after relapse. Although the autograft procedure substantially reduces relapse risk, its impact on overall survival is less clear. The lower relapse rate observed after autoBMT tended to be offset by a greater treatment-related mortality, including some late deaths in remission due to refractory thrombocytopenia; this mortality rate was observed in larger and more experienced centers as well as smaller and less experienced institutions. Improved outcome in future studies must address strategies to decrease this unacceptably high rate of procedure-related deaths.

These collaborative clinical investigations share difficulties in the conduct of these trials and emphasize the challenges inherent in large-scale, multi-institution clinical research with complex patients. For example, approximately 35% of patients eligible for randomization to either chemotherapy or autoBMT actually were randomized, and other factors led to further attrition of patients from the randomized groups. Although intention-to-treat analysis is the most rigorous and appropriate means to evaluate the results of these studies, one must also appreciate the limitations inherent in such analyses.

Analyses of these multicenter studies provide an evolving concept of stratification by risk group with regard to outcome of and indications for autoBMT for AML in first remission. Variables such as tumor cytogenetics, leukocyte count at diagnosis, and response (or lack thereof) to initial remission-induction therapy have all been used to assign risk groups (low-, standard- and high-risk) to response of

AML to conventional chemotherapy, and it now appears that similar stratifications may be useful in the autoBMT setting. Risk-group assignment might identify good-risk AML patients in whom autoBMT in first remission might not provide additional benefit (while exposing the patient to risk of death from the procedure) and in whom autoBMT might instead be considered in early first relapse or in second remission. Conversely, this stratification process might identify patients at exceptionally high risk for relapse of AML, in whom neither chemotherapy, autoBMT nor even alloBMT is likely to be curative. These patients might benefit from matched related or unrelated alloBMT applied early in first remission. The issues of efficacy and logistics of collection of autologous stem cells (from bone marrow or peripheral blood) in patients with AML in first remission, to be used for transplantation at early relapse or in second remission, are still unresolved.

Several studies strongly indicate that cytogenetic features of AML at diagnosis may provide one of the most important predictors (arguably, maybe the single most important prognostic factor) of the outcome of autologous stem cell transplantation and might identify especially high-risk groups. For example, abnormalities of chromosomes 5 or 7 or hypodiploidy, and possibly complex cytogenetic abnormalities may define an especially poor-prognosis group. It is likely that these presentations will have piqued the interest of Symposium attendees to analyze their own series and determine whether they too can confirm these associations of cytogenetic abnormalities and outcome of autoBMT for AML.

The apparent plateau in relapse-free survival after intensive combination chemotherapy or autoBMT for AML underscores the need to explore new regimens for remission-induction and consolidation and for pre-transplant cytoreduction. As the best results of autologous transplant for AML obtain in a state of minimal disease, more effective antileukemic induction/consolidation (representing a more effective in vivo purge) may complement or offset the need for ex vivo purging of the autologous marrow product. Not surprisingly, there is still no consensus with regard to preparative regimens for autoBMT in AML; adherents to either total body irradiation or busulfan-based pretransplant conditioning, or to other combination chemotherapy (e.g., BACT), have advocated the efficacy of their respective regimens. The initial report of a pilot trial of an intravenous busulfan preparation is encouraging and may provide a more rational basis for dosing and administration of this agent which currently is limited to an oral form.

Interest in the application of ex vivo treatment, or purging, of autologous marrow with chemotherapeutic or immunological agents as methods has been stimulated by reports from the European BMT Group, single-institution trials and the North American Autologous Blood and Marrow Transplant Registry (ABMTR). Elegant and seminal studies of gene marking of autologous normal marrow and AML cells support but by no means prove the need for autograft

purging. The similar outcomes of autoBMT for AML in second remission despite different purging regimens (e.g., 4-hydroperoxycyclophosphamide [4HC] versus monoclonal antibodies and complement) or no purging (as reported by the Rome group) is of interest. The data from the ABMTR suggest benefit of purging, but also a downward trend in use of purging for autoBMT for AML in remission. It is not clear whether this represents a genuine lack of interest on the part of investigators in North America or a practical limitation of chemopurging due to the lack of availability of agents such as 4HC.

These presentations and discussions emphasize the need to conduct two important prospective randomized trials of autologous transplantation for AML. A prospective trial comparing purged versus unpurged marrow has yet to be undertaken; although logistically challenging with regard to patient accrual and loss from randomization (as discussed above), this study is now even more timely. Investigators at the University of Pittsburgh intend to conduct a multicenter North American trial of autoBMT with immunopurged marrow or unpurged marrow in patients with AML in second remission. With the increased application of autologous peripheral blood stem cell transplantation (PBSCT) in lymphoma, breast cancer and solid tumors, there is interest in prospective comparison of autoBMT and PBSCT in AML. To this end, the EORTC/GIMEMA AML 10 Study may address this question.

ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

In autoBMT for ALL, the high post-transplant relapse rate (at least 55% in first remission and at least 70–75% in second or third remission) is the major challenge. Consequently, there appears to be limited interest in exploring new ex vivo marrow purging regimens, suggesting, as in the Book of Ecclesiastes, there may be "nothing new under the Sun." However, in adults with Philadelphia-chromosome (Ph^1)-positive ALL, one autoBMT trial has an encouraging relapse-free survival of 30%, albeit with short follow-up, after transplantation of marrow treated by immunomagnetic purging with an anti-CD10, anti-CD19 and anti-HLA-DR combination; this method provides at least a 2-log depletion of ALL cells.

The poor outcome of autoBMT and the increased likelihood of identification of a matched unrelated donor (URD) from national and international registries has led many centers to pursue URD BMT for high-risk ALL. A single-institution comparison of immunopurged autoBMT versus URD BMT in ALL indicated that the inherent delay when pursuing URD BMT may be improving in terms of shorter time from donor search to BMT. This study showed that the higher regimen-related toxicity with URD BMT, especially due to graft-versus-host disease, was offset by a lower relapse rate. The EFS was better after URD BMT in first- or second-remission ALL, and was better in males. For patients who lack suitable matched

unrelated donors, autoBMT may be an option, but other alternative stem cell transplants (e.g., umbilical cord blood, mismatched related donors) may compete with autoBMT. The prospects of enhancing autologous antitumor responses to decrease post-autoBMT relapse have not been impressive; in one randomized study of adult ALL patients undergoing autoBMT with unpurged marrow, administration of interleukin-2 (IL-2) did not improve EFS or decrease the relapse rate.

New avenues for autoBMT in ALL include methods to improve cytoreduction before marrow collection and autoBMT, thus attaining a state of minimal residual disease (improved ex vivo purge). Encouraging novel biotherapeutic agents being evaluated in this setting include immunotoxin conjugates, e.g., those including the pokeweed antiviral protein (PAP). More recently, a novel conjugate of the tyrosine kinase inhibitor genistein with anti-CD19 monoclonal antibody has been developed to provide targeted therapy directed against a molecular cellular pathway. This agent is currently under study for its anti-ALL activities in both in vivo and ex vivo settings.

CHRONIC MYELOGENOUS LEUKEMIA (CML)

The role of autologous stem cell transplantation for CML is not established. From the presentations at this Symposium, interest for autotransplants in CML in Europe is substantially greater than in North America. The ability of α-interferon α-IFN) to prolong survival and hematologic remissions in CML further justifies the need for randomized trials to evaluate survival, EFS and quality of life after stem cell autotransplants versus α-IFN for newly-diagnosed CML, as proposed by the Genova group. As with autoBMT for AML and ALL, relapse is the major problem of autologous stem cell transplants for CML. Therefore, proceeding to transplant in a state of minimal residual disease and evaluation of post-transplant modalities to further decrease or eradicate CML are important principles to consider in autografting for CML.

The use of PBSCT for CML is gaining favor. Investigators concur that earlier procurement of PBSC products in the chronic phase of the disease is preferable, and that the best yields and purity of Ph-negative PBSCs are obtained in early (days 1–3) apheresis products. The effect of previous α-IFN treatment on yields of cells at apheresis is not clear. Similar results have been reported using either G-CSF alone or combination chemotherapy (ICE, mini-ICE) may be used to mobilize PBPCs for collection in CML. Even with cytoreduction and pretreatment with α-IFN, 67% of the Ph1-negative PBPC products may still be *BCR-ABL*-positive. Preclinical studies with the tyrosine kinase inhibitor genistein suggest the potential use of this agent for negative pharmacological selection of *BCR-ABL*-positive CML cells in the context of ex vivo purging.

Finally, post-transplant immunomodulation or administration of other biological response modifiers to eradicate residual leukemia is especially worthy of

exploration in autotransplants for CML. The high relapse rate observed after T cell-depleted related alloBMT supports the strong allogeneic graft-versus-leukemia effect in CML, and preclinical studies suggest potential mechanisms by which leukemic cells demonstrate "immune escape" after BMT. Investigators have recommended the use of both α-IFN and IL-2 after PBSCT for CML to try to effect further cytoreduction. Roquinimex (linomide), which enhances function of natural killer cells, T cells and macrophages, has been administered to CML patients given autoBMT with Ph-positive unpurged marrow. The cytogenetic responses in roquinimex recipients were often prolonged, especially after autoBMT in first chronic phase. The appearance in some patients of clinical and histopathological manifestations similar to acute graft-versus-host (GVH) disease raises questions whether this agent actually induces an immunological autologous GVH reaction with concurrent anti-CML activity. These or other post-transplant strategies, combined with optimum pre-transplant cytoreduction, may favorably influence EFS after autografting for CML.

SUMMARY OF LYMPHOMA AND MULTIPLE MYELOMA SESSIONS

Arnold S. Freedman

In recent randomized trials, high-dose (HD) therapy and autologous bone marrow transplantation (autoBMT) has been shown to prolong disease-free survival (DFS) and overall survival (OS) in these diseases. A subset of patients with multiple myeloma, relapsed aggressive non-Hodgkin's lymphoma (NHL) with sensitive disease, and patients with high-risk and high-intermediate risk diffuse aggressive lymphomas clearly benefit from HD therapy. The data presented at this meeting have added further information which will likely further impact on the treatment of these diseases. This summary will focus in a disease-specific fashion on two issues: what we know presently and what directions need to be taken.

NON-HODGKIN'S LYMPHOMA

Indolent lymphomas

A major question raised in the meeting concerned the current results of HD therapy. In several series (Bierman, Haas, Gorin, Freedman) the DFS is consistently about 40% at 5 years with an OS of 70%. What remains unclear is whether autoBMT in first remission will yield better results than in subsequent remission.

The second issue is whether ex vivo marrow treatment affects outcome. In the studies from Dana-Farber, the detection of minimal residual disease after bone marrow purging and consistently in the marrow following autoBMT predict for relapse. In an analogous fashion, the number of CFU-GEMM in the marrow after mafosphamide purging is inversely related to the risk of relapse of lymphoma (Gorin). For future studies, it remains unclear precisely what role HD therapy has in indolent lymphomas (and chronic lymphocytic leukemia). Similarly, the issue as to whether there is any benefit for stem cell purging is unclear, although the CUP trial (Schouten) may help with insights into these two issues.

Aggressive lymphomas

The studies presented at this meeting focused on the identification of patients who might benefit from HD therapy and whether the earlier use of this approach is appropriate. In lymphoblastic lymphoma, patients in first CR have DFS of over 70% (Santini). In the diffuse aggressive lymphomas conflicting data was presented. The results of the GELA trial (Haioun) demonstrated that patients with high and high-intermediate risk lymphoma in first CR have improved DFS and overall

survival when consolidated with autoBMT, when compared with continued combination chemotherapy. In contrast, Porcellini et al. showed no benefit for autoBMT. However, patient selection and the length of follow-up differ in these two studies. Additional support for autoBMT in first CR or PR was presented by Gorin, where superior results were seen when compared with second or greater remission. It remains uncertain in lymphoblastic lymphoma as to whether HD therapy adds to conventional treatment, and a randomized trial (Santini) is being undertaken to address the question. There is still a great need to improve the results for high and high-intermediate risk patients with aggressive lymphomas. Additional prognostic studies that employ biologic markers of aggressive disease and therapy beyond autoBMT will likely impact in the treatment of these patients.

HODGKIN'S DISEASE

The role of HD therapy in early Hodgkin's disease patients and who are in fact high-risk patients was the major focus of the studies presented. Although "unfavorable" patients have a better DFS with early HD therapy (Carella), the IBMTR data showed no difference in time to relapse or on OS in early transplanted patients. It still remains unclear who are high-risk patients at diagnosis. The International Index identified hemoglobin, albumin and stage as being important prognostic factors; however, no poor prognostic subgroup of patients could be identified with certainty. Important directions were presented to move the field beyond CBV as the HD recipe of choice in Hodgkin's disease. Double transplants, radioimmunoconjugates and more selective use of HD therapy may well have an impact. To better utilize this approach, factors that predict for unfavorable outcome at diagnosis and at relapse need to be identified.

MULTIPLE MYELOMA

The recently published French randomized trial demonstrated that high-dose therapy leads to improved DFS when compared with conventional-dose therapy. The approach of "total therapy" from University of Arkansas, with two intensive consolidation courses, suggests that one may obtain benefit beyond that seen with a single high-dose regimen. The issue of source of stem cells also was discussed in the myeloma presentations, including marrow, peripheral blood stem cells and allogeneic marrow. Peripheral blood stem cells are significantly less involved with malignant cells than marrow (Berenson). Although the role of contaminating malignant cells in the autograft is unknown, CD34 selection (Berenson) provides 3 to 4 log of depletion of malignant cells. The treatment related mortality was quite high (36%) with allogeneic transplantation. Considering the encouraging results of adoptive immunotherapy, allogeneic BMT may be appropriate for a

subset of patients when applied earlier in the course of the disease. The questions that were raised for the future focused on the role of more than one high-dose treatment, whether CD34 enrichment reduces tumor cell contamination and if this impacts on outcome of high dose treatment, and finally whether adoptive transfer can be expanded to better harness what has been clearly shown to be a graft-versus-myeloma effect.

FUTURE DIRECTIONS

There are four areas where future studies will need to concentrate in order to improve the treatment of these diseases. These are: patient selection, the treatment itself, tumor contamination of the graft and generating a host-versus-tumor response.

Patient selection

For the aggressive NHLs, significant progress has been made in identifying patients at presentation and at relapse who may benefit from HD therapy. For the indolent lymphomas and Hodgkin's disease the indices used to date are not optimal, and we therefore need to better integrate what is known about these diseases biologically and apply them in prognostic models. Presently, we may be both overtreating and undertreating subsets of patients.

Therapy

In these hematologic malignancies, there is a need to be developing new ablative regimens. We have relied on cyclophosphamide and total-body irradiation for NHL and CBV for Hodgkin's disease for a very long time. Perhaps in clinical situations where the benefit of high-dose therapy with CBV or CY/TBI is limited, certain patients should be offered new ablative regimens. A topic that was not discussed but has become an increasingly bigger problem is late myelodysplasia and acute myelogenous leukemia post-autotransplant. Once again, further studies into ablative regimens may alter this problem, which is currently observed in 5–10% of patients.

Tumor cells in the graft

Although the contamination of the graft with tumor may only partially contribute to relapse, the ability to purge is a very strong surrogate marker for outcome in the studies from our institution. The encouraging results of CD34 selection followed by negative selection and/or stem cell expansion are ideally suited to help address the problem of the role of tumor cells in the graft. Finally randomized trials (CUP) may help answer the questions of the role of purging in these diseases.

Host-versus-tumor response

This area is perhaps the most exciting direction being undertaken in these diseases. Expansion and activation of effector cells with cytokines (IL-2, IL-12, interferon-γ) is already being used in clinical studies. However, to make this work optimally, one needs to incorporate specificity into the response. Since tumor cells are not well "seen" by the host, one has to make the malignant cells more immunogenic. This may involve the generation of tumor specific T cells in vivo through vaccination or ex vivo, followed by expansion and activation in vivo in order to optimize an anti-tumor response.

In order to improve the outcome of patients with these hematologic malignancies we need a multimodality approach with improved induction treatment, ablative therapy and therapy to eliminate minimal residual disease.

SUMMARY: SOLID TUMORS

Roger Herzig

GENERAL

Dose-intensive therapy, with and without stem cell rescue, is being used more frequently in patients with solid tumors; however, definitions are changing. There currently is a changing definition of dose-intensity; conventional doses are now becoming higher, and doses that were considered high are becoming even higher. In considering the outcome of trials, the doses must be clearly identified, and further, the concept of dose-over-time, i.e., dose-intensity, must be considered. This changing and increasing "conventional" dose will affect the definition of what are considered "chemosensitive" tumors. With higher standard doses, more "chemosensitive" tumors may be identified. The previous observation of chemosensitivity to standard doses as a positive predictor of good results with dose-intensive therapy will have to be re-examined. Ultimately, it will depend on where the tumor responses are on the dose-response curve. If still in the log-linear portion, the observation may still be valid; if not, the further addition of dose may be unimportant.

Tumor contamination of the stem cell product was discussed and presented. This problem still is mostly theoretical, since contamination currently has not been clinically or biologically important. With more sensitive methods for detecting tumor cells and with improving methods for positive and negative selection of stem cells, the answer to the question may be avoided.

Finally, a number of regimens were discussed. In the past, the regimens identified toxicity and the maximum tolerated doses. Now, regimens are multiple courses of the same agents or, even more interesting, multiple courses with multiple agents, taking advantage of the non-crossreactive nature of the alkylating agents.

BREAST CANCER

Historically, the role of dose-intensive therapy for metastatic disease was among the first studied. With promising results, the role of dose-intensive therapy for the primary treatment of breast cancer is being examined. In general, dose-intensive therapy provides longer disease-free and overall survival compared with standard dose therapy. Specifically in metastatic disease, the current approaches are standard dose therapy followed by dose-intensive therapy, or dose-intensive therapy followed by another dose-intensive therapy. This latter approach has used multiple cycles of the same drugs with only modest success. Multiple cycles of different drugs probably offers a better approach. The strategy for dose-intensive therapy has been somewhat confused by Dr. Peter's recent

abstract, where after initial chemotherapy for metastatic breast cancer, patients were either observed and then received dose-intensive therapy at recurrence, or had dose-intensive therapy as consolidation as part of the initial treatment. At the present time, the observation followed by dose-intensive therapy provided: better strategy, with improved overall survival (compared to the dose-intensive therapy consolidation). Thus, the strategy for responsive metastatic disease could be standard therapy to maximum response, followed by dose-intensive therapy with further progression. However, dose-intensive therapy may still offer better initial treatment of metastatic disease. For inflammatory breast cancer, the studies are currently underway. Presently, there does not appear to be an advantage for dose-intensive therapy, but the use of dose-intensive therapy may be needed as part of the primary therapy, maybe even before surgery. In the adjuvant setting, most investigators would advocate the use of dose-intensive therapy for high-risk stage II–III breast cancer with 10 or more lymph nodes involved. The final analysis from the intergroup study is still pending. A new trial of women with 4–9 nodes involved is open. This trial will examine the randomization between standard, but high-dose, therapy and a more dose-intensive therapy with stem cell rescue as consolidation in the primary adjuvant treatment. Ultimately, other risk factors may be considered in selecting patients for dose-intensive therapy in addition to nodal status, for example, the overexpression of HER2 *neu.*

GERM CELL TUMORS

For refractory and relapsed patients, the use of dose-intensive therapy has been the focus, but there is initial salvage therapy that can be curative (vinblastine, ifosphamide, cisplatin [VIP]). There has been a subset of patients identified who will benefit from dose-intensive therapy: 1) platinum sensitive; 2) not extranodal and 3) relapse occurring within two years. Currently, there is a randomized trial within the United States that compares conventional therapy for four cycles of chemotherapy versus the same conventional therapy for two cycles followed by two cycles of high-dose therapy. The patients who are included here are the patients with visceral metastases, unfavorable markers and mediastinal nonseminomatous disease. In Europe, VIP versus VIP with dose-intensive therapy in first relapse is underway. The role of dose-intensive cyclophosphamide, ifosphamide or paclitaxel remains unclear. After the initial randomized trails are completed, the use of additional agents, for example etoposide and carboplatin and/or cyclophosphamide, will be explored.

OVARIAN CANCER

Ovarian cancer has always demonstrated a good response to dose-intensive therapy. It was the first tumor in which high-dose therapy was tried. Overall,

around 15% of the patients will have long-term survival. The best results are in patients who are treated in complete remission, who are platinum sensitive, and who have been optimally debulked: 25% progression-free survival at two years. In ovarian cancer, most of the drugs, especially the alkylating agents, are active, suggesting a sequential approach may be applicable. The cooperative group trials have been slow in initiation and slow in their accrual, in part due to referral patterns and the skepticism of the gynecologic oncologists.

SMALL CELL LUNG CANCER

There are currently trials being conducted in patients with small cell lung cancer, but the trials are too early to evaluate. The problems associated in this group of patients include the older age of the patients with co-morbid problems and frequent marrow and stem cell contamination with small cell lung cancer. The dose-intensive therapy approach will be most applicable in younger patients with limited disease without significant cardiovascular or other pulmonary problems. The Dana Farber group has the largest experience. They have used the combination of dose-intensive cyclophosphamide, cisplatin, and carmustine. The high incidence of interstitial pneumonitis with the nitrosourea suggests other drugs in the combination should be considered and may be better tolerated. Applications of novel post dose-intensive therapy treatment may be used to improve survival.

NEUROBLASTOMA

Neuroblastoma is one of the most sensitive tumors, and one of the earliest studied using dose-intensive therapy. Promising results, however, have led to more questions, namely comparing dose-intensive therapy with consolidation with higher standard doses than previously used (again highlighting the problem of our changing definition of "standard dose"). Neuroblastoma, because of its marrow involvement, has been the focus of purging for marrow, with a variety of methods being explored. With the ability to collect peripheral blood stem cells in children, and with the use of allogeneic marrow, some of the problems with purging may be avoided. Additional questions in the use of total body irradiation are being examined. Finally, tandem or sequential therapy studies are underway.

BRAIN TUMORS

Glioblastoma is a sensitive tumor, but long-term improved survival has not been demonstrated. Long-term disease-free survival in stage III and IV glioblastoma has approached 10%. While most of the studies have shown an early survival benefit for dose-intensive therapy with time, it has not made a substantial difference in

overall survival. The treatment of children with medulloblastoma or primitive neuro-ectodermal tumors has shown a significant benefit in about one-third of the patients. In children with brain tumors, dose-intensive therapy may be a way to avoid radiotherapy, providing comparable results with less long-term toxicity.

CONCLUSION

Dose-intensive therapy has made a significant impact in our therapy successes. The more successful treatment of the patients with metastatic disease has prompted earlier treatment in high-risk patients, particularly in breast cancer, ovarian cancer and germ cell tumors. The future will involve other methods to continue to prolong and improve the survival following dose-intensive therapy. As the biology of the stem cell and supportive care issues are further addressed, the use of dose-intensive therapy will continue to move from highly specialized centers to routine community practice.

NEW AVENUES SUMMARY

Peter J. Quesenberry and Armand Keating

The New Avenues Session was designed to showcase research areas that are likely to lead to clinical advances in the near future.

The first presentation by van Bekkum (Rijswijk) on "Bone Marrow Transplantation in Autoimmune Diseases" provided a perfect example of a rapidly developing new clinical area. Adjuvant arthritis and chronic remitting experimental allergic encephalitis represent animal models of autoimmune disease. In these models the fully developed disease responds to treatment with allogeneic or autologous bone marrow transplantation. The key appears to be adequate levels of cytotoxic therapy which eradicate T lymphocytes. Furthermore, anecdotal experience with the treatment of aplastic anemia and leukemia indicates the possibility of prolonged complete remissions of rheumatoid arthritis, psoriasis, ulcerative colitis, regional enteritis and myasthenia gravis. A debate ensued over whether there were enough data available to justify clinical trials of autologous or allogeneic bone marrow transplantation for autoimmune disease—no contest, of course there are—and trials in multiple sclerosis are underway. This will be one of the important areas in BMT over the next decade and may even save the fading field of rheumatology. Dr. Vriesendorp (Houston) presented data on the use of radiolabeled immunoglobulins for human solid tumors. Careful distribution studies were carried out after intralesional injection in a nude mouse-human tumor model and clearly demonstrated retention in the tumor. This may provide a new treatment approach for delivery of high-dose radiation to isolated tumor foci—its use for wider-spread disease would depend on advances in tumor targeting.

Dr. Stewart (Worcester) presented intriguing data that very high levels of chimerism can be obtained in mice with doses of whole-body irradiation as low as 50 cGy when 40 million marrow cells are infused.

These data, along with those on engraftment into nonirradiated mice, suggest that stem cell engraftment is dependent not on the creation of marrow space or opening of niches, but rather on competition with residual host stem cells. The potential of such low dose treatment for gene therapy or the creation of allogeneic chimeras in such diseases as sickle cell anemia is exciting and should open up new areas of clinical investigation.

Dr. Bodine (Bethesda) presented advances in methods to transfer gene into repopulating hematopoietic stem cells. Effective gene transfer in hematopoietic stem cells has been achieved in some, but not all murine models, but has been very disappointing in primates and humans. Pretreatment of primate hematopoietic cells with SCF and G-SCF appears to result in a population of stem cells which can be

transduced in vitro and effectively engraft in vivo. The observation that 10% of peripheral blood cells carry the gene marker is very impressive and suggests that effective gene therapy will eventually be achieved.

Dr. Jones (Denver) presented a scholarly dissertation on the evaluation of high dose chemotherapy with particular emphasis on evaluating nonlinear pharmacokinetic effects (PK). Interpatient variability in BCNU PK, the relationships of AUC to acute lung injury and the existence of drug interactions between BCNU, cisplatin and cyclophosphamide all produce variability in the response of breast cancer patients to cyclophosphamide, cisplatin and BCNU. Long-term evaluation of treated breast cancer patients suggests a relationship between BCNU, AUC and relapse-free survival. If economics and managed care allow this type of analysis to be conducted, we should guide our chemotherapy in the future using this approach.

Dr. Sutherland (Toronto) presented data indicating that using a set of guidelines for accurate detection of $CD34^+$ cells by four parameter flow cytometry (CD45, FITC/CD34 PE staining, side- and forward-light scatter) one $CD34^+$ cell in 10,000 could be detected. This is a nice forward step for "blood bank quantitation." However, the question of whether the $CD34^+$ number is the best indicator of short- and long-term engraftment remains under investigation.

Dr. Long (Ann Arbor) presented data on the role of extracellular matrix-growth factor "complexes" ion hematopoietic stem cell growth, differentiation and adherence. $CD34^+DR\text{-}CD15^-$ progenitors adhere to recombinant cytokines, and there appear to be specific attachment patterns by different progenitors to matrix molecules. Thrombospondin in conjunction with c-*kit* ligand seems to provide synergetic modulation of stem cell function—a complex research area that continues to evolve and is perhaps only a few steps from clinical application.

The presentation by Dr. Quesenberry (Worcester) and colleagues focused on the relationship of the proliferative status of hematopoietic stem cells (exposed to cytokines) and their ability to engraft. Early engraftment (1–3 weeks) appeared unaffected, but engraftment at longer time intervals was defective at 48 hours post-cytokine exposure. These data indicate that the phenotype of the engrafting stem cell may in part be cell cycle phase-dependent. These observations have implications for clinical gene therapy and stem cell "expansion."

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