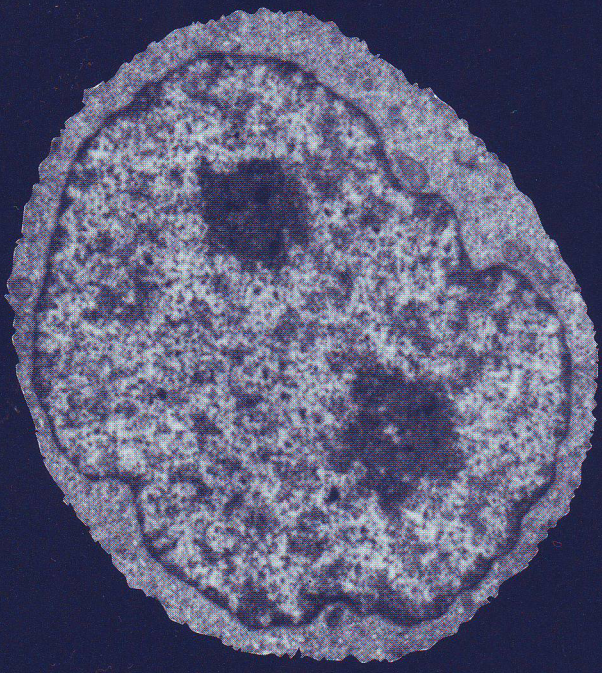
# Autologous Blood and Marrow Transplantation

## Proceedings of the Ninth International Symposium Arlington, Texas



Edited by

Karel A. Dicke & Armand Keating

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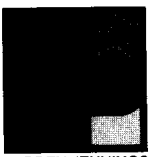


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# ACKNOWLEDGMENTS

We wish to thank Willem Dicke for expert editorial assistance, our wives for their understanding and support, and Kathy White for her exemplary dedication to make this project possible.

# PREFACE

The Ninth International Symposium of Blood and Marrow Transplantation provided an extraordinarily dynamic overview of the field in just over two days. We hope that the sense of enthusiasm, the high level of scientific discourse, and the comprehensiveness of topic coverage evident during the Symposium is conveyed, at least in part, by these Proceedings.

During the Symposium, Dr. Hans Kolb was honored as the third recipient of the van Bekkum Stem Cell Award in recognition of his outstanding contributions to the field.

We hope that these Proceedings will continue to serve as a guide to the progress we have achieved and to the major advances we have yet to make on behalf of our present and future patients.

*Karel A. Dicke*
*Arlington Cancer Center*
*Arlington, Texas*

*Armand Keating*
*The Toronto and Princess Margaret Hospitals*
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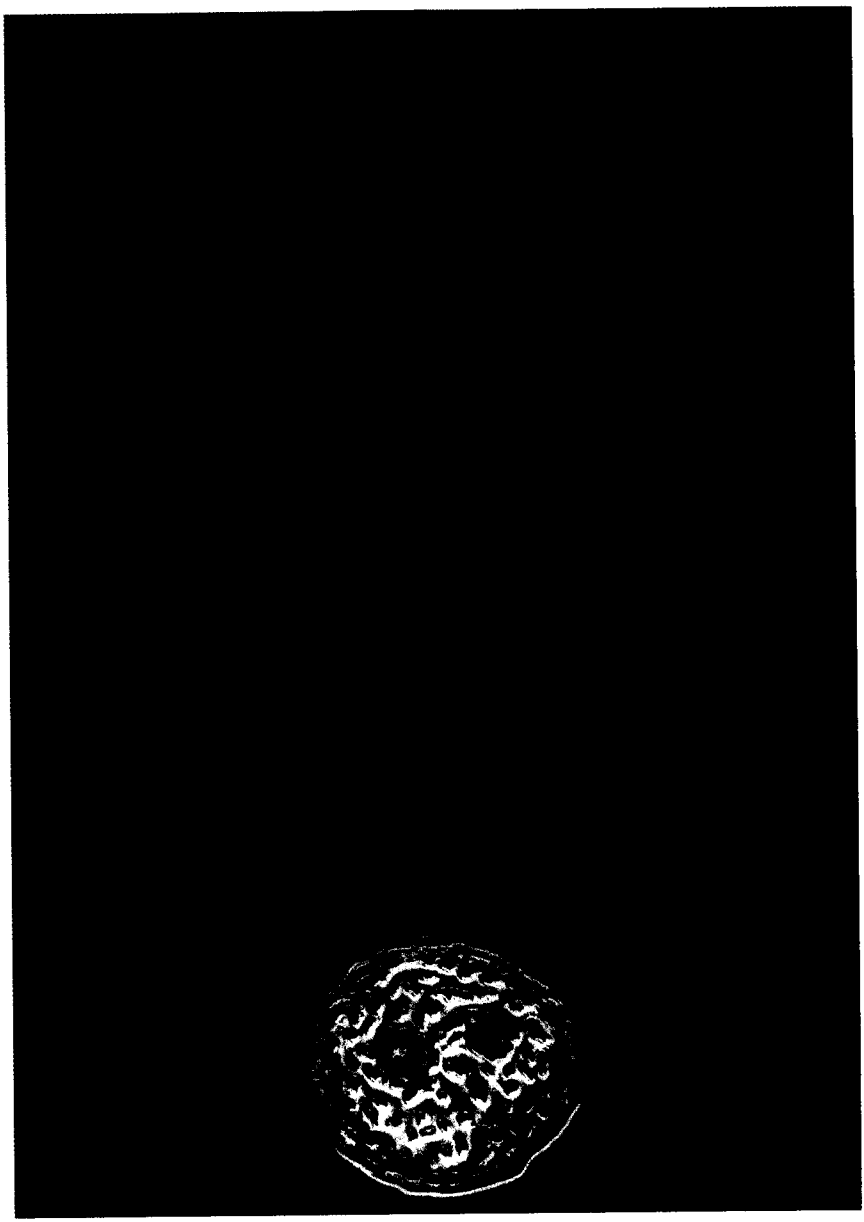
*Toronto, Ontario*



# THE VAN BEKKUM STEM CELL AWARD

Professor Hans Kolb is the third recipient of this award that is bestowed on individuals who have made major contributions for a prolonged period of time to the field of hematopoietic stem cells and transplantation.

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# LIST OF ABBREVIATIONS

## 0–9

2CDA .....2-chlordeoxyadenosine
4HC .....hydroperoxycyclophosphamide

## A

ABMTR-NA ...Autologous Blood and Marrow Transplant Registry of North America
ABVD .....doxorubicin, bleomycin, vinblastine, daccarbazine
AILD .....angioimmunoblastic lymphadenopathy-like T cell lymphoma
ALL .....acute lymphoblastic leukemia
alloBMT .....allogeneic bone marrow transplantation
AML .....acute myeloid leukemia
ANC .....absolute neutrophil count
AP .....accelerated phase
APC .....antigen-presenting cell
Ara-C .....cytosine arabinoside
ASO .....allele-specific oligonucleotide
ATG .....antithymocyte globulin
autoBMT .....autologous bone marrow transplantation

## B

BAVC .....BCNU, M-AMSA, VP16, cytosine arabinoside
BC .....blast crisis
BCNU .....carmustine (nitrosourea)
BEAC .....BCNU, etoposide, cytosine arabinoside, cyclophosphamide
BEAM .....BCNU, etoposide, cytosine arabinoside, melphalan
BEP .....bleomycin, etoposide, cisplatin
BFU-E .....burst forming unit-erythroid
BMMNC .....bone marrow mononuclear cells
BMT .....bone marrow transplantation

## C

CAE .....cyclophosphamide, doxorubicin, etoposide

CAV .....cisplatin, adriamycin, etoposide
CBP .....cyclophosphamide, carmustine, cisplatin
CBV .....etoposide
CBVP .....etoposide, cisplatin
CCG .....Children's Cancer Group
CDR .....complementary-determining region
CFU-GM .....colony-forming unit-granulocyte/macrophage
CHAQ .....child health assessment questionnaire
CI .....confidence interval
CMA .....cyclophosphamide, mitoxantrone, alkeran
CML .....chronic myeloid leukemia
CMT .....cyclophosphamide, mitoxantrone, thiotepa
CP .....chronic phase
CR .....complete remission
CRP .....C-reactive protein
CTCb .....cyclophosphamide, thiotepa, carboplatin
.....cyclophosphamide, doxorubicin, vincristine

## D

DEPC .....diethylpyrocarbonate
DFS .....disease-free survival
DHAP .....dexamethasone, cytosine arabinoside, cisplatin
DHMC .....Dartmouth-Hitchcock Medical Center
DLBC .....diffuse large B cell lymphoma
DLI .....donor lymphocyte infusion
DLT .....donor lymphocyte transfusion
DMSO .....dimethylsulfoxide
DTH .....delayed-type hypersensitivity
DTT .....dithiothreitol

## E

EATL .....enteropathy-associated T cell lymphoma
EBDIS .....European Breast Dose Intensity Study
EBMT .....European Group for Blood and Marrow Transplantation
EBMTG .....European Bone Marrow Transplant Group
EC .....epirubicin, cyclophosphamide
ECOG .....Eastern Cooperative Oncology Group
ECP .....early chronic phase
EDTA .....ethylene diamine tetraacetic acid

EFS .....event-free survival
EORTC-LCG . . .Leukemia Cooperative Group of the European Organization for Research and Treatment of Cancer
EP .....etoposide, cisplatin
Epo .....erythropoietin
ESR .....erythrocyte sedimentation rate
EULAR .....European League Against Rheumatism

## F

FAB .....French-American-British
FACS .....fluorescence-activated cell sorter/sorting
FDA .....Food and Drug Administration
FEC .....5-fluorouracil, epirubicin, cyclophosphamide
FITC .....fluorescein isothiocyanate

## G

G-CSF .....granulocyte colony-stimulating factor
GCT .....germ cell tumor
GELA .....French Lymphoma Trials Group
GIMEMA .....Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto
GM-CSF .....granulocyte-macrophage colony-stimulating factor
GOELAM .....Groupes Ouest Est Leucemies Aigues Myeloblastiques
GPI .....glycosyl phosphatidyl inositol
GVHD .....graft-vs.-host disease
GVL .....graft-vs.-leukemia
GVM .....graft vs. multiple myeloma

## H

HAM .....cytosine arabinoside, mitoxantrone
HCG .....human chorionic gonadotropin
HD .....high dose
HDM .....high-dose melphalan
HDS .....high-dose sequential
HLA .....human leukocyte antigen
HSA .....human serum albumin
HSCT .....hematopoietic stem cell transplantation

## I

IBC .....inflammatory breast cancer
ICC .....intensive consolidation chemotherapy
ICE .....ifosfamide, carboplatin, etoposide
ICT .....intensive chemotherapy
IFM .....Inter Groupe Français du Myelome
IFN .....interferon
IGR .....Institute Gustave-Roussy
IL .....interleukin
IMUST .....International Marrow Unrelated Search and Transplant
ISHAGE .....International Society of Hematotherapy and Graft Engineering
IV .....intravenous

## J

JCA .....juvenile chronic arthritis

## K

KIR .....kill cell inhibitory receptor

## L

LBL .....lymphoblastic lymphoma
LDH .....lactate dehydrogenase
LFS .....leukemia-free survival
LTC-IC .....long-term culture–initiating cells
LVEF .....left ventricular ejection fraction

## M

mAb .....monoclonal antibody
MAC-G .....mitoxantrone, cytosine arabinoside, cyclophosphamide, G-CSF
MACOP-B ....methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin
MBP .....myelin basic protein
MDS .....myelodysplastic syndrome
MHC .....major histocompatibility
MITT .....mitoxantrone, thiotepa
MMF .....mycophenolate mofetil

MOPP .....mechlorethamine, vincristine, procarbazine, prednisone
MP .....melphalan, prednisone
MRC .....Medical Research Council
MVPP .....mechlorethamine, vinblastine, prednisone, procarbazine

## N

NCI .....National Cancer Institute
NCIC-CTG ....National Cancer Institute of Canada, Clinical Trials Group
NHLCSG .....Italian Non-Hodgkin's Lymphoma Study Group
NIH .....National Institutes of Health
NK .....natural killer
NMDP .....National Marrow Donor Program
NRM .....nonrelapse mortality
NS .....not significant

## O

ORF .....open reading frame
OS .....overall survival

## P

PAP .....pulmonary arterial pressure
PBMNC .....peripheral blood mononuclear cell
PBPC .....peripheral blood progenitor cell
PBSC .....peripheral blood stem cell
PBSCT .....peripheral blood stem cell transplantation
PCI .....prophylactic cranial irradiation
PCR .....polymerase chain reaction
PE .....phycoerythrin
PFS .....progression-free survival
Ph .....Philadelphia chromosome
PLP .....proteolipid protein
PML .....polymorphonuclear leukocytes
PMN .....polymorphonuclear
POG .....Pediatric Oncology Group
PPD .....purified protein derivative
PR .....partial response
PSC .....peripheral stem cell
PSCT .....peripheral stem cell transplant

PSS .....progressive systemic sclerosis
PTK .....protein tyrosine kinase

## R

R1 .....first relapse
RCR .....replication-competent retrovirus
RDI .....relative dose intensity
RFLP .....restriction fragment length polymorphism
rh .....recombinant humani
RI .....relapse incidence
RT-PCR .....reverse-transcription polymerase chain reaction
RTOG .....Radiation Therapy Oncology Group

## S

SCLC .....small cell lung cancer
SD .....standard deviation
SE .....standard error
STAMP V .....cyclophosphamide, thiotepa, carboplatin
SWOG .....Southwest Oncology Group

## T

TBI .....total-body irradiation
TdT .....terminal deoxynucleotidyl transferase
TNF .....tumor necrosis factor
TNT .....paclitaxel, mitoxantrone, thiotepa
TRM .....treatment-related mortality
TRT .....thoracic radiotherapy
TTP .....thrombotic microangiopathy

## U

URD .....unrelated donor
URI .....upper respiratory illness

## V

VACOP-B .....etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin

VOD .....veno-occlusive disease
VPC .....virus-polycation complex

## W

WBC .....white blood cell
WHO .....World Health Organization

# CHAPTER 1
# AML

# 4HC-Purged Autologous Stem Cell Transplant in Acute Myelogenous Leukemia After Relapse

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## ABSTRACT

In patients who have relapsed after conventional chemotherapy, cure with chemotherapy alone is rare. Autologous stem cell transplant offers the potential for cure; however, the need for an adequate leukemia-free stem cell graft may limit the use of this treatment. For acute myelogenous leukemia (AML) in second clinical remission (CR2), we have shown a 30% actuarial disease-free survival in patients transplanted with autologous bone marrow grafts purged with 4-hydroperoxycyclophosphamide (4HC), a cyclophosphamide congener that is active ex vivo. Relapse was the major cause of failure. Duration of neutropenia was prolonged, with a median day to absolute neutrophil count (ANC) > 500/mm$^3$ of 45 days (range 20–285); deaths related to delayed engraftment were seen in only two patients. Overall transplant-related mortality was 15%. These data are comparable to the ABMTR database, in which actuarial disease-free survival at 3 years in 64 patients with AML in CR2 treated with 4HC-purged marrows grafts was 38%, compared with 4% in 22 unpurged comparison patients. The data from this study suggest a benefit for purging in this patient population; however, a randomized trial of purging has not yet been done, and delayed engraftment with purged marrow contributes to morbidity and cost of transplantation. To overcome delayed engraftment, we propose a randomized trial of 4HC-purged vs. unpurged peripheral blood progenitors in patients with AML in CR2.

## DISCUSSION

Patients who relapse after conventional chemotherapy are rarely cured with chemotherapy alone. Autologous stem cell transplant offers the potential for cure; however, the need for an adequate leukemia-free stem cell graft potentially limits the use of this treatment. Brenner et al.[1] used gene marking studies to show that tumor cells (leukemia or neuroblastoma cells) present in autologous marrow grafts

contribute to relapse. Purging the marrow ex vivo may decrease this tumor cell contamination, but the issue remains controversial in AML because of the lack of randomized trials. Cyclophosphamide congeners (4HC and mafosfamide) have been extensively studied as purging agents in AML, since these drugs have significant antileukemic activity and are relatively sparing of the earliest hematopoietic progenitors.[2,3] Cyclophosphamide cannot be used ex vivo because it requires metabolism in the liver, while 4HC and mafosfamide do not require this metabolic conversion to generate an active alkylating agent. Aldehyde dehydrogenase is the enzyme that inactivates the intermediary metabolite of cyclophosphamide and 4HC into an inactive form. The primitive hematopoietic stem cell contains high levels of this enzyme, and thus is relatively spared from toxicity due to these agents.

Mafosfamide is currently approved in Europe as a purging agent and in retrospective studies has shown effectiveness in this role in patients with AML. The European registry data demonstrated that the use of purged marrow was associated with a decrease in relapse and an improvement in disease-free survival. The beneficial effect of purging was seen in patients transplanted within 6 months of complete remission and who received a radiation containing preparative regimen.[4]

4HC has been extensively studied in both phase I and phase II trials in the U.S. The initial phase I trials using 4HC as a purging agent in patients with acute leukemia established the maximal tolerated dose using buffy-coated mononuclear cells (100 μg/mL for 30 minutes at 37°C). Two of seven patients treated at this dose remained in remission at the time of publication.[5] The phase II trial in patients with AML in second or third remission showed a 30% disease-free survival.[6] We have since reviewed the 10-year experience at Johns Hopkins Oncology Center with 4HC-purged autotransplants in 70 patients with AML, 62 in second and eight in third remission.[7] Median follow-up of survivors is 8 years. In this high-risk patient population, the overall disease-free survival was 31% at 5 years. Figure 1 shows the actuarial disease-free survival in second and third remission patients. There is no difference in disease-free survival between the patients transplanted in second or third remission; however, the number of third remission patients is small. Relapse was the major cause of failure, with an actuarial relapse rate of 55%. Duration of neutropenia was prolonged, with a median day to ANC >500/mm$^3$ of 45 days (range 20–285), although deaths related to delayed engraftment were seen in only two patients. Overall transplant-related mortality was 15%. Although the disease-free survival is comparable to that seen with allogeneic transplant at Johns Hopkins over the same time period (data not shown), the cause of transplant failure was different. Relapse was uncommon after alloBMT in second remission, whereas transplant-related mortality was higher, especially in older patients.

Data from the ABMTR also demonstrate effectiveness of purging with 4HC for autoBMT in AML. We performed a prospectively defined analysis of 4HC purging

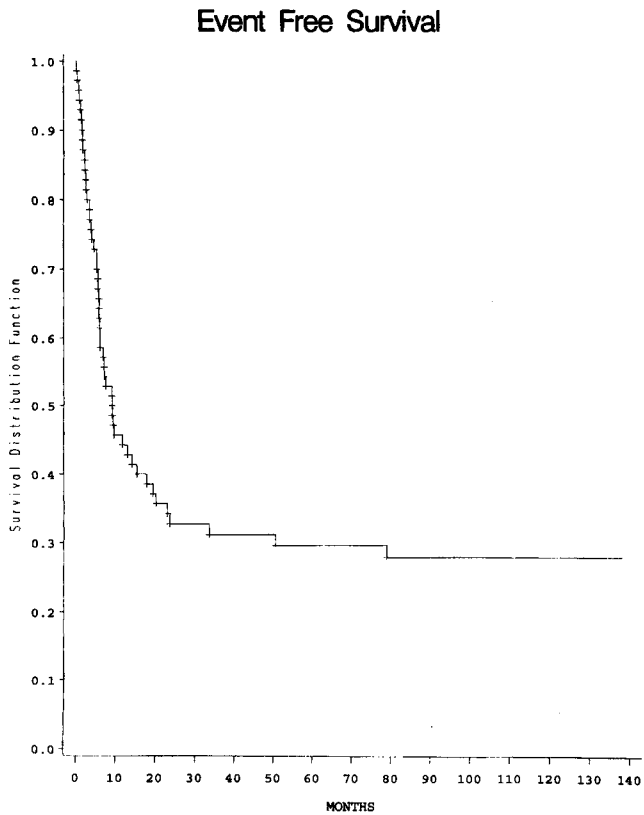


**Figure 1.** *Disease-free survival of 70 patients with AML in CR2 or CR3 who received autografts purged with 4HC at Johns Hopkins.*

vs. no purging that included 295 patients in first or second remission who were transplanted between 1989 and 1993. Initial analysis of the data was performed in 1995 when the median follow-up was 25 months. Several baseline characteristics were significantly different between the two groups. Patients in first remission who received 4HC-purged grafts were more likely to be younger, were less likely to have received consolidation chemotherapy, and were transplanted earlier in remission. Patients in second remission receiving 4HC-purged autograft were more likely to have relapsed within 1 year of first remission compared with those receiving an unpurged autograft (62 vs. 19%). To account for these differences, a forward stepwise model was built to test the effect of purging on time to treatment failure. Using this model, the risk of treatment failure was significantly lower using 4HC-purged grafts compared with unpurged grafts. Other significant covariates for treatment failure were first remission duration, number of cycles of consolidation

**Table 1.** Purging effect

| | *RR (95% CI)* | *P value* |
|---|---|---|
| 0–1 year posttransplant (4HC-purged vs. unpurged) | 0.6 (0.38–0.97) | 0.04 |
| >1 year posttransplant (4HC-purged vs. unpurged) | 0.21 (0.10–0.44) | <0.0001 |
| Other significant covariates | | |
| Age | | 0.06 |
| ≤20 years | 1.00 | |
| 20–40 years | 0.97 (0.61–1.54) | |
| >40 years | 1.55 (1.02–2.37) | |
| Cytogenetic abnormalities | | 0.06 |
| None | 1.00 | |
| Good | 0.48 (0.23–0.99) | |
| Intermediate | 0.58 (0.27–1.28) | |
| Poor | 1.37 (0.77–2.43) | |
| Unknown | 0.98 (0.65–1.49) | |
| Number of consolidation cycles | | 0.01 |
| 0 | 1.00 | |
| 1 | 1.04 (0.67–1.61) | |
| 2 | 0.55 (0.33–0.91) | |
| ≥3 | 0.40 (0.19–0.85) | |
| Unknown | 0.49(0.22–1.49) | |
| Duration of CR1 (≥1 vs. <1 year) | 0.38 (0.20–0.74) | <0.01 |
| Conditioning regimen | | <0.01 |
| BuCy ± other | 1.00 | |
| TBI ± other | 0.47 (0.29–0.81) | |
| Other drugs | 0.33 (0.17–0.65) | |

*Bu, busulfan; RR, relative risk; TBI, total-body irradiation.*

therapy, conditioning regimens, age, and cytogenetic abnormalities. Effects of purging and these other covariates are summarized in Table 1. Adjusted disease-free survival, stratified by remission status, is shown in Fig. 2A and B. In first remission, the adjusted leukemia-free survivals were 59 ± 10% after purged autografts and 18 ± 18% after unpurged autografts. In second remission, the adjusted leukemia-free survival in the purged group was 38 ± 17% compared with 4 ± 17% in the unpurged group. There was a ~10-day delay in neutrophil and platelet recovery related to 4HC purging that did not affect transplant-related mortality. These data are now being updated.

This retrospective, controlled study supports the role of 4HC purging in patients with AML, yet a randomized trial has not been done. Data from both Johns Hopkins and the ABMTR demonstrate no increase in transplant-related mortality attributable to 4HC purging; however, the delay in engraftment may contribute to longer

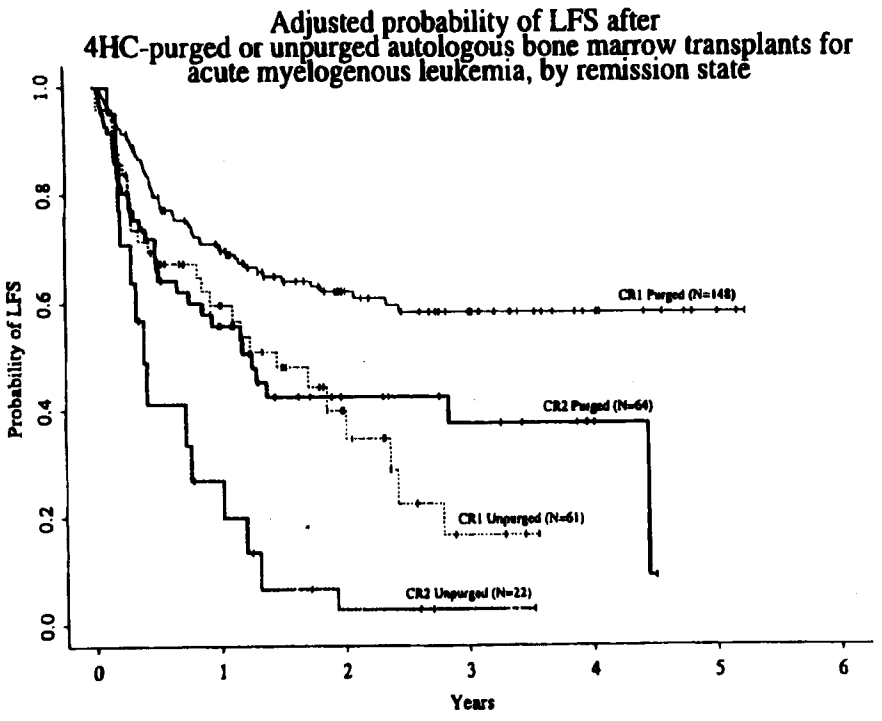


**Figure 2.** *Adjusted disease-Free survival of 295 patients reported to the ABMTR registry, stratified by remission status and purging.*

hospital stay and increased resource utilization. Many studies have shown that the use of mobilized peripheral blood stem cells (PBSC) speeds engraftment compared with bone marrow stem cells.[9–11] Several investigators have studied the use of PBSC as a source of stem cell grafts in AML, and long-term disease-free survival data are being collected. One study suggested a higher relapse rate with unpurged PBSC compared with purged bone marrow stem cell in patients with AML, due to a higher relapse rate in the unpurged PBSC arm.[11] Mobilized PBSC are also likely to be contaminated with leukemia cells, suggesting a benefit to purging. There are no published trials of the clinical use of purged PBSC in patients with AML. An in vitro study[12] compared the efficacy of purging normal and leukemic progenitors from bone marrow or PBSC. The data indicated no difference in the in vitro efficacy of purging clonogenic leukemia or normal myeloid progenitors related to stem cell source. Our preliminary data support the notion that myeloid progenitor recovery is similar to 4HC-purged PBSC and bone marrow.

The use of purged PBSC is attractive in that the delay in engraftment with purging may be partially overcome by the increased progenitor cells in the PBSC

graft. We plan to begin a randomized, double-blind trial of 4HC- vs. sham-purged PBSC in patients with AML in second remission.

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# Who Benefits From Autograft in AML in First Remission?

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Since younger patients with acute myeloid leukemia (AML) can reliably be induced to enter disease remission[1–3] the current priority is to prevent relapse of disease. Autologous bone marrow transplantation (autoBMT) has been extensively used in first and second remission and offers long-term survival of 45–55% and 25–39%, respectively.[4] Much of this experience has been derived from single-center or registry data. A number of prospective clinical trials have now prospectively evaluated this approach using cyclophosphamide with either total body irradiation or busulfan as myeloablation and bone marrow as the source of stem cells.

The two pediatric trials reported so far, conducted by the Pediatric Oncology Group[5] and the Italian AIEOP group,[6] randomized 304 patients. No improvement in disease-free or overall survival was noted between patients randomized to receive autograft or further intensive chemotherapy. Of the three trials of this design in adults, the results have been conflicting.

The EORTC-GIMEMA trial[2] showed a superior relapse-free interval and disease-free survival in the autograft arm compared with further chemotherapy. This was explained by the suboptimal effect of the chemotherapy arm but did not result in an improved overall survival because some patients who relapsed after chemotherapy were salvaged. The GOELAM study[7] had high-dose cytosine arabinoside (Ara-C) as the comparison arm and could not demonstrate a benefit for autograft in either disease-free or overall survival. The recently reported trial conducted by the US Intergroup,[8] which was very similar in design to the GOELAM protocol, similarly could not show any advantage in disease-free or overall survival for the autograft arm.

Two further trials conducted by the Medical Research Council (MRC) in the U.K.[9] and the Dutch HOVON group had a different design. In these trials, transplantation was being evaluated as an additional modality in consolidation. That is, the randomization was to receive or not an autograft (or allograft) after intensive chemotherapy. The HOVON trial is not yet reported, but the large MRC trial was able to demonstrate a significant improvement in disease-free survival in favor of adding an autograft. However, it was only in patients followed beyond 2

years that a significant survival advantage emerged. In summary, therefore, of six major trials aimed at evaluating the role of autografting in AML in first remission, overall survival benefit has been demonstrated in only one trial, where follow-up was prolonged.

These results do not at first sight suggest that autografting has a routine role in first remission. There are a number of features of this extensive trial experience that deserve closer examination. The single-center and registry data suggested that 45–55% of patients who received an autograft would become long-term survivors. Even on the intent-to-treat analysis, this has been exceeded in most trials. In virtually all trials, the autograft resulted in a significant reduction in relapse risk even though not all patients allocated to autograft actually received it. Perhaps the clearest example of this was in the MRC trial. Here the survival at 7 years from diagnosis with chemotherapy alone was 40%, which is at least equivalent to that achieved by high-dose Ara-C schedules.[1] However, the addition of autograft as an extra treatment course was able, on an intent-to-treat analysis, to reduce the relapse risk from 58 to 37%, even though one-third of the patients allocated by randomization to autograft did not receive it. So the substantial antileukemic effect was provided by only two-thirds of the patients. Arguably if they all had received it, the relapse risk could have been reduced further. Compliance with allocated treatment in these trials varied between 54 and 88%. In some cases, this was partially explained because after randomization patients had to undergo a further course of chemotherapy before receiving the autograft (as in the MRC trial). In some cases there was a delay in delivering the autograft, which resulted in some patients relapsing (as in the US Intergroup Trial).

The beneficial effect on relapse was counteracted by two factors. First, there were more deaths in the autograft arm. Most, but not all, followed autografting. The risk of death varied from 3 to 15%, which is higher than that expected (6–8%) from the single-center reports. In the MRC trial, where 12% of recipients of autograft died, the reasons were largely associated with poor quality of hematologic engraftment. If autografting is to fulfill its antileukemic potential, it has to be made safer. It remains to be seen whether the change to peripheral blood stem cells will improve the situation.

The second main confounding factor was that in several of the trials, patients who relapsed from the chemotherapy arm were more successfully rescued by further treatment. This is well illustrated in the EORTC-GIMEMA trial and to some extent in the US Intergroup trial. In general, previous experience in the treatment of AML suggests that once a patient has relapsed, the prognosis is very poor. On the other hand, registry data persistently demonstrate a durable survival of about 25–35% in patients who received a transplant in second remission. What has been unknown to date is how representative patients transplanted in CR2 were of all patients who relapsed. In other words, what was the denominator against

which to set the transplant experience? In the EORTC-GIMEMA trial, where this salvage effect was most pronounced, 22 of 36 who relapsed received an autograft. In the US Intergroup trial, the equivalent figure was 43 of 73, and in the MRC trial, 35 of 60. In all cases, approximately one-third of patients who actually received a transplant in second remission survived. This all suggests that, at least for some patients, delaying autograft (and allograft) for second remission is a viable option. Most investigators believe that transplantation should be used in remission rather than as primary treatment of relapse. Only one study has prospectively examined this strategy.

It is very important that patients apparently salvaged in this way are subject to prolonged follow-up. Most of the trials have limited follow-up of this cohort of patients, so the actual cure rate for salvage is not yet known. With longer follow-up, these patients in the EORTC-GIMEMA trial, where most received an autograft, have durable survival. No prospective studies have delineated which treatment option is superior in CR2, although a cohort comparison suggested that this was a complex issue and that there were patient subgroups in which transplantation was not superior.[10]

## Identifying who should have delayed transplant

Since the prospective trials appear to suggest that some patients who relapse can reliably be salvaged, it now becomes important to identify this subset. The other feature that is important in this equation, but very difficult to define, is the possibility that patients who relapsed after chemotherapy were more energetically treated than those who relapsed after an autograft.

### Prognostic factor index

A number of prognostic factors can reliably predict the relapse risk for patients in first remission. This is helpful in two respects. First, it is possible to compare treatment modalities within these risk categories. Second, risk assignment may provide valuable information about which patients can be reliably retreated when they relapse. The large MRC trial attempted to define such parameters.

Cytogenetic risk groups are the most powerful and the most universally accepted. The MRC trial defined t(8:21), inv(16), and t(15:17) as good risk cases, and abnormalities of chromosome 5 or 7, 3q- or complex changes as bad risk.[11] All other cases were designated as intermediate risk. The respective risks of relapse for more than 1600 patients were 34, 34, and 51%. A second important factor was the extent to which the marrow blast percentage was reduced by the first treatment course. However, the inclusion of the morphologic definition did not change the relapse risk in each group but did include more patients.[12]

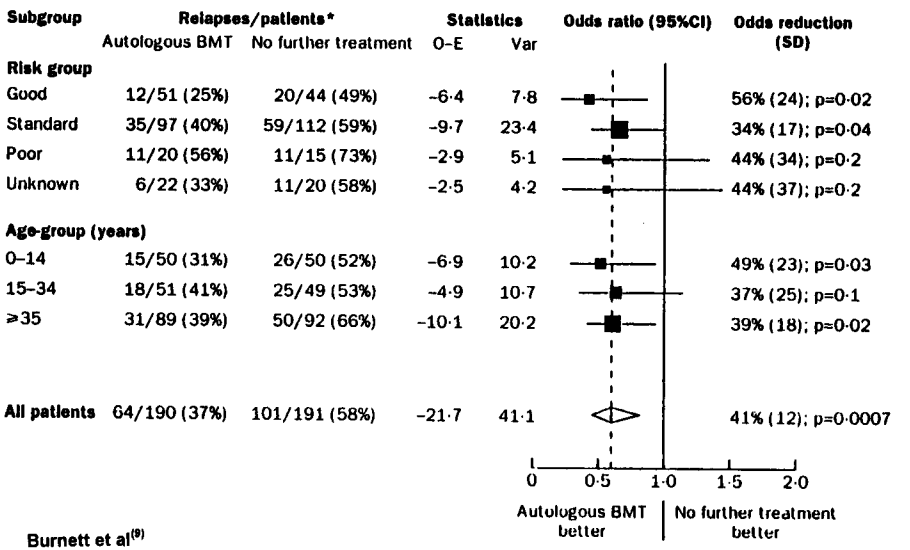


**Figure 1**

Whichever risk score is used dictates the patient's prognosis, irrespective of treatment approach used as consolidation. The third relevant factor was patient age.

## Which risk groups benefit from autograft?

Just as most of the randomized trials demonstrated a reduced risk of relapse, so within the risk and age categories in the MRC trial, autograft reduced the risk of relapse in all categories (Fig. 1). The numbers in each subgroup became too small to show a significant *P* value in a subset, but the odds ratio plot shows a consistent effect with an overall risk reduction of 41% ($P$=0.0007). This effect was proportionately equally important in each risk category. So while the ultimate survival is dependent on the risk group, relapse can be reduced by adding an autograft in all risk categories. So far other trials have not been analyzed in this way.

## Predicting salvage after relapse

If a strategy of delaying transplant is to be adopted to avoid unnecessary morbidity or risk of procedural mortality, it is crucial to identify patients who will reliably be successfully reinduced into a second remission. In the MRC AML10 trial, it was patients in the favorable risk category (Table 1) who had a high chance of entering CR if relapsing after chemotherapy. The survival of such patients at 3 years from relapse was 34%. While it will be necessary to review these patients in long-term follow-up

**Table 1.** Outcome after relapse in MRC AML 10

| | *AutoBMT* (*n*) | *No BMT* (*n*) | *% in CR2* | | *% Survival at 2 years* | |
|---|---|---|---|---|---|---|
| | | | *AutoBMT* | *No BMT* | *AutoBMT* | *No BMT* |
| All | 60 | 101 | 34 | 59 | 15 | 18 |
| Risk group | | | | | | |
| Good | 12 | 20 | 67 | 90 | 38 | 38 |
| Standard | 35 | 59 | 31 | 54 | 12 | 9 |
| Poor | 11 | 11 | 10 | 45 | 0 | 15 |
| Unknown | 6 | 11 | 33 | 45 | 17 | 9 |
| Age group (years) | | | | | | |
| 0–14 | 15 | 26 | 27 | 65 | 18 | 35 |
| 15–34 | 18 | 25 | 56 | 48 | 7 | 16 |
| >35 | 31 | 50 | 26 | 62 | 17 | 12 |

*From Burnett et al.*[9]

to be sure they are cured, it does seem a reasonably secure strategy to delay transplant in these groups. The trial data demonstrate that 40% will become long-term survivors with chemotherapy only, and about a third of those who relapse can be rescued.

These data preceded the introduction of all *trans*-retinoic acid for patients with acute promyelocytic leukemia. This development improves the prospect of cure without transplantation and further endorses the strategy of delaying transplant in this subgroup (Burnett AK, Grimwade D, Solomon E, Wheatley K, Goldstone AH, manuscript submitted).[13,14]

For patients in other risk groups, e.g., poor- or standard-risk or patients >15 years old, the prospects of salvage after relapse are much less reliable (Table 1). While there remains uncertainty about the best approach to consolidation, it is probably unwise to rely on rescuing patients who relapse. In contrast, it remains a priority to optimize first-line treatment. The relapse risk can be reduced in these patients by more treatment than the four intensive courses given in MRC AML10, but it is important to harness this effect with minimal toxicity. In standard risk, or patients >35 years old, the mortality in the autograft patients was 16 and 18%, respectively, so there is an opportunity to reduce treatment-related risk and thereby harness the antileukemic effect of autograft. While the MRC trial provides important evidence that more is better, it remains to be tested whether this is best achieved with a transplant or further chemotherapy. This is the central question on the ongoing MRC12 trial.

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# Immunotherapy in AML: No Positive Effect of Linomide *A Role for IL-2 + Histamine?*

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## ABSTRACT

Linomide has broad immunomodulatory properties in acute myeloid leukemia (AML) after autotransplantation. It was therefore tested in a phase III multicenter, double-blind placebo-controlled study in this patient group. Two hundred seventy-eight patients were randomized. Treatment was twice weekly for up to 2 years. Linomide had no effect on leukemia-free or overall survival. Platelet recovery was delayed in the linomide group. Thus, the short-term effect on natural killer (NK) cells and T cells could not be confirmed during long-term linomide treatment and may be due to NK and T cell inhibition by monocytes. The interleukin (IL)-2 effect on NK cell AML blast killing in vitro is also inhibited by monocytes. This inhibition can be inhibited by histamine. We therefore tested histamine and IL-2 in AML patients in remission. In a phase II study, toxicity was acceptable and clinical outcome is promising. We are now testing this drug combination in a worldwide phase III study in AML patients in remission.

## DISCUSSION

Linomide, a kinoline derivative, was originally synthesized by Pharmacia-Upjohn. The drug was found during screening for anti-inflammatory agents and shown to have potent immunostimulatory properties. Linomide in mice increased B and T cell function, enhanced NK cell activity, and had antitumor effects in lung cancer, colon cancer, and melanoma.[1,2]

In clinical studies on patients with AML after autologous bone marrow transplantation (autoBMT), linomide given in intermittent courses increased the numbers and activity of NK cells and number of monocytes, as well as increasing the T cell response and serum levels of IL-6.[2] Because this immunologic stimulation could be of potential therapeutic benefit in leukemia, we performed a

placebo-controlled phase III trial of linomide after autoBMT for AML. The rationale was that immune function has a bearing on prognosis in AML.

The study was conducted as a randomized, parallel, double-blind, multicenter phase III study. Study objectives were, as the primary end point, time to relapse, and as secondary end points, relapse rate after 1, 2, and 3 years; overall survival; immunologic reconstitution; hematologic reconstitution; and toxicity.

Between February 1991 and May 1994, 278 patients (from 39 centers in 10 European countries) were randomized and analyzed. They received linomide or placebo escalated to 0.2 mg/kg twice a week for 2 years. Two hundred sixty-six patients received at least one dose of study medication.

Surviving patients have been followed for 2–5 years. The total number of relapses were 58 in the linomide group and 63 in the placebo group. At present, there is no significant difference in time to relapse between the two treatment groups. Leukemia-free and overall survival were also similar between the two groups. Platelet recovery was significantly delayed in the linomide group, and this was the only event that differed significantly between the two treatment groups.

To study the immunologic effect, the maximum absolute number of circulating immune cells was calculated. There were significantly lower numbers of CD19-, CD16-, and CD56-positive cells in the linomide group. Thus, the earlier clinical pilot study findings on B cells and NK cells could not be confirmed. A possible explanation is that linomide has a different effect when given over a long interval compared with the previous short treatment duration. It is also possible that different results could have been obtained with another dose or dose schedule, such as intermittent administration.

Since linomide in this study had no effect, one could ask in which direction we should proceed with immunotherapy for AML. IL-2 alone seems not to be the solution. This compound has also been tested in AML. Despite some encouraging phase II studies in MRD,[4] randomized phase III studies have not shown benefits of IL-2 in AML.

IL-2, like linomide, also activates T cells and NK cells and promotes their expansion. One theoretically possible reason for the apparent lack of benefit from both linomide and IL-2 in AML could be monocyte/macrophage activation. Thus, Hellstrand and colleagues[5–7] showed that monocytes inhibit NK and T cell activation and NK cell–mediated AML blast killing in vitro and also that histamine protects NK cells and T cells from monocyte inhibition. This inhibition is, among others, thought to be mediated via production of reactive oxygen species (ROS). A sizeable fraction of NK cells undergo fragmentation of nuclear DNA and die by apoptosis after contact with monocytes. Histamine inhibits ROS formation in monocytes and thereby allows activation of NK cells by IL-2 in the presence of the suppressive monocytes.

In an animal model, histamine and IL-2 tested on melanoma gave greater tumor reduction than IL-2 or histamine alone. In vitro, it has been shown that monocytes

inhibit IL-2 induced killing of AML blasts and that histamine maintains this IL-2 induced killing despite increases in monocyte numbers.

AML patients have been treated with histamine dihydrochloride (0.3–0.7 mg b.i.d.) and IL-2 (1 μg s.c. b.i.d.) in repeated courses of 21 days. Thirty-six patients with AML in CR1 ($n$=24) and CR>1 ($n$=12) have been included. The courses of histamine + IL-2 have been continued until relapse or until a CR duration of 24 months. Most patients treated in CR >1 have also received low-dose chemotherapy (cytarabine and thioguanine) between the initial courses.

In CR1, median time to relapse has not been reached after a median follow-up of 23 months. For patients in CR 2–4, median time to relapse is 21 months after a median follow-up of 32 months. Eight of 11 evaluable patients have achieved a longer remission compared with their own previous remission.

Although there were local inflammatory reactions, fever, musculoskeletal discomfort, fatigue, short-lasting flush, headache, transient hypotension, and tachycardia, this treatment did not seem to have an impact on the quality of life of these patients. We conclude that treatment with IL-2 + histamine is safe and feasible and gives promising clinical results in a phase II study. These results, however, remain to be confirmed. We have therefore initiated a phase III study in which IL-2 + histamine is compared with no treatment in AML in CR1 or CR >1. The study has been initiated in the U.S., Canada, Australia, New Zealand, Israel, and Europe.

The general conclusion is that we were unable to confirm a positive effect of linomide in AML after autoBMT. This lack of efficacy may be due to inhibition of NK and T cell activity by monocytes. Since histamine inhibits the effect of monocytes on NK and T cells in vitro and based on a promising phase II study, we have initiated a multinational study to evaluate the role of IL-2 combined with histamine in prolonging leukemia-free survival in AML.

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# Autologous Bone Marrow Transplantation for Acute Myeloid Leukemia in Remission or First Relapse Using Monoclonal Antibody–Purged Marrow

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## ABSTRACT

One hundred thirty-eight patients with acute myeloid leukemia (AML) underwent autologous bone marrow transplantation (autoBMT) with monoclonal antibody (mAb)-purged marrow between 25 August 1984 and 28 March 1997. One hundred ten patients were in complete remission (CR) (CR1, 23; CR2 or CR3, 87) and 28 were in first relapse (R1) at autoBMT. Marrow was purged with complement and two mAbs (PM-81, anti-CD15; AML-2-23, anti-CD14) and cryopreserved. Preparative regimens included busulfan (16 mg/kg) and cyclophosphamide (Cy) (120 mg/kg) ($n$=93), Cy (120 mg/kg over 2 days) with total body irradiation (TBI) (1200 cGy in six fractions) ($n$=35), and busulfan (16 mg/kg) plus etoposide (60 mg/kg) ($n$=10). The median age was 40 years (range 2–66). Examining outcomes by remission status and preparative regimen, we found the following: for patients in CR1 treated with Cy/TBI ($n$=7), 3- and 5-year disease-free survival (DFS) rates were 71 and 57%. For CR1 patients treated with BU/Cy ($n$=12), 3- and 5-year DFS rates were both 45%. Three- and 5-year DFS for patients in CR2/3 treated with Cy/TBI ($n$=26) were both 23%. Three- and 5-year DFS for patients in CR2/3 treated with BU/Cy ($n$=55) were 31 and 28%, respectively. Three- and 5-year DFS for patients in R1 treated with BU/Cy ($n$=26) were both 37%. Increased age was associated with greater risk of death or relapse or both. In patients in CR2/3 treated with BU/Cy ($n$=55), a significant predictor of both DFS and OS was the length of time of CR1 ($P$=0.002 and 0.003, respectively). The use of autologous peripheral blood stem cells (PBSC) may accelerate engraftment. We have taken two approaches to this issue. First, if the number of $CD34^+$ cells/kg harvested from bone marrow was $<10^6$, patients underwent

apheresis after G-CSF mobilization. In five patients, the mean $CD34^+$/kg obtained from one to three (median 2) aphereses was $6.8\times10^5$. Three of the patients underwent autoBMT supplemented with PBSC. The median time to neutrophil recovery was 13 days. In addition, we have studied the use of PM-81 mAb in conjunction with the CellPro CEPRATE column to isolate $CD34^+$ cells from peripheral blood in patients with AML in remission. This protocol involves an initial selection for $CD34^+$ cells. In AML patients with CD34-blasts, this step results in the elimination of leukemic cells, while in $CD34^+$ AML, it will concentrate the CD34/$CD15^+$ cells. The second step is mAb purging, which eliminates any remaining $CD15^+$ leukemic cells. Three patients (one in CR1, one in CR2, and one in second relapse) have been treated with this purging method. Median time to neutrophil recovery was 15 days. Further experience with this technique is needed to evaluate its safety and efficacy.

## INTRODUCTION

The treatment of AML in children and adults has improved over the past two decades.[1–3] Combination chemotherapy can induce CR in 50–80% of patients. However, at least 50% of patients subsequently relapse and ultimately die of their disease.

Allogeneic bone marrow transplantation (alloBMT) has been shown to reduce relapse rates significantly in patients with AML in first CR.[4] Due to treatment-related mortality, overall DFS is approximately 50% in CR1[4] and, in CR2 and CR3, 20–30% at 5 years.

A limitation of alloBMT is that it can be applied only to a minority of patients with AML. Only about 40% of patients with AML have histocompatibility antigen (human leukocyte antigen [HLA])-matched donors, and most patients >55 years are considered too old to tolerate this procedure. Therefore, other treatment strategies are necessary for the majority of patients with AML.

Autologous BMT (autoBMT) is a promising therapy for the treatment of AML and has several advantages over alloBMT. The lack of a bone marrow donor does not preclude treatment, it can be applied to patients as old as 65 years, and there is no graft-vs.-host disease. The relapse rate with autoBMT has been higher than in allogBMT, due to either the reinfusion of marrow contaminated with leukemia cells or the absence of the graft-vs.-leukemia effect. However, numerous studies have shown that autoBMT can result in outcomes that compare very favorably to those of alloBMT.[5–12] In an attempt to increase the efficacy of this treatment, methods of purging autologous marrow using mAbs or cytotoxic drugs have been evaluated.[13–18]

We have been using cytotoxic mAbs that react specifically with myeloid cells and recognize antigens expressed on AML blast cells.[19,20] The mAbs PM-81 (anti-

CD15) and AML-2-23 (anti-CD14) bind to leukemia cells from >95% of AML patients.[19,20] These mAbs are cytotoxic to cells in the presence of complement (C′) and thus can lyse leukemia cells from almost all patients with AML.[21]

From 25 August 1984 until 10 February 1997, 140 purged autotransplants were performed on 138 patients who were in CR or first relapse at the time of transplant. Sixty of these patients were described in a previous report.[17] We now report on the long-term results of these phase II studies.

## PATIENTS AND METHODS

### Patients

Patients <65 years of age with a Karnofsky performance status of 80–100% and an expected survival time of >2 months were eligible for this protocol. At the time of bone marrow harvest, remission had to be documented by bone marrow aspirate and biopsy. Patients underwent harvest in first, second, or third CR. Transplants were performed in CR1, CR2, CR3, or first relapse (R1). Leukemia blast cells obtained at 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.

One hundred thirty-eight AML patients (with two patients undergoing retransplantation) ranging in age from 2 to 57 years in CR or first relapse were transplanted between 25 August 1984 and 10 February 1997 (Table 1). All but 11 patients had de novo AML at the time of initial diagnosis. Eleven patients had a myelodysplastic syndrome (MDS) before the diagnosis of AML. Twenty patients were treated on CALGB protocols 8882 or 8781. Four patients were transplanted at the Scripps Clinic (La Jolla, CA) and Children's Hospital (San Diego, CA), 59 at the Dartmouth-Hitchcock Medical Center (DHMC, Hanover, NH), 14 at Bowman-Gray School of Medicine (Winston-Salem, NC), four at the Medical Center of Delaware (Newark, DE), six at the University of Iowa Hospitals, 23 at the University of Pittsburgh, four at the University of Alabama, two at Children's Hospital of Pittsburgh, one at the University of Colorado, four at Miami Children's Hospital, four at Shands Hospital (Gainesville, FL), 10 at Stanford University Medical Center, and one at the Harris Methodist Hospital (Ft. Worth, TX).

The FAB subclasses of the cases were as follows: M1/M2, 61; M3, 16; M4/M5, 51; M6/M7, three; biphenotypic, one; unknown, seven. Cytogenetic analysis on the leukemia cells at presentation were available from the records of 103 patients.

The median time between the current remission or relapse and autoBMT ($n$=137) was 53 days (range 3–420). The median time between the current

**Table 1.** Clinical characteristics of patients

| | |
|---|---|
| Sex | |
| Male | 79 |
| Female | 59 |
| Median age (years) | 40 |
| FAB* | |
| M1 | 22 |
| M2 | 39 |
| M3 | 16 |
| M4 | 42 |
| M5 | 9 |
| M6 | 1 |
| M7 | 2 |
| Previous history | |
| MDS or secondary AML | 11 |
| Extramedullary disease | 17 |
| Cytogenetics† | |
| Abnormal 16q | 9 |
| 7q– | 5 |
| +8 | 4 |
| t(15;17) | 9 |
| t(8;21) | 8 |
| Normal karyotype | 52 |

**Frequency missing =7. †Frequency missing = 34.*

remission or relapse and autoBMT for patients in CR1 was 138 days (24–421). The median time between the current remission or relapse and autoBMT for patients in CR2 was 64 days (3–391). The median time between the current remission or relapse and autoBMT for patients in CR3 was 36 days (24–83). The median time between the current remission or relapse and autoBMT for patients transplanted in R1 was 15 days (4–374). Sixty-eight patients were harvested in CR1, 64 in CR2, and seven in CR3. One marrow harvest was performed in a patient with an extramedullary relapse (breast chloroma, marrow was normal).

## Marrow harvesting and purging

Bone marrow was harvested as described.[21] An effort was made to harvest $6\times10^8$ cells/kg from each patient. A mean of $5.37\times10^8$ cells/kg were actually harvested. Bone marrow mononuclear cells were prepared before treatment with mAb + C′ as described.[22,23] A mean of $1.4\times10^8$ cells/kg were treated with a mean recovery of 47%. An average of $6.59\times10^7$ cells/kg were used for the transplant.

The ex vivo treatment was performed on the Haemonetics cell processor for patients treated after May 1987 at DHMC, Bowman Gray Medical Center, and University of Pittsburgh. Before that date at DHMC, Scripps Clinic and Children's Hospitals, Stanford, and the Medical Center of Delaware, the marrow cells were treated in plastic or Teflon vessels (Savillex, Minnetonka, MN) with gentle shaking. For these treatments, two separate incubations with mAbs and C′ were performed as previously described.[23] As of March 1996, the AML-2-23 mAb was eliminated from the purging regimen based on in vitro experiments demonstrating that it did not add to the cytotoxicity mediated by PM-81 alone.

## PBPC collection

After determining that $CD34^+$ cell numbers were quite low in some patients, attempts to mobilize PBPC were made in several patients. Five patients not included in the analysis above underwent apheresis with granulocyte colony-stimulating factor (G-CSF) (5 μg/kg) mobilization, resulting in a mean collection of $1.0\times10^6$ $CD34^+$ cells/kg body weight (one to three aphereses; median two). These collections were purged in the same manner as bone marrow. Three patients underwent transplantation with bone marrow supplemented with PBPC receiving 0.7, 1.5, and $4.3\times10^6$ $CD34^+$ cells/kg.

PBPC from three additional patients were treated ex vivo using a two-step procedure: first, a $CD34^+$ cell collection using the Ceprate column, followed by a single treatment with PM-81 plus complement as described.[24]

## CD34 analysis

The total numbers of harvested cells were enumerated by particle counting. A measured aliquot was stained with two FITC-labeled anti-CD34 antibodies, one directed against 8G12 and the other against Qbend epitope. To eliminate cells committed to mature lineages ($Lin^+$), the suspension was counterstained with a cocktail of phycoerythrin (PE)-labeled antibodies including CD3 (T cells), CD19 (B cells), CD11b (neutrophils), and CD14 (monocytes). Particles <6 μm in diameter were excluded by use of a standard bead gate. Regions were established using unstained U937 cells to set the vertical axis and PE-stained U937 cells for the horizontal axis, and 20,000 events/sample were analyzed. This procedure was used until October 1995 on 39 patient samples.[25]

After October 1995, the method of Sutherland et al. was used for CD $34^+$ analysis.[26] Total nucleated white blood cells (WBC) were quantified by staining with fluorescein isothiocyanate (FITC)-conjugated CD45 antibody on eight patient samples. Simultaneous staining by phycoerythrin (PE)-conjugated CD34 antibody defined the progenitor/stem cell fraction. Using the fact that progenitor/stem cells

exhibit low-density CD45 expression and low side-scatter characteristics, a series of four gates were used to generate events considered to be true progenitor/stem cells. Cells within the final progenitor/stem cell gate can be distinguished from lymphocytes, monocytes, granulocytes, and other events that can contaminate the $CD34^+$ population.

## Preparative regimens

Thirty-five patients were treated with the following preparative regimen: Cy (60 mg/kg intravenously for 2 days, days −5 to −3) and fractionated total body irradiation (TBI) (200 cGy twice daily for 3 days, total dose of 1200 cGy, dose rate 5 to 10 cGy/min, days −2 to 0). In 1988, the preparative regimen was changed from Cy/TBI to busulfan (BU)/Cy2. Ninety-three patients were treated with BU (4 mg/kg/d orally for days −8 to −5) and Cy (60 mg/kg/d intravenously for 2 days, days −4 and −3). One patient in CR2 was treated with BU (4 mg/kg/d orally for 4 days, days −9 to −6) and Cy (50 mg/kg/d intravenously for 4 days, days −5 to −2). Ten patients (Stanford) were conditioned with BU (4 mg/kg/d orally, days −7 to −4) and VP-16 (60 mg/kg, day −3).

## Statistical methods

Two patient sets were included in this analysis: the first was the total set of 138 patients and the second was the set of 55 patients who underwent autoBMT in CR2/3 and received BU/Cy as their conditioning regimen. For both analyses, the last transplant represented was performed on 10 February 1997 and the last follow-up contact was 25 September 1997.

We estimated rates of relapse, overall survival (OS), and disease-free survival after autoBMT (and 95% confidence intervals [CIs]) using the product-limit or Kaplan-Meier method.[27]

In addition, proportional hazards regression (Cox regression, relative risk regression) was used to investigate the association of factors of interest with OS and DFS in these patients.[28] The factors listed in Table 2 were allowed to enter the OS and DFS models in a forward stepwise fashion (i.e., factors were evaluated first individually to see whether they contributed substantially to predicting outcome). The most significant univariate factor was then forced into the model, other factors being allowed to enter if they contributed significantly given the presence of the first. Additional factors were allowed to enter the models with interactions as appropriate.

Some factors were transformed before analysis using logarithms to make their distributions more symmetric. Body mass index was calculated as weight (kg)/height $(m)^2$.

**Table 2.** Factors investigated using proportional hazards regression

| |
|---|
| Remission status |
| Conditioning regimen |
| Age |
| Age ≥30 |
| Log10 BMI |
| Female sex |
| FAB |
| Hx MDS |
| Secondary AML |
| Hx MDS or secondary AML |
| Extramedullary disease |
| Log10 event to autoBMT |
| PM81 |
| Log10 WBC at diagnosis |
| Abnormal 16q |
| 7q– |
| +8 |
| t(15;17) |
| t(8;21) |
| Normal karyotype |
| Karyotype prognosis |
| Length of time in CR1 (CR2/3 patients only) |

## RESULTS

### Engraftment

A median number of $4.0\times10^7$ cells/kg body weight (range $2.30–8.23\times10^7$) were infused into each CR1 patient. The median number of cells transfused into the CR2/3 group was $2.80\times10^7$ (range $0.075–1.16\times10^8$). A median number of $4.10\times10^7$ cells/kg (range $2.38–59.6\times10^7$) were infused into each R1 patient.

Median observed recovery time for neutrophils to 500 cells/μL was 33 days. Median times to reach platelet counts of >20,000 and >50,000/μL independent of platelet transfusions were 51 and 79 days, respectively.

### Disease-free and overall survival

The DFS and OS from transplant of all patients as of 25 September 1997 are shown in Figures 1–6 by status at transplant (CR1, CR2/3, and R1).

For patients in CR1 treated with Cy/TBI ($n$=7), 3- and 5-year DFS rates were 71 and 57% (95% CI 38–100% and 20–94%) (Tables 3 and 4). For CR1 patients treated with BU/Cy ($n$=12), 3- and 5-year DFS rates were both 45% (95% CI

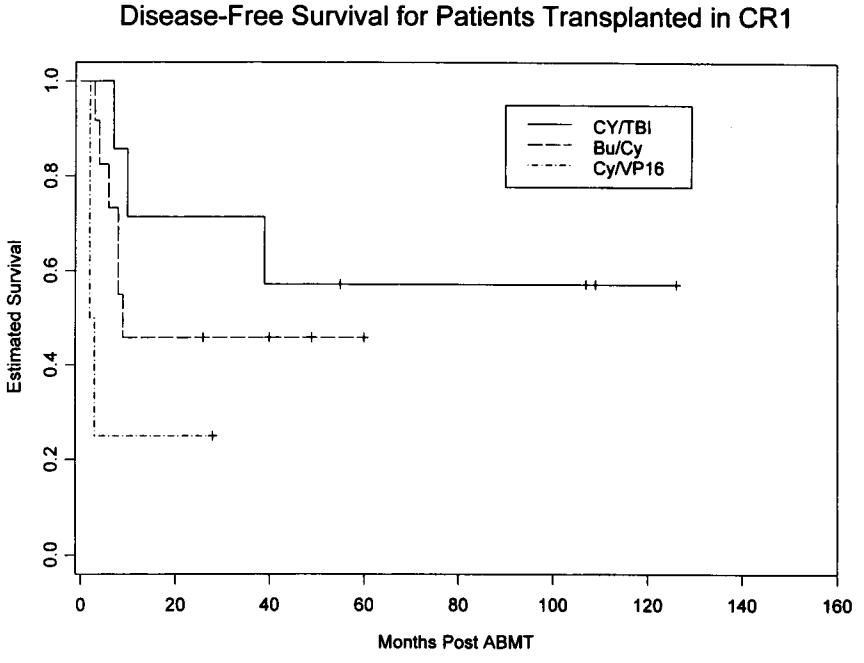


**Figure 1**

16–75%). Overall survival at 3 and 5 years for the patients treated with Cy/TBI was 71 and 57% (95% CI 38–100% and 28–94%). OS for patients treated with BU/Cy at 3 and 5 years was 46% (95% CI 16–75%).

Three- and 5-year DFS for patients in CR2/3 treated with Cy/TBI ($n$=26) were both 23% (95% CI 7–40%) (Tables 3 and 4). Three- and 5-year DFS for patients in CR2/3 treated with BU/Cy ($n$=55) were 31 and 28%, respectively (95% CI 17–44% and 14–41%). OS for patients treated with Cy/TBI at 3 and 5 years were both 27% (95% CI 10–44%). OS for the patients treated with BU/Cy at 3 and 5 years were 38 and 30% (95% CI 24–52% and 16–45%).

Three- and 5-year DFS for patients in R1 treated with BU/Cy ($n$=26) were both 37% (Tables 3 and 4). OS for the patients treated in R1 with BU/Cy at 3 and 5 years were both 38% (95% CI: 18–58%).

Eleven CR1 patients survive disease-free at a median follow-up time of 49 months (range 3–126). Twenty-seven patients transplanted in CR2/3 survive disease-free at a median time of 55 months (range 1–149). Ten patients transplanted in R1 survive disease-free at a median time of 47 months (range 5–96). For 48 of 115 CR2/3 and R1 patients, the duration of the post-autoBMT remission exceeded the duration of CR1 or CR2.

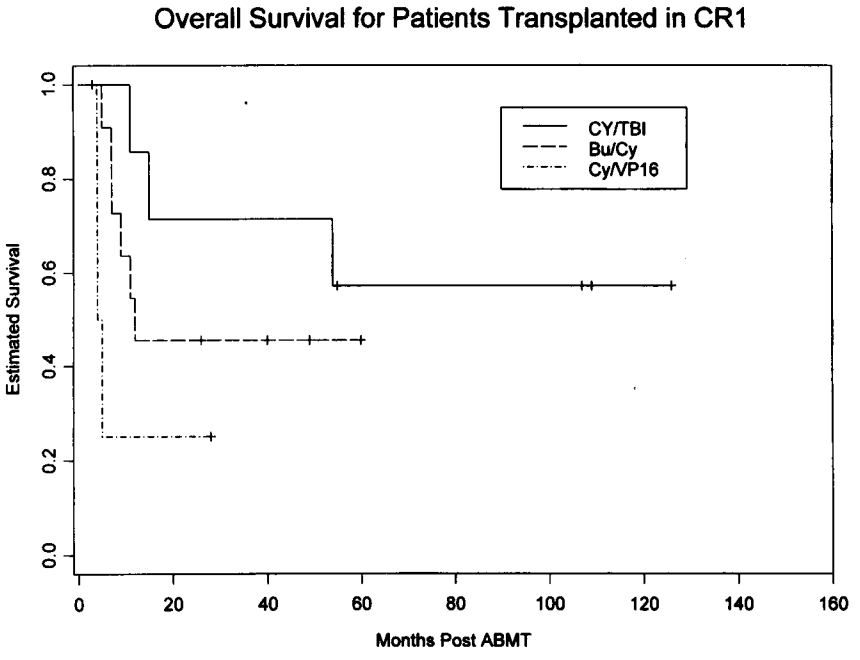


**Figure 2**

## Relapse

The actuarial relapse rates for different patient groups were calculated using the Kaplan-Meier method. For all patients ($n$=138), the relapse rates were 58 and 61% at 3 and 5 years. For patients in CR1, the relapse rates were 50 and 55% at 3 and 5 years. For patients in CR2/3, the rates were 62 and 65% at 3 and 5 years. For patients in R1, the rates were 56 and 56% at 3 and 5 years.

Examined by regimen, the results were as follows: CR1: Cy/TBI, 29 and 43% at 3 and 5 years; BU/Cy, 55 and 55% at 3 and 5 years. CR2/3: Cy/TBI, 56 and 56% at 3 and 5 years; BU/Cy, 62 and 65% at 3 and 5 years. Patients in R1 treated with the BU/Cy regimen: 54 and 54% at 3 and 5 years.

Median time to relapse for patients who relapsed ($n$=65) was 5.7 months. Median time to death or relapse ($n$=92) was 4.5 months.

## Multivariate analysis of risk factors for survival

*All patients (n=138).* When the factors in Table 2 were evaluated in a univariate fashion in all patients, age and age ≥30 were the only variables significantly related to OS ($P$=0.047 and 0.025, respectively) and DFS ($P$=0.026 and 0.015, respec-

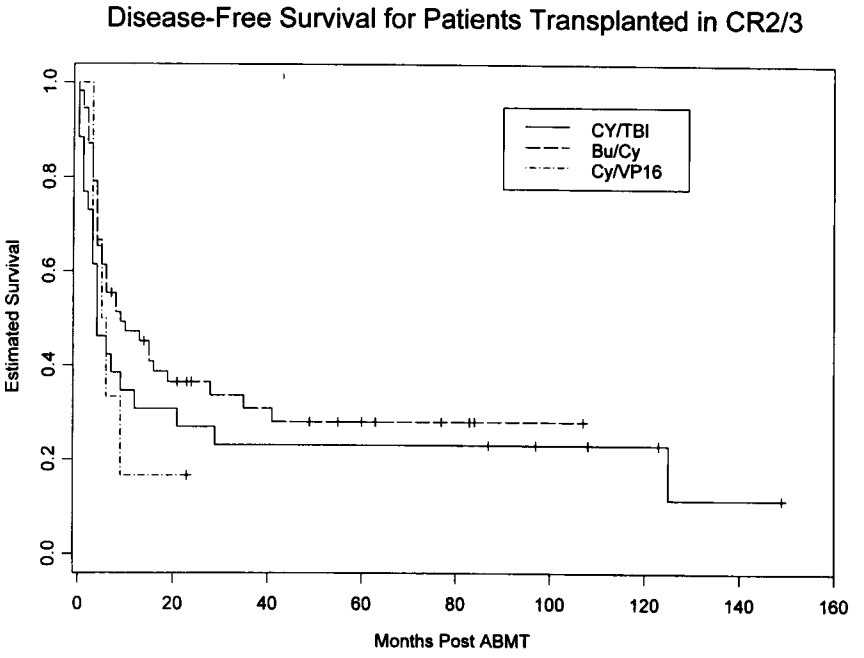


**Figure 3**

tively). As would be expected, higher age was associated with increased risk of death and death or relapse. When age ≥30 was forced into the OS and DFS models (i.e., in a multivariate analysis), no additional factors were found to be significant.

*CR2/3 patients conditioned with BU/Cy (n=55).* Among these patients, the strongest predictor of both OS and DFS was time in CR1 ($P$=0.002 and 0.003, respectively) (Tables 3 and 4). Longer time in CR1 (i.e., >184 days) was associated with decreased risk of death and death or relapse. In addition, male patients had an increased risk of death ($P$=0.045).

When time in CR1 was forced into the OS model, body mass index (BMI) ($P$=0.047), male sex ($P$=0.035), FAB 1,2 vs. others ($P$=0.022), and an extra chromosome 8 ($P$=0.048) were all associated with risk of death. However, as shown in Table 2, their effects varied according to the time in CR1. High BMI was associated with increased risk of death among patients with short and long CR1 times (i.e., CR1 <184 or >287 days). However, high BMI was associated with decreased risk of death among patients with intermediate CR1 times. Females with intermediate and long CR1 times had decreased risk of death compared with males. However, the opposite was true for females with short CR1 times. In patients with

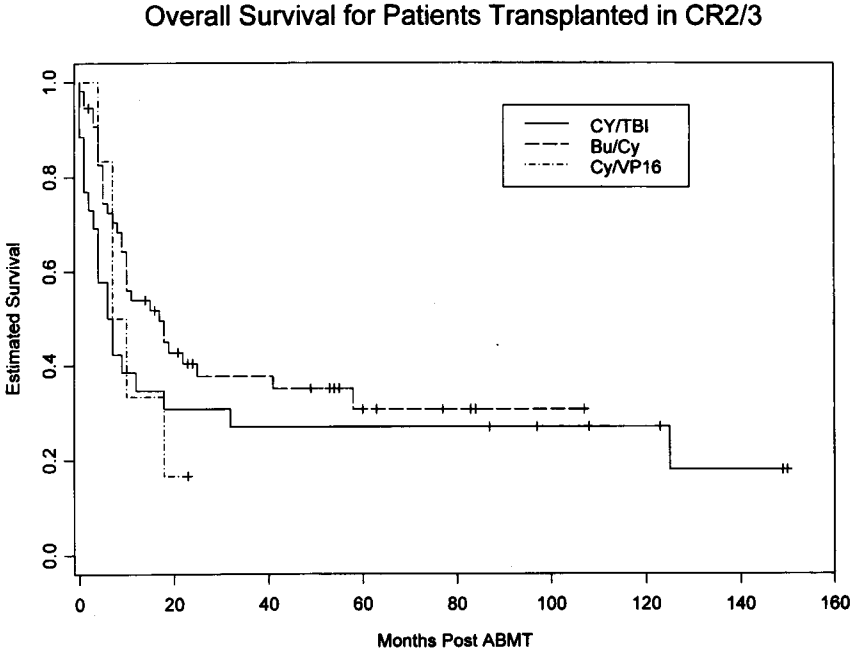


**Figure 4**

intermediate and long CR1 times, FAB 1 or 2 was a risk factor for death. The opposite was true for patients with short CR1 times. There was apparently an effect of CR1 time on the risk of death due to an extra chromosome 8. However, because of model fitting problems, it is not possible to describe the interaction between those factors.

When CR1 time was forced into the DFS model, FAB 1,2 vs. others ($P$=0.045), MDS or secondary AML ($P$=0.017), and PM81 ($P$=0.025) were associated with risk of death or relapse. As shown in Table 8, MDS or secondary AML was strongly associated with increased risk of death or relapse. There was interaction between the other two factors and time in CR1, although the relationship between PM81 and DFS was obscured by model fitting problems. FAB 1 or 2 patients with short CR1 times (<184 days) had lower risk of death or relapse than did patients in the remaining FAB categories. In contrast, FAB 1 and 2 patients with longer CR1 times had higher risk of death or relapse than did patients in the other FAB categories.

*Peripheral blood stem cell transplants.* In five patients, the mean $CD34^+$ cell/kg obtained from one to three (median two) aphereses was $6.8\times10^5$. Three patients

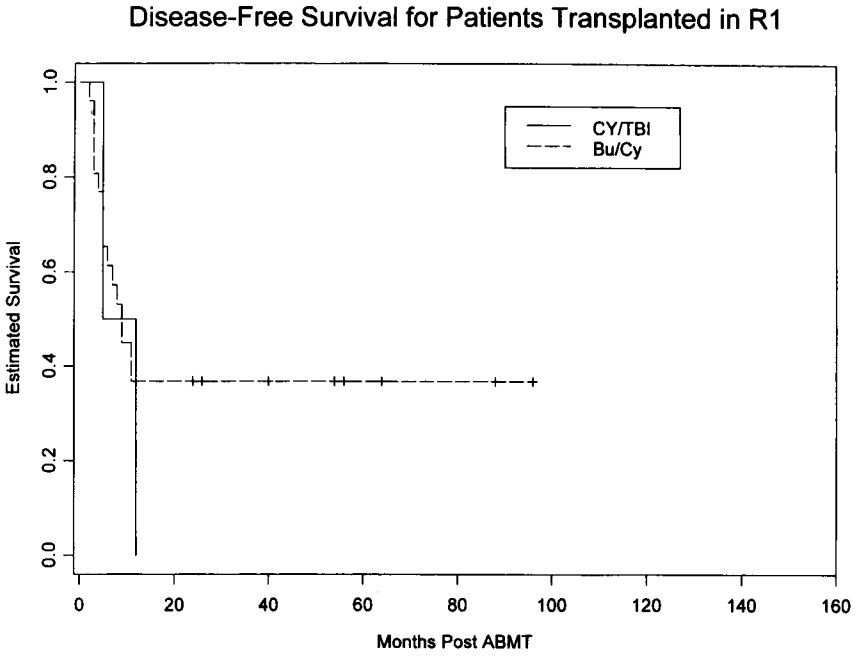


**Figure 5**

underwent autoBMT with supplemental PBSCT. Each engrafted neutrophils at a median time of 13 days (11–23 days). Two patients have relapsed at 8 and 14 months posttransplant in CR2.

Three additional patients were infused with cells first purified with the CellPro CEPRATE column to isolate $CD34^+$ cells followed by PM-81 mAb plus complement. Three patients (one in CR1, one in CR2, and one in second relapse) have been treated with this purging method. Median time to neutrophil recovery was 21 days.

## DISCUSSION

Bone marrow transplantation offers the potential for complete elimination of occult leukemia cells after initial remission induction and is probably the only curative treatment for patients with AML after first relapse.[4] Allogeneic BMT is established as a potentially curative therapy for patients in first remission, but the majority of patients with AML cannot undergo this therapy due to lack of an HLA-matched donor or advanced age.[4] This report and others[5–18] show that autoBMT is a viable alternative.

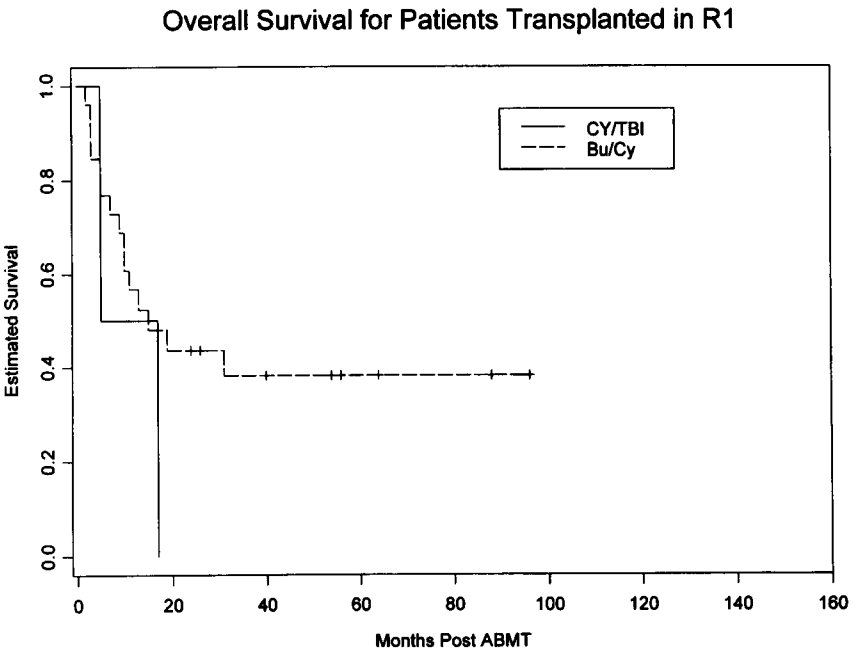


**Figure 6**

Since reinfused marrow may be contaminated with residual malignant cells after autoBMT, we have used ex vivo purging to eliminate residual neoplastic cells from the graft. Long-term survival for AML patients after autoBMT using various methods for removing occult leukemia cells has been reported.[12–18] The benefit of mafosfamide purging for patients transplanted in first CR within 6 months of attaining CR has been reported.[16] Chao et al.[9] published phase II studies showing that patients who received purged bone marrow (4-hydroperoxycyclophosphamide [4HC] or etoposide or both) had an actuarial DFS of 57% compared with a DFS of 32% in patients who received unpurged bone marrow. Yeager et al.[13] have reported favorable results similar to alloBMT with 4HC marrow purging in patients with AML who underwent autoBMT. Miller et al.[29] recently demonstrated that 4HC-purged autoBMT reported to the Autologous Bone Marrow Transplantation Registry (ABMTR) had significantly better outcomes than unpurged autoBMT in both CR1 and CR2/3.

Brenner et al.,[30] in a gene marking trial in AML autografting, demonstrated that autologous marrow harvested from leukemia patients in 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.

**Table 3.** Probability of 1-, 2-, and 3-year survival by regimen and remission status

| | | *Overall survival* | | | *Disease-free survival* | | |
|---|---|---|---|---|---|---|---|
| *Factor* | *n* | *1 year* | *2 years* | *3 years* | *1 year* | *2 years* | *3 years* |
| Remission status | | | | | | | |
| CR1 | 23 | 0.54 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| CR2/3 | 87 | 0.46 | 0.36 | 0.33 | 0.40 | 0.32 | 0.27 |
| R1 | 28 | 0.56 | 0.40 | 0.35 | 0.33 | 0.33 | 0.33 |
| Conditioning regimen | | | | | | | |
| Cy/TBI | 35 | 0.46 | 0.37 | 0.34 | 0.37 | 0.34 | 0.31 |
| BU/Cy | 93 | 0.53 | 0.42 | 0.39 | 0.44 | 0.37 | 0.34 |
| BU/VP16 | 10 | 0.30 | 0.20 | — | 0.20 | 0.20 | — |
| Conditioning regimen/status | | | | | | | |
| CR1 | | | | | | | |
| Cy/TBI | 7 | 0.87 | 0.71 | 0.71 | 0.71 | 0.71 | 0.71 |
| BU/Cy | 12 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 |
| BU/VP16 | 4 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | — |
| CR2/3 | | | | | | | |
| Cy/TBI | 26 | 0.35 | 0.31 | 0.27 | 0.31 | 0.27 | 0.23 |
| BU/Cy | 55 | 0.54 | 0.49 | 0.38 | 0.47 | 0.36 | 0.31 |
| BU/VP16 | 6 | 0.33 | 0.17 | — | 0.17 | 0.17 | — |
| R1 | | | | | | | |
| Cy/TBI | 2 | 0.5 | — | — | 0 | — | — |
| BUCy | 26 | 0.56 | 0.43 | 0.38 | 0.37 | 0.37 | 0.37 |

mAb-based techniques using antimyeloid mAbs have been used to purge AML marrow. This report updates our >10-year multi-institutional clinical data of autoBMT in AML with mAb and C′–mediated purging. As with allogeneic BMT, the results are dependent on remission status. Five-year DFS for patients transplanted in R1 and CR2/3 who were conditioned with Bu/Cy2 were 37 and 28%, respectively. These results compare favorably with those of allogeneic BMT.[4,31]

Significant prognostic factors operating in our study were length of first CR (for patients transplanted in R1 or CR2/3) and sex. Interestingly, patients with secondary AML or extramedullary disease did not fare worse. Nor did cytogenetics of AML cells at diagnosis affect outcome (although some of the patient subgroups were small). These results are surprising, since prior central nervous system (CNS) disease and cytogenetic abnormalities have been associated with worse outcomes in previous studies.[32–34]

We have conducted a phase II clinical trial of monoclonal antibody purging of bone marrow in patients with AML in remission at the time of harvest and in remission or relapse at the time of transplant. The overriding question raised by the

**Table 4.** Actuarial overall and disease-free survival at 5 years in patients grouped by preparative regimen and remission status at time of autoBMT

| *CR* | *n* | *Preparative regimen* | *5-year DFS* | *5-year OS* |
|---|---|---|---|---|
| 1 | 7 | Cy/TBI | 71 (38–100) | 57 (20–94) |
| | 12 | Bu/Cy | 46 (16–75) | 46 (16–75) |
| 2/3 | 55 | BU/Cy | 28 (14–41) | 30 (16–45) |
| | 26 | Cy/TBI | 23 (7–39) | 27 (10–44) |
| R1 | 26 | BU/Cy | 37 (18–55) | 38 (18–58) |

favorable outcomes reported for purged autologous bone marrow transplants is how much the purging contributed to the outcomes. This is stimulated by the occasional reports that unpurged marrow transplants have been associated with results comparable to purged transplants. However, recent evidence has been reported supporting the notion that purging is probably effective in reducing late relapses in AML.[29] Thus, we propose to conduct a phase III randomized trial comparing purged to unpurged stem cell transplants in patients with AML in remission.

We propose to conduct this trial in patients with AML in first remission. There are compelling reasons to target this group of patients. One is that this group of patients has a cure rate of <25%. Thus, improvements in this result would be welcome. Two, this patient population is well represented in major medical centers, thus ensuring timely accrual. Third, this population of patients is less encumbered with cumulative toxicities (overt or occult) from previous therapy.

Moreover, we propose to use peripheral blood stem cells in this trial. Others have reported the use of unpurged peripheral blood progenitor cells in the treatment of AML.[35–41] The survival results using PBSCT have been comparable to those using bone marrow, while, as expected, engraftment kinetics have been superior with PBSC. It is unknown whether purging of peripheral blood progenitors will affect engraftment or the relapse rate after high-dose chemotherapy and PBSCT. Our preliminary experience with purged PBSC shows that satisfactory engraftment times can be achieved with relatively small numbers of $CD34^+$ cells. A randomized trial of purged PBSC vs. unpurged PBSC will address the larger question of whether purging affects relapse and survival.

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# Autologous Blood Cell Transplantation in Acute Myeloid Leukemia (AML) in First Remission: The BGMT Experience

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Since 1984, the BGMT group has conducted three consecutive prospective studies aiming to prospectively compare allogeneic and/or autologous stem cell transplantation (SCT) with intensive chemotherapy in patients with AML in first remission. From these studies, it was found that allogeneic SCT was superior to autologous SCT or intensive chemotherapy, and disease-free survival (DFS) of patients undergoing either autologous SCT or intensive chemotherapy was equivalent. For autologous SCT, the patients underwent either bone marrow or blood cell transplantation (BMT or BCT). From prospective and retrospective analyses, it was concluded that *1*) hematopoietic recovery was significantly shorter after BCT than after BMT; *2*) DFS and relapse incidence (RI) were not statistically different after either BCT or BMT; and *3*) the timing of leukaphereses and the use of growth factors for blood cell collection did not influence DFS or RI in BCT patients.

# Can Autologous Stem Cell Transplantation Cure Myelodysplastic Syndromes?

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## ABSTRACT

Conventional, multidrug chemotherapy, such as that applied to induce complete remission (CR) in de novo acute myeloid leukemia (AML), has been demonstrated to be effective in myelodysplastic syndromes (MDS), with CR rates varying from 15 to 64%. Prolonged survival rates are disappointingly low and were reported to be <10% at 4 years after chemotherapy. The experience with autologous bone marrow transplantation (autoBMT) in patients with MDS or secondary AML is limited. Until now 173 recipients of autologous marrow grafts with MDS or AML after MDS have been reported to the registries of the European Group for Blood and Marrow Transplantation (EBMT). The 2-year survival of the 126 patients transplanted in first CR was 38%, the disease-free survival (DFS) was 33%, and the actuarial relapse rate was 55%. Patients <40 years old had a better DFS compared with patients with ≥40 years. This difference could be mainly explained by the higher treatment-related mortality of 39% in the older age group compared with 16% in the patients <40 years. Sixteen patients received autologous peripheral blood stem cell transplantation (SCT) in a prospective study of the European Organization for the Research and Treatment of Cancer (EORTC) Leukemia Cooperative Group and the EBMT. Peripheral stem cells were collected during the recovery phase of the first consolidation course using 300 μg of filgrastim daily until completion of peripheral blood stem cell (PBSC) collections. Repopulation was much faster compared with autoBMT. AutoSCT has emerged as a treatment option for patients with myelodysplastic syndromes. About one-

quarter of the patients with autologous stem cells may be free of disease for 3 years or longer.

## INTRODUCTION

The myelodysplastic syndromes are a heterogeneous group of disorders with a variable prognosis. The outlook of patients with refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEBt), therapy-related myelodysplastic syndromes, or secondary acute myeloblastic leukemia (sAML) is poor if untreated, with median survival durations of <12 months.[1–3] The cornerstone of therapeutic management for most patients with MDS is supportive care, mainly in view of the average advanced age in MDS and the poor response to therapy. Young patients can be treated successfully by allogeneic BMT if a histocompatible sibling donor is available.[4–7] Two-thirds of the patients with MDS who may benefit from allogeneic stem cell transplantation lack a suitable family donor. These patients may be transplanted with stem cells from unrelated donors or autologous stem cells. Intensive chemotherapy followed by autologous SCT may provide an alternative therapy for patients not eligible for allogeneic SCT.

## INTENSIVE CHEMOTHERAPY

Conventional, multidrug chemotherapy, such as that applied to induce CR in de novo AML, has been demonstrated to be effective in MDS, with CR rates varying from 15 to 64%.[8–12] CR rates of patients with MDS or sAML appear lower than those of patients with de novo AML treated with similar chemotherapy regimens. The higher failure rate of remission-induction therapy can be explained partly by the longer duration of hypoplasia after chemotherapy,[8,12,13] but also by a higher intrinsic biological drug resistance of the leukemic clone.[14,15] Addition of drugs that are less dependent on P-glycoprotein, such as idarubicin, or induction into proliferation of leukemic stem cells by granulocyte colony-stimulating factor (G-CSF) may overcome this higher drug resistance.[16–19] Some patients with MDS in CR after combination chemotherapy may achieve prolonged, disease-free survival,[9,10] but overall median remission duration appeared to be short and usually <12 months.[8,20] Prolonged survival rates are disappointingly low and were reported to be 8% at 4 years after chemotherapy[21] and 7% after 3 years.[8] The use of G-CSF after remission-induction chemotherapy may increase the response rate but does not prolong remission duration and survival.[16]

## PROGNOSTIC FACTORS ON OUTCOME AFTER INTENSIVE CHEMOTHERAPY

Patients with the morphologic picture of RAEB[9] and RAEBt[10] appeared to respond favorably to intensive chemotherapy, approaching remission rates of de

novo AML. Auer rod–positive patients, regardless of karyotype, appeared to have a better prognosis than Auer rod–negative patients when treated with intensive chemotherapy.[21] Patients with secondary AML evolved from MDS respond less well to chemotherapy than do those with de novo leukemias. Long-term remissions are rare.[8,20] The clinical outcome after intensive chemotherapy for therapy-related MDS and AML (t-MDS and t-AML) is usually poor, with only an exceptional patient surviving beyond 1 year.[9,22] A minority of the patients with specific cytogenetic rearrangements of de novo AML or no cytogenetic abnormalities who present with AML not preceded by MDS seem to have a high remission rate and a more prolonged CR duration compared with the average patient with MDS.[22–24]

Children with MDS respond poorly to intensive chemotherapy. The proportion of complete remission in 20 patients with MDS was 35% compared with 74% in 35 children with AML treated with the same protocols.[25] Patients younger than 45–50 years appeared to respond better to combination chemotherapy than did older patients. CR rates in patients <45–50 years old ranged from 71 to 86% in several studies, and the remission rates in the older patients ranged from 25 to 43%.[8,11,20,26]

The presence of cytogenetic abnormalities specific for MDS, such as abnormalities of chromosomes 5 or 7, has a major negative impact on the prognosis after combination chemotherapy. Fenaux[10] observed a CR rate of 57% in MDS patients with a normal karyotype, contrasting with a CR rate of 31% in patients with rearrangements of chromosomes 5 or 7. None of the five patients with multiple chromosomal abnormalities achieved complete remission in a Leukemia Cooperative Group study of the EORTC.[18] Remission duration is extremely short in patients with cytogenetic abnormalities of chromosomes 5 and/or 7, with all patients relapsing within 5 months.[10]

## AUTOLOGOUS BONE MARROW TRANSPLANTATION

Maintaining remission after remission-induction chemotherapy is a difficult issue. Some patients may achieve prolonged, disease-free survival if treated with postremission chemotherapy, but overall median remission duration was usually <12 months.[8,10,27] The feasibility to collect normal polyclonal hematopoietic stem cells in patients with MDS has been questioned until recently. However, the majority of patients with MDS who reach remission after intensive chemotherapy appeared to achieve a cytogenetic remission.[18,19] Moreover, the remission appeared to be polyclonal when tested with X-linked polymorphic genes.[27] Polyclonality was also observed in marrow progenitor cells of patients with MDS in remission.[29] The experience with autoBMT in patients with MDS or sAML is limited, and the literature contains only case reports.[30,31] Until now, 114 recipients of autologous marrow grafts with MDS or AML after MDS have been reported to the registries

of the EBMT.[32] An update of this report on 173 reported patients was presented at the Dallas meeting in 1998 (Ninth International Symposium on Autologous Blood and Marrow Transplantation). The overall survival at 2 years of the 126 patients transplanted in first CR was 38%, the DFS was 33%, and the actuarial relapse rate was 55%. Nineteen patients were transplanted for MDS that had not progressed to AML before autoBMT. The actuarial DFS at 2 years after ABMT was 40%, and the relapse rate 58%. Thirty-nine MDS patients had progressed to AML before chemotherapy and autoBMT. DFS of these patients was 30%, and the relapse rate 68% (Table 1). Twenty-one patients were transplanted for MDS or AML that had developed after treatment with chemotherapy for other malignancies or autoimmune diseases. Actuarial DFS of these patients was 36%, and the relapse rate 60%.[32] Patients <40 years old had a better DFS compared with patients of age ≥40 years. This difference could be explained partly by the higher treatment-related mortality of 39% in the older age group compared with 16% in the patients <40 years (Table 1). The results were compared with a matched control group of 110 patients with de novo AML. The DFS at 2 years was 28% for the cohort of 55 patients transplanted for MDS/sAML and 51% for those transplanted for de novo AML ($P$=0.025). AutoBMT for MDS or sAML resulted in a lower DFS due to a higher relapse rate.[32]

Transplant-related mortality and death due to regeneration failure did not appear to occur more often than after ABMT for de novo AML. The hematopoietic engraftment was slower despite the sufficient number of CFU-GM collected ($5\times10^4$/kg).[30] The median time to engraftment was 37 days for the white blood cells and 75 days for the platelets in the first retrospective analysis of the EBMT on 17 autografted MDS patients.[30] Laporte reported the results of ABMT with mafosfamide-treated marrow in seven patients with AML after MDS. The

**Table 1.** Three-year actuarial probability of disease-free survival (DFS), survival, treatment-related mortality (TRM), and relapse of patients treated with autologous transplants: influence of age on treatment outcome

| *Autologous transplants* | *Number* | *DFS* | *Survival* | *TRM* | *Relapse* |
|---|---|---|---|---|---|
| All patients | 173 | 30 | 32 | 29 | 58 |
| CR-1 | 126 | 33 | 38 | 25 | 55 |
| No CR-1 | 47 | 18 | 14 | 51 | 64 |
| *P* value | | 0.06 | 0.01 | 0.07 | 0.32 |
| <20 years* | 12 | 46 | 58 | 17 | 44 |
| 20–40 years* | 48 | 36 | 41 | 15 | 58 |
| >40 years* | 66 | 29 | 29 | 39 | 51 |
| *P* value | | 0.08 | 0.05 | 0.22 | 0.27 |

**Only patients in first complete remission.*

hematopoietic engraftment was also slower in these seven patients, but all patients engrafted except for one patient who died early before engraftment of treatment-related causes. Two patients were alive and well at 10 and 28 months after autoBMT.[30]

Demuynck et al.[33] investigated the feasibility of collecting peripheral stem cells in 11 patients with myelodysplasia. This resulted in seven patients with an adequate yield ($>1\times10^6$/kg) of CD34 cells.[32] Three patients with a normal to excellent stem cell harvest were demonstrated to be polyclonal by PCR techniques based on X-chromosome inactivation patterns.[27] Sixteen patients received autologous peripheral blood SCT in a prospective study of the EORTC Leukemia Cooperative Group and the EBMT.[34] Peripheral stem calls were collected during the recovery phase of the first consolidation course using 300 µg filgrastim (subcutaneously) daily until completion of PBSC collections. Preliminary data indicate that the repopulation was much faster compared with autoBMT.[27,33–35] Autologous SCT has emerged as a treatment option for patients with myelodysplastic syndromes or acute myeloid leukemia evolved from MDS. Only patients who are in complete remission after intensive chemotherapy are considered as candidates for autoSCT. About one-quarter of the patients with autologous stem cells may be free of disease for 3 years or longer. The high treatment-related mortality contributes substantially to this result, which is inferior to the results obtained by transplantation for de novo AML. Prospective, multicenter studies may reveal which categories of patients with MDS will benefit from intensive chemotherapy followed by autoSCT. This treatment approach has to be assessed against the merits and disadvantages of allogeneic stem cell transplantation with donors other than HLA-identical siblings.

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# Immunotherapy With IL-2 After Autologous Stem Cell Transplant for Acute Myelogenous Leukemia in First Remission

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## ABSTRACT

Despite improvement in the proportion of patients achieving durable remission with dose-intensive consolidation strategies, leukemic relapse still occurs in 40–70% of patients who achieve remission. Interleukin (IL)-2 is a cytokine that has a broad range of antitumor effects and has been used in some patients undergoing autologous transplant for a variety of malignancies. We have conducted a study to determine the toxicity and efficacy of high-dose consolidation followed by stem cell collection and the use of a radiation-based transplant regimen followed by intensive IL-2 therapy in a phase II trial of 54 patients who had achieved first remission of their disease. The treatment consisted of consolidation postinduction with high-dose cytosine arabinoside (Ara-C) with or without idarubicin followed by peripheral blood stem cell collection. This was followed by autologous transplant using FTBI 12 g, VP-16 60 mg/kg, cytoxan 75 mg/kg, and IL-2 on hematologic recovery. The IL-2 schedule was $9\times10^6$ IU/m$^2$ for 24 hours days 1–4 and $1.6\times10^6$ IU/m$^2$ for 24 hours days 9–18. Eight patients were unable to proceed to transplant because of either toxicity from consolidation or inadequate stem cell collection. Forty-six patients underwent transplant at a median of 4 months after complete remission. There was one septic death during neutropenia in consolidation and one during neutropenia for transplant for an overall mortality of the program of 4%. Thirty-five of 46 patients were able to receive posttransplant IL-2 at a median of 36 days after transplant. With a median follow-up of 25 months (1.2–47) the 2-year probability for all 54 patients is 75% (95% confidence interval [CI] 62–86%) and 83% (95% CI 69–92%) for the 46 patients undergoing ASCT. Toxicities from IL-2 were mainly thrombocytopenia, leukopenia, fluid retention,

and fever. No patient required intensive care unit (ICU) or ventilatory support. These results suggest that high-dose IL-2 is associated with a very low regimen-related mortality after autologous transplant for acute myeloid leukemia (AML) in first complete remission. Longer follow-up is required to determine the impact of this strategy on long-term disease-free survival.

## INTRODUCTION

Since the mid-1970s, interest in autologous hematopoietic cell transplantation for AML has increased substantially in transplant centers around the world. This increase has been due in part to the limited success of standard-dose chemotherapy in achieving long-term disease-free survival for the vast majority of adults with AML. New techniques for hematopoietic cell procurement, combined with the expanded knowledge of the cellular and molecular biology of AML, have allowed refinements in the interpretation of clinical results for AML in trials using either chemotherapy or autologous hematopoietic cell transplantation.

Studies using unpurged marrow, purged marrow, or peripheral blood stem cells have reported disease-free survival for patients transplanted in first CR of between 34 and 70%.[1–6] Although each trial demonstrates the potential efficacy of the approach chosen, many of the studies have been criticized for including patients who had received widely varying induction therapies, types and numbers of consolidation cycles for autologous transplant, duration of CR before transplant, and relatively short follow-up times as well as differences in stem cell product manipulation and preparative regimens.

Unlike allogeneic transplantation for AML in first remission, where the major causes of failure are complications of the therapy, the major cause of failure after autologous transplant is leukemic relapse. Both twin transplants and cell marking studies have documented that the source of relapse after autologous transplant is related to the residual body burden of disease and/or to the infusion of leukemic cells contained in the stem cell graft.[7,8] These observations support the concept that an immunotherapeutic effect of the allograft contributes to prevention of relapse after an allogeneic transplant (graft-vs.-tumor effect).[9]

IL-2 is a cytokine that has antitumor activity in selected tumors. Based on data suggesting that the activation of natural killer and/or cytotoxic T lymphocytes is also active against leukemia and lymphoma cells, IL-2-based therapies have also been under active investigation for hematologic malignancies. IL-2 has been administered to patients following recovery from autologous transplant in an effort to reproduce the graft-vs.-malignancy effect seen in allogeneic transplant.[10] A graft-vs.-host-like clinical phenomenon has also been reported in patients receiving IL-2 following autologous bone marrow or stem cell transplant as well as in patients treated with combinations of low-dose cyclosporin A with or without interferon.[11]

Several centers have explored the use of IL-2 either as part of a marrow purging approach with in vitro incubation of the graft with IL-2, concomitant with administration of IL-2 in the early posttransplant phase, or as consolidation therapy following hematologic recovery.[12,13] Robinson et al. reported on 22 patients with acute leukemia in relapse or beyond first remission who underwent autologous BMT or peripheral blood stem cell transplant using cells that were harvested during first complete remission.[14] IL-2 was given by continuous intravenous infusion after hematologic recovery at doses ranging from 9 to 12 million IU/m$^2$/d for 4–5 days followed 1 week later by a 10-day infusion of 1.6 million IU/m$^2$/d. Among 17 patients with AML, four remained in continuous remission from 12 to 25 months after therapy and four of five ALL patients were also in remission 15–25 months after therapy. This regimen is currently undergoing evaluation in a randomized study at the Southwest Oncology Group in which patients with lymphoma undergoing a peripheral blood stem cell transplant are randomized to receive a single course of IL-2 vs. no therapy.

Based on the laboratory studies indicating induction of effector cells that have the capacity for lysing autologous tumor cells and the clinical trials indicating a potential therapeutic effect in patients undergoing autologous transplant for relapsed disease, we have explored the feasibility of administering posttransplant IL-2 in patients undergoing autologous transplant following high dose Ara-C consolidation and mobilization of stem cells and who were then treated with a radiation-based transplant regimen. The goals of this study were to determine the feasibility, toxicity, and therapeutic effect of a treatment program that began with consolidation therapy of AML in first remission.

## MATERIALS AND METHODS

### Patient population

Between August 1994 and April 1998, 54 patients with AML in first remission were entered onto the study. The median age of this adult population was 46 (21–60). Cytogenetic analysis of the leukemia at diagnosis included favorable T8;21, inversion 16 = 13 (24%), intermediate (normal) = 23 (43%), unfavorable 7 (13%), and indeterminate and unknown 11 (20%). The median time from achievement of complete remission to entry on study was 27 days (7–327).

### Treatment program

Patients with AML in first complete remission were treated with consolidation therapy using a regimen of high-dose Ara-C with or without idarubicin. Ara-C was given at 3 g/m$^2$ over 3 hours for eight doses, and idarubicin was given at 12 mg/m$^2$

after doses 1, 3, and 5 of Ara-C. Granulocyte colony-stimulating factor. (G-CSF) was started on day 7 after completion of chemotherapy at a dose of 5 μg/kg until completion of stem cell collection. Stem cell collection was performed on hematologic recovery with a target collected cell dose of $2\times10^6$ CD34 cells/kg.

After collection of stem cells, patients underwent autologous transplant using a preparative regimen of fractionated total body irradiation (1200 rads in 10 divided fractions), VP-16 60 mg/kg on day –4, and cyclophosphamide 75 mg/kg on day –2.[15] All patients received an unpurged stem cell product on day 0.

After stem cell reinfusion, G-CSF was given at 10 μg/kg until the absolute neutrophil count (ANC) was >500 for 3 days. After clinical and hematopoietic recovery (white blood cells (WBC) >1000 and platelets >20,000 with one platelet transfusion per day for 3 days), IL-2 was administered in the following schedule: $9\times10^6$ IU/m$^2$ for 24 hours on days 1–4 as an inpatient and $1.6\times10^6$ IU/m$^2$ for 24 hours on days 9–18 by infusion pump in the outpatient department.

## RESULTS

Of the 54 patients entered on study, 46 patients underwent autologous stem cell transplantation at a median of 4 months (1.8–10.2) after achievement of hematologic remission. The reasons for eight patients failing to proceed to autologous BMT from consolidation were toxicity (five) and inadequate stem cell collection (three) including one septic death during consolidation.

Hematopoietic recovery to an ANC of 1000 and platelets of 20,000 after autologous stem cell transplantation was 11 (8–58) and 20 (7–183) days, respectively. There was one septic death during neutropenia after BMT for an overall mortality of 2 of 54 (3%). Thirty-five of 46 patients (76%) were able to receive post–autologous stem cell transplant IL-2 at a median of 36 days (25–75) following autologous stem cell transplant. Currently, with a median follow-up of 25 months (1.2–47), the 2-year disease-free survival probability for all 54 patients is 75% (95% CI 62–86%), and 83% (95% CI 69–92%) for the 46 patients undergoing autologous stem cell transplant. Toxicities from the IL-2 were mainly thrombocytopenia, leukopenia, fluid retention, and fever, which resolved with discontinuation of IL-2. No patient required ICU or ventilatory support.

## DISCUSSION

Relapse is still the major cause of failure after autologous transplantation for leukemia. Several studies, including our own pilot trials and the most recent report of the U.K. Medical Research Council (MRC) trial, have demonstrated that the major cause of failure after autologous transplant is related to relapse, in all cytogenetic risk groups.[15,16] Because relapses after autologous transplant tend to

occur within the first year, and IL-2-responsive lymphocytes have been detected in the circulation within 2 or 3 weeks after transplantation of autologous marrow or peripheral blood stem cells, IL-2 has been administered early after the patients have recovered from transplant-related toxicities at a time when the tumor burden is still minimal. Several phase I studies have identified the maximum tolerated dose of IL-2 that can be administered after autologous transplant and have documented that these doses have immunostimulomodulatory effects.[17,18] We therefore conducted this study to determine the feasibility of giving high-dose IL-2 after a radiation-based transplant regimen early after transplant and have observed tolerable toxicities and encouraging disease-free survival for such patients. Patients usually exhibit transient early lymphopenia followed by a rebound of lymphocytosis after stopping IL-2, something that was also observed in our own patient population. This rise reflects an increase in the number of cells expressing CD8-positive T cells and CD16-positive and CD56-positive activated natural killer cells with concomitant enhanced cytotoxicity for in vitro tumor targets.

In our previous studies with autologous transplant using marrow, the disease-free survival was 49% for those patients on the intent-to-treat analysis and 61% for those patients who actually underwent transplantation.[15] Disease-free survival in that study was not correlated with cytogenetic results of the leukemia at the time of diagnosis. Patients who required two courses of induction therapy had an inferior outcome to those patients with good risk cytogenetics. To try to improve the efficacy of the autologous transplant procedure, several modifications were made in the current protocol. The first was the addition of idarubicin to high-dose Ara-C consolidation. Most patients on the study reported here underwent consolidation with Ara-C and idarubicin in an attempt to provide a better in vivo purge before the collection of peripheral blood stem cells. Although some investigators hypothesized that peripheral blood stem cells have a higher relapse rate than marrow, this was not seen in our own study, and is consistent with observations from other studies.[19] One of the issues in this study was the feasibility of collecting adequate numbers of stem cells in patients who underwent consolidation of this intensity. Approximately 50% of the patients had also received induction chemotherapy with high-dose Ara-C. The fact that most patients could undergo collection of stem cells suggests that this is a feasible way of reducing the tumor burden in a patient who is undergoing stem cell collection in preparation for autologous transplant.

Once patients had completed cell collection, they were treated with an autologous stem cell transplant regimen using total body irradiation, VP-16, and cyclophosphamide, a program that we have used in our previous study that is tolerable for patients up to the age of the early 60s. As noted in the Results, the recovery rate after hematopoietic cell transplantation was relatively short, with good recovery of neutrophils and adequate recovery of platelets. The delay in platelet recovery (to >50,000) reflects the thrombocytopenic effect of IL-2 given

early after the transplant regimen. Nevertheless, no patient had graft failure as a consequence of this, and all have achieved hematopoietic recovery. Most patients undergoing autologous stem cell transplant in centers around the world received a regimen of busulfan and cyclophosphamide.[1] Whether there are benefits to a radiation-based regimen compared with one using only chemotherapy cannot be assessed from this study.

One of the major questions addressed in this pilot study was whether it would be possible to administer doses of IL-2 that have been reported to induce natural killer cell activity early after transplant at a time when the disease burden was at a minimum. The use of IL-2 is designed to treat not only the residual body burden of tumor not addressed by the preparative regimen but also the leukemia cells that may have been reinfused with the stem cell graft. This study shows that with proper use of supportive care, it is possible to administer IL-2 in these doses early after recovery from transplantation with minimal toxicity to the patient. As described above, no patient required admission to the ICU and none suffered respiratory failure as a consequence, despite fever and fluid accumulation, well-recognized side effects of IL-2.

This study was also designed to assess the incidence of relapse before autologous transplant, the number of patients unable to undergo adequate collection of peripheral blood stem cells, and toxicities during consolidation that precluded proceeding to autologous transplant. In this trial, 85% of patients who were entered on protocol were able to proceed to transplant, with the most common cause of failure being failure to collect adequate numbers of stem cells. Some studies suggest that combined cytokine therapy could facilitate stem cell collection.[20] Trials are now being initiated in the Southwest Oncology Group to determine whether the addition of thrombopoietin to G-CSF will increase the quality and rate of stem cell procurement after high dose Ara-C consolidation and thereby reduce the numbers of patients who are unable to proceed to autologous transplantation because of inadequate stem cell collections.

In summary, this pilot study indicates that it is feasible to use high-dose IL-2 following a radiation-based autologous transplant program and that a program of intensive consolidation, stem cell collection, transplant, and IL-2 may improve disease-free survival for patients with AML in first remission undergoing transplantation. Further studies with larger numbers of patients will help determine the efficacy of this transplant approach and the role of IL-2 in curing patients with AML.

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# CHAPTER 2
# ALL

# Bone Marrow Transplantation for Acute Lymphoblastic Leukemia (ALL) in First Complete Remission

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Long-term survival data using conventional chemotherapy or allogeneic bone marrow transplantation in ALL in first remission are virtually superimposable on the postremission data in acute myeloid leukemia (AML). Allogeneic transplantation in first remission ALL is clearly efficacious and should probably be routinely offered to younger adults in first remission, at least those with the poorer prognosis. Phase III studies are currently underway to evaluate the role of allogeneic transplantation for patients with standard-risk ALL. Autologous transplantation in first remission is also efficacious, and the results of clinical trials are awaited to determine whether it is superior to conventional consolidation-maintenance therapy. Even if the disease-free survival is not superior, there may be an inherent advantage in autologous stem cell transplantation if a single course of such therapy produces the same disease-free survival as protracted consolidation/maintenance therapy.

Data describing disease-free survival in AML are somewhere in the range between 40 and 65% in most large studies. Almost identical data have been described among the larger studies of ALL in first remission. Beyond first remission, the published allogeneic data in ALL are, once again, at least as good as the published data in AML. At the same time, the reported data for disease-free survival in ALL using chemotherapy is virtually superimposable on the published disease-free survival data in AML (Table 1). Nevertheless, the widespread introduction of allogeneic transplantation in first remission ALL followed that of AML by about a decade.

It is important to clearly define the patient group for whom such a transplant is indicated. In general, the published reports support the performance of allogeneic bone marrow transplantation for ALL in first remission among patients with identifiable high-risk features, for example, cytogenetic abnormalities such as the 9;22 or 4;11 translocations, very high white cell counts at presentation, and a long period until first complete remission is achieved.

The earliest and largest single-institution data of allogeneic bone marrow transplantation in first remission have come from the City of Hope Medical Center.[1] Among 53 high-risk patients, disease-free survival of 61% was reported.

**Table 1.** Comparison of disease-free survival in alloBMT and chemotherapy for AML and ALL

| | *CR1 (%)* | *CR2 (early first relapse) (%)* | *>CR2 (%)* |
|---|---|---|---|
| Allogeneic BMT | | | |
| AML | 40–65 | 15–40 | 5–20 |
| ALL | 40–65 | 25–40 | 10–20 |
| Chemotherapy | | | |
| AML | 20–40 | 0–5 | 0 |
| ALL | 20–40 | 0–5 | 0 |

Several studies have been reported on patients with ALL who are beyond first remission or in relapse.[2,3] Over the past decade, several studies have reported on allogeneic bone marrow transplantation (alloBMT) in first remission, with the cumulative data suggesting a disease-free survival of 40–65% and a relapse rate of 10–40%, mostly during the first 2 years.[1,4,5]

A retrospective study from the International Bone Marrow Transplant Registry (IBMTR) with selected published data of chemotherapy using matched patients showed no significant difference between allogeneic transplants for patients in first remission when compared with standard chemotherapy. Treatment-related mortality was significantly higher in patients undergoing allotransplant but was offset by a far greater relapse among those receiving chemotherapy.[6]

Prognostic factors that impact favorably on the outcome of allogeneic transplantation in ALL are first complete remission, low tumor burden, absence of resistant disease, and the presence of mild (grade 1–2) posttransplant graft-vs.-host disease.[7]

The majority of patients do not have a suitable HLA-compatible donor. While recent reports on the use of allogeneic matched unrelated donors show promise, such a procedure cannot be justified for ALL in first remission except for those with the presence of the Philadelphia chromosome. For all other patients who do not have a histocompatible sibling, the only transplant alternative to be considered is autologous stem cell transplantation.

Unlike AML, the standard postremission therapy for ALL includes protracted consolidation/maintenance chemotherapy for up to 2 years, and a single course of autologous stem cell transplantation early in the course of disease may significantly improve the quality of life of patients in first remission, even if the long-term outcome is not superior to standard therapy but is at least as good. Obviously, the ultimate aim would be to develop a stem cell regimen that would improve on standard therapy, but the consideration for stem cell transplant must take into account the difficult period that patients undergo during their protracted standard therapy with chemotherapy.

In reviewing the data, rather surprisingly, a considerable number of autologous transplants have been performed in ALL and there is no shortage of published

**Table 2.** Autologous BMT in ALL in first remission

| *Reference* | *No. patients* | *Median age (years)* | *Disease-free survival* |
|---|---|---|---|
| EBMT, 1995[10] | 834 | 30 (18–51) | 42 ± 4 (8 years) |
| Fière, 1993[8] | 63 | NS | 51 (5 years) |
| Blaise, 1990[12] | 22 | 31 (7–47) | 40 (3 years) |
| Kantarjian, 1990[13] | 26 | 30 | 60 (3 years) |
| Carey, 1991[14] | 15 | 30 (18–51) | 57 (3 years) |
| Powles, 1995[15] | 50 | 26 (15–58) | 53 (3 years) |
| Vey, 1994[16] | 34 | 29 (16–59) | 27 (8 years) |
| Attal, 1995[17] | 64 | NS | 29 (3 years) |

reports (Table 2). The problem is that so little of this is controlled, and there has only been one previous prospective study attempting to define the role of autologous bone marrow transplantation.[8] Data from the European Transplant Bone Marrow Group describe a disease-free survival of 42% among "standard risk" patients.[7,8] However, it must be remembered that these are retrospective data taken from a multitude of centers with patient selection making interpretation difficult.

The French study reported by Fière in 1993[8] suggested, in a prospective study, that the response to autologous bone marrow transplantation may be at least as good as conventional chemotherapy. It is reassuring that the recent update[11] of this study has not changed very much, with a median follow-up now of more than 8 years (Table 3). In this prospective study, allogeneic transplantation remains statistically superior among patients with "high-risk" ALL emphasizing, with long-term follow-up, the potential for cure in this group.

Nevertheless, the entire issue of autologous bone marrow transplantation remains uncertain, as is the precise role of allogeneic BMT in first remission for patients who are not at high risk. A major prospective study is currently underway with a trans-Atlantic effort between the Eastern Cooperative Oncology Group in the United States and the Medical Research Council in Britain (ECOG 2993 and

**Table 3.** Allogeneic and autologous BMT in first complete remission French Group on Therapy of Adults ALL—Fière et al.

| Post-CR (intent-to-treat) | Allogeneic BMT | Autologous BMT | Chemotherapy |
|---|---|---|---|
| *n* | 116 | 95 | 191 |
| DFS | 43% | 39% | 32% |
| JCO, 1993[8] | | | |
| DFS | 46% | 34% | 30% |
| ASCO, 1998[11] | | | |
| High risk | 44% | 16% | 11% |
| Standard risk | 49% | 49% | 39% |

UKALL XII). This study compares, prospectively, the role of allogeneic transplant for patients who have a matched histocompatible sibling and randomizing all other patients to receive either conventional chemotherapy or an autologous transplant. This study stratifies patients by risk groups but maintains study randomization for all such groups. The only exceptions are for patients with very high-risk factors such as the Philadelphia chromosome, detected either by standard cytogenetics or by the presence of the *bcr-abl* oncogene. A search for a matched-unrelated donor is recommended for these high-risk patients in preference to standard chemotherapy or autologous transplantation. In this prospective randomized study, all patients receive identical induction and intensification therapy. A direct prospective comparison is then made between allogeneic transplantation and autologous transplantation using identical preparative regimens and conventional consolidation/maintenance therapy. Importantly, all treatment strategies are decided prior to intensification therapy to allow for an intent-to-treat analysis and an avoidance of selection biases. The study has currently accrued close to 800 patients and should be completed within the next 2 years.

Such prospective collaborative studies are critical to the understanding of the best therapies for adult ALL in first remission and are likely to influence the treatment strategies over the next decade.

The issue of peripheral stem cell transplants is fairly novel in ALL but is rapidly gaining widespread experience if not unanimous acceptance. Preliminary data that are available suggest that hematopoietic recovery is significantly improved with this modality without evidence of increasing the risk of relapse. Assuming this can be confirmed in subsequent data, the relatively low morbidity from such transplants will make this procedure even more attractive as an option to significantly shorten the traditionally long period of therapy for adult ALL.

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# Adult ALL: BMT in First Remission for Selected Patients

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## INTRODUCTION

One of the major areas of discussion in the treatment of adult acute lymphoblastic leukemia (ALL) is which patient should be treated with chemotherapy only and which should receive stem cell transplantation in addition. In relapsed and refractory adult ALL patients, bone marrow transplantation (BMT) in second or subsequent clinical remission (CR) is undoubtedly the treatment of first choice. There is, however, an ongoing debate whether BMT should be offered to all adult ALL patients in first remission (CR1) or whether it should be restricted to selected subgroups. In adult ALL, survival after chemotherapy in first remission is approximately 35%, with a wide range of 10 to >50% for specific subgroups. For subgroups such as T-ALL and B-ALL (T and B cell ALL), considerable progress has been achieved, with survival rates of approximately >50% with optimization of chemotherapy. Whereas in other subgroups such as high-risk B-lineage ALL, and particularly in Ph/*bcr-abl*-positive ($Ph^+$) ALL, no improvement has occurred and survival is still <30% and <10%, respectively.

On the other hand, the leukemia-free survival (LFS) for allogeneic BMT (alloBMT) in CR1 according to the literature is about 51%, for autologous BMT (autoBMT) 42%, and for matched unrelated donor (MUD) BMT 34%. In prospective trials comparing alloBMT in CR1 and chemotherapy there was no unequivocal advantage for BMT. For high-risk patients, however, alloBMT was mostly superior to chemotherapy and autoBMT. For favorable subgroups such as T-ALL and mature B-ALL the results of chemotherapy are at present superior to those of autoBMT or MUD BMT.

The proposed approach is therefore to select subgroups of adult ALL in which a benefit from BMT in CR1 can be expected. All high-risk ALL patients with a sibling donor should receive immediate BMT in CR1, whereas those without a donor should be treated with either autoBMT or MUD BMT. From a practical point of view, it seems advisable to collect peripheral blood stem cells (PBSC) from all patients, to search for an unrelated donor in parallel and to make a choice depending mainly on the time needed to find a donor and on the patient's age.

A change in BMT strategy in adult ALL may go in several directions. There is a general trend to the use of PBSC from selected related and unrelated donors and particularly in autologous stem cell transplantation (autoSCT). The use of

minitransplants with lower toxicity may offer a treatment opportunity also for low-risk and elderly patients. Finally the detection of minimal residual disease (MRD) may provide an indication for BMT in patients with an MRD status that is associated with high relapse probability, e.g., after 6–12 months of chemotherapy.

## RESULTS OF AlloBMT IN CR1

In a total of 1496 adult ALL patients with alloBMT in CR1 collected from 14 trials in the literature, the weighted mean for LFS was 51% with a wide range, from 21 to 80%.[1] The relapse incidence (RI) was 26% (9–50%) and the treatment-related mortality (TRM) 29% (12–42%) (Table 1). In Ph$^+$ ALL, the overall LFS after alloBMT in CR1 was about 40% because of a somewhat higher RI (34%). In more recent studies, survival after alloBMT is increasing, most probably because of improvement of supportive care and prophylaxis for GVHD (graft-vs.-host disease) leading to a reduction of TRM. Thus, the European Group for Blood and Marrow Transplantation (EBMT) reported a reduction of TRM from 39% in patients transplanted before 1986 to 25% for those transplanted between 1987 and 1991,[2] and similar results were reported by the International Bone Marrow Transplantation Registry (IBMTR).

GVHD, however, also contributes to a reduction of relapse rate. RI is lower in patients with limited GVHD compared with those without or with extensive GVHD, resulting in a better LFS for the former.[3] In an analysis by the IBMTR of 349 ALL patients transplanted in CR1, the relapse rate was highest in patients without GVHD after allogeneic BMT without T cell depletion (44%), after syngeneic BMT (41%), or after allogeneic BMT with T cell depletion (34%). In patients with acute GVHD (17%), chronic GVHD (20%), or both types of GVHD (15%), the relapse rates were significantly lower.[4] Thus, a GVL effect (graft-vs.-

**Table 1.** Results of bone marrow transplantation in adult ALL in CR1

| *BMT* | *Patients (n)* | *TRM* | *Relapse probability* | *LFS* |
|---|---|---|---|---|
| Allogeneic | | | | |
| Overall | 1496 | 29% | 26% | 51% |
| Ph/*bcr-abl*$^+$ | 75 | 32% | 34% | 40% |
| Autologous | | | | |
| Overall | 1336 | 2–8% | 51% | 42% |
| Ph/*bcr-abl*$^+$ | 37 | 17% | 44% | 37% |
| MUD | | | | |
| Overall | 180 | 59%* | 22% | 34% |

*Results are pooled data from literature; data for Ph/bcr-abl$^+$ ALL are from Hoelzer.[1] *One trial ≥CR2, one trial high risk.*

leukemia) appears to be present in ALL, although it is less pronounced than in CML or AML.

Age is another significant prognostic factor for survival after alloBMT. In patients >40 years old, TRM (37 vs. 28%) as well as relapse incidence (38 vs. 29%) are higher compared with younger patients,[5] leading to a less favorable LFS.

## RESULTS OF AUTOLOGOUS BMT IN CR1

The LFS after autoBMT in CR1 of approximately 42% (15–75%) is somewhat inferior compared to alloBMT (Table 1). Several trials comparing allo- and autoBMT demonstrated an advantage of alloBMT in terms of LFS and RI, whereas TRM after autoBMT was clearly lower.[6,7]

AutoBMT can be employed in elderly patients up to approximately 65 years of age due to its low TRM (2–8%). The major disadvantage of autoBMT is the high RI (51%) probably caused to a lesser extent by the reinfusion of leukemic blasts but more by the lack of GVL effects. Purging of the marrow graft with monoclonal antibodies, chemotherapeutic drugs or immunomagnetic beads is of interest since it may reduce the leukemia cell burden. Thus, purging with immunomagnetic beads can reduce tumor load by 2 log either for BM or for PB grafts in ALL.[8] However, no comparative studies with purged and unpurged autoBMT have been reported to date.

In addition to purging, the administration of maintenance treatment after autoBMT may contribute to a reduction of RI. A favorable LFS of 53% has been achieved in a single-center trial with autoBMT or PBSCT in 50 adult ALL patients in first CR followed by a 2-year maintenance treatment with 6-mercaptopurine and methotrexate.[9] Other options for maintenance therapy after autoBMT are biological response modifiers such as interferon-α or interleukin-2. The reported results, however, are not conclusive.

AutoBMT is a reasonable treatment option for a substantial number of $Ph^+$ patients >50 years old without a sibling donor, for whom a MUD transplant is not considered. Thus, a large proportion of $Ph^+$ ALL patients remain candidates for autologous transplants. The LFS in 37 patients after autoBMT from several small series was 37% (Table 1).

## RESULTS OF PERIPHERAL BLOOD STEM CELL TRANSPLANTATION (PBSCT) IN CR1

Transplantation of PBSC instead of bone marrow is increasingly employed in adult ALL since TRM may be reduced due to faster bone marrow recovery. Even more importantly, experience in $Ph^+$ ALL shows that the tumor load is lower in PBSC grafts compared to bone marrow. Since in heavily pretreated ALL patients, it may be difficult to collect a sufficient number of stem cells from the peripheral

blood, early scheduling of stem cell apheresis after induction/consolidation treatment is recommended. In a preliminary report from the EBMT, in ALL patients in first remission, LFS was 41% after autoPBSCT compared with 35% after autoBMT with a similar RI of 60%. [10]

## RESULTS OF MISMATCHED AND MATCHED UNRELATED BMT

Mismatched (MM) BMT from related donors or MUD BMT has been increasingly employed to extend the possibilities of alloBMT by enlarging the number of bone marrow donors available. With BMT from partially MM family donors, two groups have reported quite favorable results (LFS 38–53%) in pediatric and adult patients with relapsed ALL. Comparing results of fully matched and partially MM alloBMT in ALL >CR1 (including children), the LFS was similar (38 vs. 38%), whereas TRM was higher (38 vs. 31%) and RI lower (31 vs. 25%) after MM BMT. It remains an open question as to whether MM BMT may be a treatment option in adult patients without a matched sibling or unrelated donor.[11]

In retrospective analyses of MUD transplants in adult ALL, the weighted means for LFS, TRM, and RI were 34, 59, and 22%, respectively (Table 1). However, adult ALL patients with all stages of disease were included, and the median age was low. In one retrospective analysis with a higher median age of 35 years (18–51) in patients with high-risk ALL, the overall LFS was 20% with a TRM of 65%. Outcome was clearly better in patients transplanted in CR1, with an LFS of 42% compared with 7% in those transplanted in subsequent disease stages.[12] Results of MUD BMT in $Ph^+$ ALL are only available from small patient cohorts. In the largest report on 18 patients (age 1.5–51 years) in CR1, the LFS was 48%, which is quite promising.[13]

## RESULTS OF TRIALS COMPARING BMT AND CHEMOTHERAPY

The overall LFS after alloBMT is superior to that obtained with chemotherapy alone. However, when these results were adjusted for age, risk factors, and time to BMT (thereby excluding early relapses), the differences between BMT and chemotherapy were no longer statistically significant, although there was a trend toward better results for alloBMT. In a comparative analysis of BMT patients from the IBMTR and chemotherapy patients treated according to the GMALL (German Multicenter Adult ALL Studies) protocol, results for alloBMT and chemotherapy were comparable (34 vs. 32%). This was predominantly due to a higher TRM in the transplant group (53 vs. 5%), whereas RI was higher in the chemotherapy group (30 vs. 66%).[14] Similar results were reported for chemotherapy results of the JALSG chemotherapy protocol compared with BMT results of the IBMTR.[15] A

recent update, however, demonstrated an advantage in terms of LFS for alloBMT compared with chemotherapy in patients <30 years old (53 vs. 30%), whereas in patients >30 years old, results of chemotherapy and BMT were similar (30 vs. 26%).[16] It seems that with better management of BMT complications and a reduction in TRM, the BMT indications in adult ALL in CR1 may be extended.

Several groups also failed to demonstrate a statistically significant advantage for allo- or autoBMT in first CR compared with chemotherapy in prospective trials.[6,15,17–19] The French study group conducted a randomized trial with autoBMT vs. chemotherapy in patients without a sibling donor and alloBMT for all patients with sibling donors. LFS in patients treated with alloBMT (44%) was not significantly superior to those treated with chemotherapy (32%). In high-risk patients, however, there was a significantly better LFS for patients treated with alloBMT (39%) compared with those in the control group (14%) treated with either chemotherapy or autoBMT (Table 2).[20]

It remains an open question whether autoBMT or chemotherapy is superior in adult ALL patients in CR1. Generally there was a trend in favor of autoBMT compared with chemotherapy which did not reach statistical significance. Thus, two groups reported a higher LFS after autologous BMT compared with chemotherapy, 54 vs. 35%[21] and 48 vs. 20–30% (depending on chemotherapy protocol), respectively.[19] Both trials, however, included small patient numbers of 26 vs. 19 patients[21] and 19 vs. 72 patients.[19] In the large prospective, randomized French trial, autoBMT was not superior to chemotherapy with similar TRM (4 vs. 4%), RI (57 vs. 61 %), and LFS (39 vs. 32%).[6]

## IDENTIFICATION OF HIGH-RISK PATIENTS

ALL is not a uniform disease but can be separated into subgroups with considerably different LFS ranging between <10% and >50%. In addition to the "standard" clinical risk factors (white blood cell [WBC] count, age, time to CR)[22]

**Table 2.** Comparison of alloBMT and chemotherapy as postremission therapy in adult ALL

| | *DSF* | | *TRM* | | *RI* | |
|---|---|---|---|---|---|---|
| *Group* | *AlloBMT* | *Chemo* | *AlloBMT* | *Chemo* | *AlloBMT* | *Chemo* |
| Fiere et al. 1993[6] | 44% (116) | 32% (96) | 16%+ | 4%+ | 41% | 61% |
| Zhang et al. 1995[4,14] | 34% (234) | 32% (484) | 53% | 5% | 30% | 66% |
| Mrsic et al. 1993[19] | 52% (22) | 20–30% (43) | NR | NR | 20% | 70–80% |
| Oh et al. 1995[16] | 33% (250) | 27% (80) | 54% | 12% | 28% | 69% |
| Forman et al. 1995[18] | 66% (37) | 55% (66) | NR | NR | 9% | 38% |

*NR, not reported; RI, relapse incidence.*

**Table 3.** Risk factors for the definition of high-risk ALL

| |
|---|
| Conventional |
| Age >35 years, >50 years |
| WBC |
| B lineage >30,000/μL |
| T lineage >100,000/μL |
| Treatment response |
| Complete remission >2 weeks, >4 weeks |
| Immunophenotype |
| Immature: pro B-ALL, pro T-ALL |
| subtypes |
| Cytogenetic/molecular markers |
| t(9;22)/*bcr-abl* |
| t(4;11)/MLL-AF-4 |
| New markers? |

biological risk factors such as immunophenotype, cytogenetics, molecular genetics, and finally minimal residual disease have been more recently included in risk models for adult ALL (Table 3). In mature B-ALL, the outcome after chemotherapy alone is quite favorable, with an LFS >50%. In T-ALL and B precursor ALL, risk factors can be used to identify standard- and high-risk patients. In standard-risk patients, the LFS with chemotherapy is ~50% or more. In high-risk patients, the LFS with chemotherapy is generally <30% and thus clearly inferior to BMT results. Very high-risk patients ($Ph^+$ ALL) achieve no long-term survival with chemotherapy alone.

## RISK-ADAPTED BMT INDICATIONS

The proposed approach is therefore to select subgroups of adult ALL that clearly profit from BMT in CR1. The decision depends on the patient's age on one hand and specific prognostic factors on the other.

Thus, high-risk ALL patients with a sibling donor should receive immediate BMT in CR1. AutoBMT may be a reasonable treatment option for adult high-risk ALL patients without a compatible sibling donor or in patients where MUD BMT is still not indicated. Ongoing trials compare MUD BMT or autoBMT in CR1 with intensified chemotherapy, and the results are still open.

For very-high-risk patients ($Ph^+$ ALL), any kind of BMT is superior to chemotherapy. Depending on age, availability of a donor, and general status of the patient, preferably MUD BMT or autoPBSCT should be attempted in all patients without a sibling donor. From a practical point of view, it seems advisable to collect peripheral blood stem cells (PBSC) from all patients, to search for a donor

in parallel, and to make a choice depending on the results and the time delay of donor search. PBSCT could be considered in patients for whom a matched unrelated donor is not found within 3 months from diagnosis.

For favorable subgroups, such as mature B-ALL as well as T-ALL and B precursor ALL without risk factors, the results of chemotherapy are at present superior to overall results of auto- or MUD BMT and most probably also to alloBMT, although BMT results for ALL subgroups have so far not been reported. BMT should, therefore be employed in first relapse or second or subsequent CR (Table 4).

The indications for BMT in CR1 in adult ALL need to be continuously redefined depending on BMT results, chemotherapy results, and new prognostic factors.

## FUTURE INDIVIDUALIZED BMT INDICATIONS BASED ON MINIMAL RESIDUAL DISEASE

In the future, additional BMT indications will most probably arise from prospective evaluation of minimal residual disease (MRD).[23] Still, 40–50% of the patients with standard-risk ALL eventually relapse. These patients at present cannot be identified with known prognostic factors. The evaluation of MRD may provide new, individual prognostic parameters. The majority of reports demonstrate a strong correlation between relapse risk and MRD status in ALL.

Thus, patients with a high MRD level ($>10^3$) after induction therapy, independent of other risk factors, have a significantly higher relapse risk compared with patients with lower or negative MRD status.[24,25] According to cumulative data from six trials after induction treatment, 10% of MRD-negative, 18% of "low MRD," and 79% of "high MRD" patients eventually relapse. If these results are confirmed in prospective trials in adult ALL, early BMT might be a reasonable

**Table 4.** Risk-adapted BMT indications in ALL

| | |
|---|---|
| CR1 | |
| BMT | |
| High risk B-lineage ALL (WBC >30,000/μL, CR >4 weeks, proB, $Ph^+$) | |
| High risk T-lineage ALL (WBC >100,000/μL, CR >4 weeks, proT) | |
| No BMT | |
| Standard risk B- or T-lineage ALL (LFS 40–50%) | |
| Mature B-ALL (LFS >50%) | |
| CR2<br>Relapsed<br>Refractory | BMT for all patients |

treatment option for those with high MRD levels. The course of MRD during the first year of treatment is another independent risk factor. A review of 14 studies from the literature showed that 48% of the patients with MRD after induction therapy relapse compared with 12% of MRD-negative patients. At the end of therapy, 61% of MRD-positive patients relapsed compared with 15% of MRD-negative patients. Thus, patients with reportedly positive or increasing MRD levels carry a high risk of relapse, whereas patients with decreasing and finally low levels have a more favorable outcome. Two recently published trials in pediatric ALL patients demonstrated that MRD detection at any time point of therapy is significantly correlated with relapse risk.[26,27]

An individualized treatment strategy could be based on sequential MRD evaluations during the first year of treatment, and BMT could be scheduled for those who are still MRD-positive after 1 year. For autoBMT, MRD evaluation offers the possibility to evaluate the leukemic blast proportion in the graft and assess remission control after transplantation. This approach may provide reasonable indications for the intensity and duration of post-BMT maintenance treatment.

Thus, two major alternatives for MRD-adapted BMT indications are available:

- the indication for early BMT according to the quantitative MRD status after induction treatment; and
- the indication for delayed BMT according to the course of MRD during the first year of treatment.

Both approaches may lead to an individualized treatment strategy, with treatment intensity adapted to the relapse risk of the individual. Additional candidates for BMT in CR1 will be identified and, most importantly, in patients with repeatedly negative MRD status, treatment could be stopped after 1 year.

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# Autologous Hematopoietic Stem Cell Transplantation as Treatment for Adult Acute Lymphoblastic Leukemia

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## ABSTRACT

Between October 1986 and November 1997, 79 adult acute lymphoblastic leukemia (ALL) patients received an autologous stem cell transplant (autoSCT) at two institutions in Barcelona. In 48 cases, bone marrow (BM) was purged with monoclonal antibodies and complement or immunomagnetic beads (P-autoBMT group). Nineteen patients received an unpurged BMT (Unp-autoBMT) due to unavailability of the leukemic immunophenotype ($n$=2) or harvesting of an insufficient number of BM cells ($n$=17). Autologous peripheral blood stem cell transplant (autoPBSCT) was performed in the remaining 12 cases. Forty-eight patients were autografted in first complete remission (CR1), 19 in CR2, and 12 in other disease stages. The intensive therapy regimen was cyclophosphamide and total-body irradiation in all but one patient. Graft failure was observed in two patients. Hematologic recovery was significantly faster in the autoPBSCT group. Six patients died in the first 4 weeks after transplantation due to causes other than leukemia (NLD), three between 4 weeks and 6 months, and one thereafter. At a median follow-up of 62 months, 28 patients remain alive and disease-free. Leukemia-free survival (LFS) in patients autografted in CR1 was best in the autoPBSCT group, intermediate in the P-autoBMT recipients, and worst after Unp-autoBMT: 75 ± 21, 40 ± 8 and 12 ± 11%, respectively. However, this last group could be unfavorably selected. Relapse was the main cause of treatment failure. When analyzing P-autoBMT in CR1, factors associated with outcome in patients treated with chemotherapy also affected transplant results. Adverse features were high leukocyte counts, delayed CR achievement, and poor-prognosis cytogenetic abnormalities. Our results confirm that long-term LFS may be achieved after autoSCT in a substantial proportion of adults with ALL. The role of this treatment vs. chemotherapy and the clinical efficacy of purging have to be explored in large prospective studies.

## INTRODUCTION

Two-thirds of ALLs develop in children, a patient population where a substantial improvement in prognosis has been achieved.[1] ALL represents 20% of all adult leukemias, and the outcome in this setting is less encouraging.[2,3] Complete remission (CR) rates range between 74 and 95% in a review including data from 10 studies.[4] Significant therapeutic advances have been made in specific immune subtypes such as T-ALL and B-mature ALL and are the consequence of more tailored intensive postremission chemotherapy.[4,5] Despite this, adult ALL is curable in only 20–35% of all patients.[4] Since leukemia recurrence is the main cause of treatment failure, high-dose therapy with hematopoietic rescue has been performed in relatively young patients with the intention to eradicate the disease. Allogeneic bone marrow transplantation probably improves the outcome of adult ALL patients in first complete remission (CR1) with very high risk features at diagnosis.[6,7] After relapse, adult ALL is unlikely to be cured with chemotherapy, and SCT is usually indicated. Only 30% of patients who are candidates for high-dose therapy and SCT have an HLA-identical sibling. AutoSCT is the most frequent option for the remaining 70% of cases. The role of this therapy in adult ALL and the optimal conditions for the procedure (preparative regimen, source of stem cells, need of purging to remove malignant cells from the graft) are not well established.

We herein describe the 11 years' experience on 79 consecutive autoSCT for adult ALL using a common protocol at two institutions in Barcelona. Special emphasis is placed on the series of patients who received purged BM and the factors that influenced the outcome of the procedure. A subset of the patients from this study has been included in a previous report.[8]

## PATIENTS AND METHODS

Between October 1986 and November 1997, adult ALL patients in remission or in early relapse, diagnosed at or referred to the Hospital de la Santa Creu I Sant Pau or the Hospital Clinic from Barcelona, were considered for SCT. Allogeneic SCT (alloSCT) was the first option if an HLA-identical sibling was available. The upper age limit for this procedure increased over time from 45 to 60 years. Unrelated SCT was performed only in very poor prognosis patients lacking a suitable relative if a compatible nonfamily volunteer was identified on a short-term basis. Patients without a donor for alloSCT and aged ≤60 years were eligible for autoSCT using purged BMT (P-autoBMT) or unpurged PBSC (PBSCT). The latter transplantation modality was the therapy for 12 recent patients. During the interim, 48 P-autoBMT were performed and 19 additional patients received an unpurged BM (Unp-autoBMT) due to unavailability of the leukemic immunophenotype ($n$=2) or

**Table 1.** Patient characteristics

| *Data* | *Unpurged autoBMT* | *Purged autoBMT* | *PBSCT* |
|---|---|---|---|
| Number | 19 | 48 | 12 |
| Age (years): median (range) | 24 (18–49) | 28 (18–52) | 28 (14–49) |
| Sex: male/female | 13/6 | 30/18 | 12/- |
| Immunophenotype | | | |
| B-lineage | 13 | 33 | 6 |
| T-lineage | 4 | 15 | 6 |
| Not available | 2 | — | — |
| Initial WBC x$10^9$/L: median (range) | 6 (0.7–295) | 14 (0.8–650) | 17 (5–70) |
| Days to CR1: median (range) | 32 (20–150) | 30 (15–150) | 45 (26–116) |
| Months CR–SCT: median (range) | 4 (0.5–15) | 5.8 (0.2–18) | 4.2 (0.5–8) |

harvesting of an insufficient number of BM cells ($n$=17). The main characteristics of the series appear in Tables 1 and 2.

As shown in Table 2, most patients receiving an autoSCT in CR1 had one or more poor prognosis features. These adverse characteristics were age >35 years ($n$=10), white blood cell counts (WBC) >30×$10^9$/L ($n$=12), null or B (surface Ig+) immunophenotype ($n$=9), complete remission obtained in more than 5 weeks ($n$=22), Philadelphia chromosome ($Ph^+$) ($n$=5), or t(4;11) ALL ($n$=1). Table 2 also reflects the number of patients autografted in first remission with none, one, and two or more poor prognosis features.

All patients in second or subsequent remission and those in relapse were considered to have poor-risk ALL, since adults in these stages are unlikely to be cured with chemotherapy. In these categories, an established prognostic factor is the duration of first remission. The number of patients autografted in CR2 with first remission ≤18 months is specified in Table 2.

Marrow cells were obtained from iliac crests by multiple needle aspirations under general anesthetic. Purging methodology using monoclonal antibodies (mAbs) and complement ($n$=37) or immunomagnetic beads ($n$=11) has been described in detail elsewhere.[9,10] Marrow was not purged if the number of nucleated cells after harvesting was <2.5×$10^8$/kg of patient body weight or the number of mononuclear cells after Ficoll separation was <3×$10^7$/kg. PBSC ($n$=12) were collected after mobilization with granulocyte colony-stimulating factor (G-CSF) in dose of 10 μg/kg subcutaneously. BM or PBSC was cryopreserved, thawed on day 0, and infused intravenously through a right atrial catheter. The intervals between achieving CR and transplant appear in Table 2. All but one patient were treated with cyclophosphamide, 60 mg/kg/day intravenously on two consecutive days, and TBI in a single dose or four to six fractions up to a total dose of 10–13.5

**Table 2.** Adverse characteristics of the patients

| *Data* | *Unpurged autoBMT* | *Purged autoBMT* | *PBSCT* |
|---|---|---|---|
| Patients in CR1 | 8 | 33 | 7 |
| Age >35 years | 2 | 6 | 2 |
| WBC $>30\times10^9$/L | 0 | 11 | 1 |
| Null or sIg $B^+$ ALL | 0 | 7 | 2 |
| Ph+ or t(4;11) | 1 | 5 | 0 |
| >5 weeks to CR | 5 | 12 | 5 |
| Adverse factors | | | |
| None | 1 (12%) | 8 (24%) | 0 |
| One | 6 | 15 | 5 |
| Two or more | 1 | 10 | 2 |
| Patients in CR2 | 6 | 11 | 2 |
| CR1 ≤18 months | 2 | 6 | 2 |

Gy. In the remaining case, the intensive therapy regimen consisted of BCNU, M-AMSA, VP-16, and cytosine arabinoside (BAVC).[11] All patients were nursed in individual rooms with protective environment including laminar air flow or positive pressure systems. Antibacterial and antifungal prophylaxis were administered, and intravenous treatment was started when clinically indicated.

Actuarial probabilities were calculated by the Kaplan Meier method, and comparisons between curves were made using the log-rank test.[7] Follow-up was updated in April 1998.

## RESULTS

### Hematologic recovery

Two of the 73 patients surviving at least 4 weeks after transplantation did not recover an absolute neutrophil count (ANC) of $0.5\times10^9$/L. The actuarial probabilities of ANC and platelet recovery at 100 days are shown in Table 3. Median times and probabilities of hematologic recovery were faster in the PBSCT group and slower in the P-autoBMT patients.

### Nonleukemic death

Six patients died in the first 4 weeks after transplantation due to causes other than leukemia, three between 4 weeks and 6 months, and one thereafter. The most common cause of NLD was pneumonia occurring in five patients. The proportion

**Table 3.** Hematologic recovery

| *Data* | *Unpurged autoBMT* | *Purged autoBMT* | *PBSCT* |
|---|---|---|---|
| ANC $>0.5\times10^9$/L | | | |
| Median (range) in days | 20 (11–31) | 21 (13–63) | 11 (10–23) |
| KM probability at 100 days (%) | 86 ( 9 | 100 | 100 |
| PLT $>20\times10^9$/L | | | |
| Median (range) in days | 32 (28–80) | 38 (20–142) | 12 (7–77) |
| KM probability at 100 days (%) | 100 | 80 ± 9 | 100 |

*The difference between BMT and PBSCT was significant at P <0.001.*

and actuarial probability of NLD according to the type of transplant are shown in Table 4. In the P-autoBMT, the number of patients with NLD in CR1 was three of 33, two of 11 in CR2, and two of four in other stages. In the PBSCT series, the values were one of seven in CR1, zero of two in CR2, and zero of three in the remaining phases. NLD was observed in two of 19 patients autografted with unpurged BM, zero of eight in CR1, one of six in CR2, and one of five in other phases.

## Relapse

Overall, 41 patients relapsed after the autograft, at a median of 6.3 months after the autograft. Twenty-one patients relapsed in the first 6 months, 11 between 6 and 12 months, and nine >12 months after SCT. Proportion and actuarial probability of relapse at 4 years according to the type of transplant are shown in Table 4. In the

**Table 4.** Transplantation outcome

| *Data* | *Unpurged autoBMT* | *Purged autoBMT* | *PBSCT* |
|---|---|---|---|
| TRM | | | |
| Death/total (%) | 2/19 (10) | 7/48 (15) | 1/12 (8) |
| KM probability (%) | 11 ± 7 | 14 ± 5 | 20 ± 17 |
| Relapse | | | |
| Relapsed/total (%) | 14/19 (74) | 24/48 (50) | 3/12 (25) |
| KM probability (%) | 84 ± 9 | 60 ± 8 | 27 ± 13 |
| LFS | | | |
| Alive disease-free/total (%) | 3/19 (16) | 17/48 (35) | 8/12 (67) |
| KM probability (%) | 14 ± 8 | 34 ± 7 | 59 ± 17 |
| CR1 | 12 ± 11 | 40 ± 8 | 75 ± 21 |

*Kaplan Meier (KM) probabilities are % at 5 years for autoBMT and at 2 years for PBSCT.*

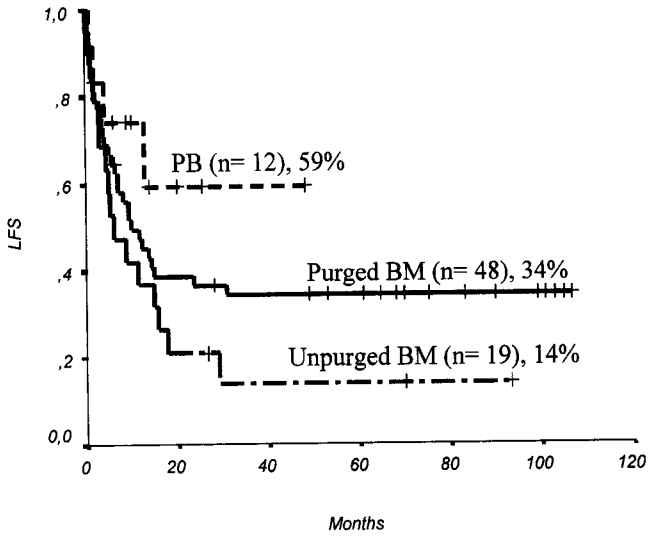


**Figure 1**

P-autoBMT, the number of patients who relapsed in CR1 was 17 of 33 and six of 11 in CR2. In the PBSCT series, the values were zero of seven in CR1 and two of three stages other than CR1 or CR2. Relapse was observed in 14 of 19 patients autografted with unpurged BM, seven of eight in CR1, three of six in CR2, and four of five in other phases.

### Leukemia-free survival

After a median follow-up of 62 months, 28 patients remain alive and disease-free and 51 have relapsed or died. LFS depending on the type of SCT is shown in Table 4 and Fig. 1. LFS in the P-autoBMT according to disease stage at transplantation and other potential prognostic factors is shown in Figs. 2–8. As may be seen, several factors previously described as predictive for outcome after chemotherapy were also relevant for prognosis after P-autoBMT in first remission. By contrast, age, immunophenotype, type of purging, and CR1 duration in patients transplanted for >CR2 ALL had no impact on the outcome. Regarding PBSCT, it is of note that only one of seven patients in CR1 failed after transplant.

## DISCUSSION

Our results confirm that long-term LFS may be achieved after autoSCT in a substantial proportion of adults with ALL. The outcome was significantly influenced by disease stage at transplantation. The results obtained in patients

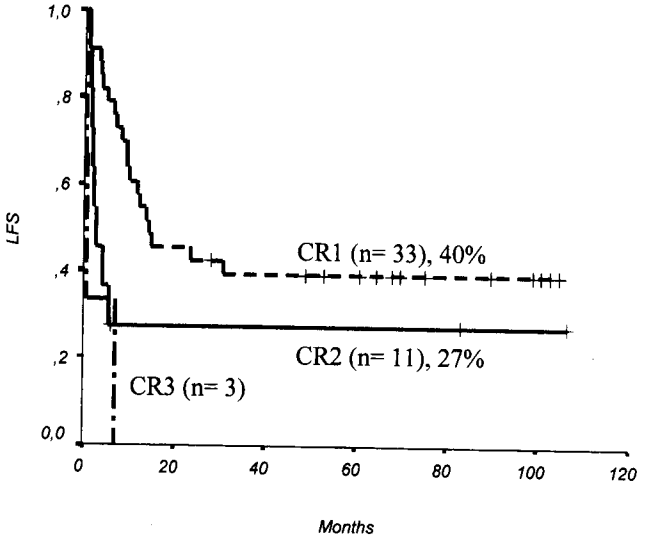


**Figure 2**

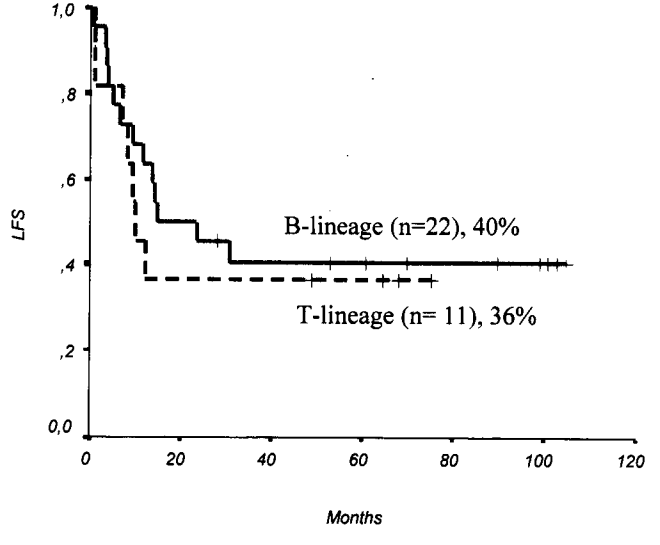


**Figure 3**

receiving purged BM or PBSC were remarkable, taking into account that most of our CR1 patients had one or more poor-prognosis characteristics. However, it is well known that SCT recipients are in part favorably selected since they must remain in remission until the procedure is performed.

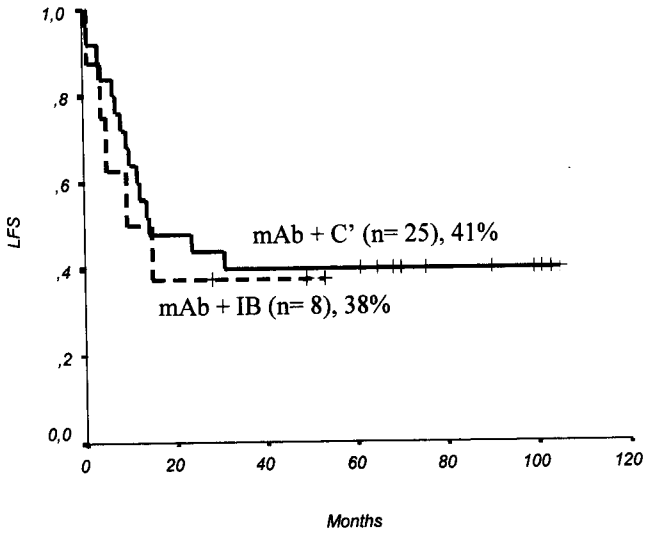


**Figure 4**

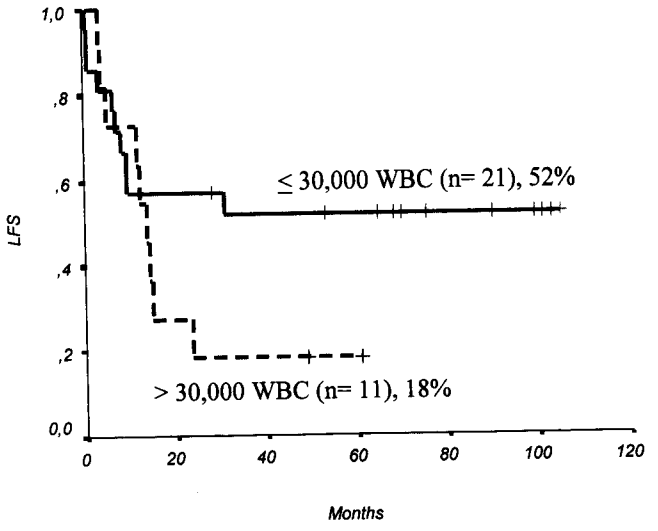


**Figure 5**

Several authors have emphasized that adult ALL with adverse prognostic factors in CR1 may be cured in only 20–28% of cases.[4] AutoSCT could be an option in these cases. However, in our experience most of the factors affecting the outcome with chemotherapy also influenced the results of P-autoBMT. Consequently, the question of whether autoSCT may benefit a subset of high-

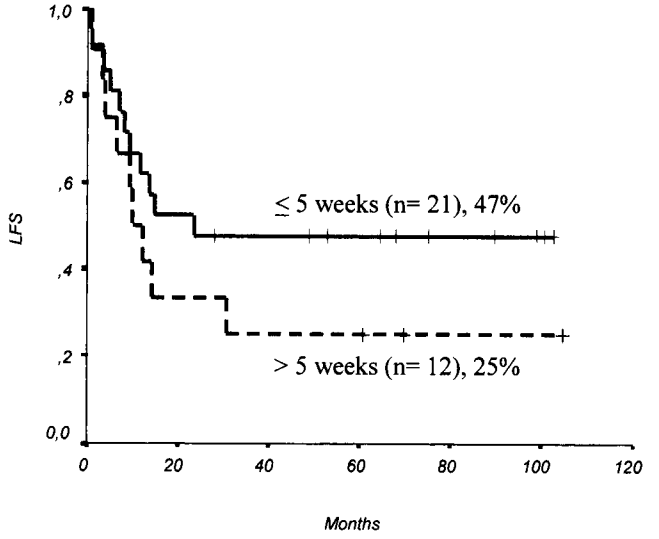


**Figure 6**

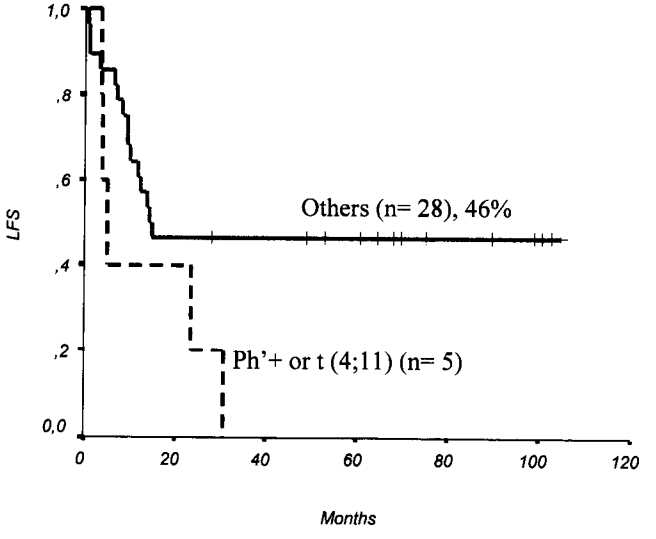


**Figure 7**

risk ALL patients in CR1 remains open. Prospective randomized studies have to date been unable to demonstrate an advantage for this procedure.[6,7] Modifications of the current technology, including double autografts and posttransplant immunotherapy, are currently being explored. Another approach in patients without an HLA-compatible relative is to expand the use of unrelated donors.

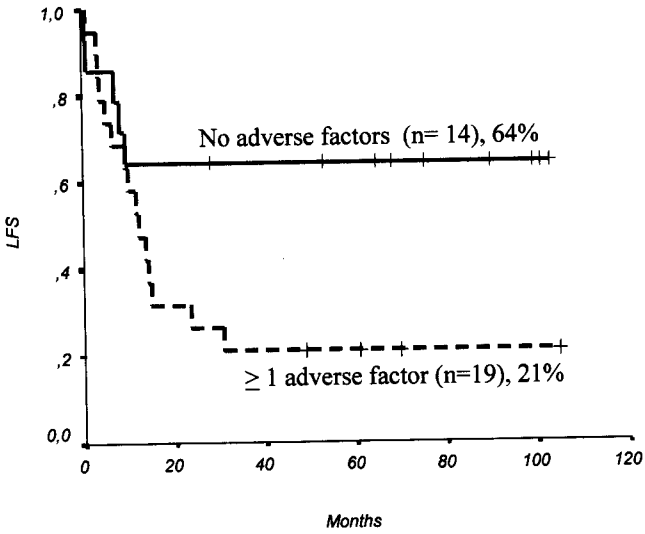


**Figure 8**

Preliminary results in very high-risk leukemia, such as $Ph^+$ ALL in CR1, appear encouraging.[12]

TRM occurred in approximately 10% of our patients. BM purging did not affect hematologic recovery or increased mortality. Although no overall improvement was noted with the use of PBSC, early TRM was not observed in this group. The only fatal episode after PBSCT was sepsis >1 year after transplant, a very uncommon event after autografts. It seems evident that to optimize autoSCT, for patients with acute leukemia TRM should approach zero.

Relapse is the main cause for failure after autoSCT. The lower relapse rate observed after P-autoBMT when compared with Unp-autoBMT could in part be due to a beneficial effect of purging. Another possibility is that the Unp-autoBMT group were selected for unfavorable prognostic patients. A minimum number of cells was required for ex vivo treatment. The worst CR marrow may be the least cellular and therefore not purged. In addition, the interval between CR and SCT, an established prognostic factor, was shorter in the Unp-autoBMT than in the P-autoBMT group. It is noteworthy that none of the seven recipients of autoPBSCT in CR1 relapsed after the procedure, and there are no data supporting that they were good-risk patients. In fact, all had at least one adverse prognostic feature. It is obvious that these results need confirmation in large series with prolonged follow-up. The possibility that PBSC products are in some instances less contaminated by tumor cells than BM has been suggested by some authors and needs further investigation.[13]

Other aspects regarding autoSCT in ALL also need clarification and include the following: *1*) Is this procedure better than chemotherapy for any subgroup of patients? *2*) Can current autoSCT technology be improved? *3*) Does purging the autograft have any clinical relevance? *4*) Are double autotransplants better than single transplants? *5*) What is the role of autoSCT vs. unrelated donor hematopoietic transplantation (BM, PBSC, or cord blood)? All these questions need prospective studies and scientific collaboration to be adequately addressed.

## ACKNOWLEDGMENTS

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# Autologous BMT/PBSCT in BCR-ABL-positive ALL: Rationale for Purging

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## ABSTRACT

The BCR-ABL rearrangement is a useful disease-specific clonal marker to detect leukemic cells at a sensitivity of about 1 malignant cell in $10^6$ cells. To quantify the burden of residual leukemia in autologous grafts, we prospectively analyzed 20 bone marrow and 30 peripheral blood autografts using a semiquantitative limited log-dilution reverse transcription polymerase chain reaction (RT-PCR) from a total of 41 patients with BCR-ABL$^+$ acute lymphoblastic leukemia (ALL) who all were in complete morphologic remission. Leukemic contamination was found in 19 of 20 unpurged bone marrow (BM) autografts at a level of median 4 (range 0–6) log above the limit of detection, while median 3 log lower BCR-ABL titers were detected in unpurged peripheral blood stem cell (PBSC) autografts. Thus all grafts were purged with CD10 and CD19 immunomagnetic beads resulting in a median 2.5 (range 1–4) log BCR-ABL signal depletion in BM and median 1 (range 0–2) log depletion in PBSC autografts. After purging, BCR-ABL-negative grafts were achieved in 10 of 21 PBSC patients but only in one of 20 BM patients. A total of 34 patients received either single ($n$=25) or double autologous transplants ($n$=9) in first ($n$=26) or in/beyond second ($n$=6) CR. The posttransplant relapse rate was 65% at 2 years for single transplants in CR1 ($n$=19). After double autologous PBSC transplantation in first CR, two of seven patients relapsed with a median follow-up of 213 (range 40–400) days. All patients transplanted beyond CR1 relapsed. Four patients remaining in complete CR for >2 years after single autologous bone marrow transplant (autoBMT) had a median 1:10 titer of residual BCR-ABL signals in the purged grafts. Overall, relapse rate after transplantation did not correlate with the BCR-ABL titer in the reinfused purged cells. In conclusion, the rationale for purging in BCR-ABL$^+$ ALL is based on the evidence of leukemia in unpurged grafts, and low titer residual BCR-ABL signals after purging are not necessarily associated with relapse.

## INTRODUCTION

The Philadelphia chromosome (Ph)-positive/BCR-ABL$^+$ ALL is the most unfavorable subgroup of adult acute lymphoblastic leukemia.[1,2] About 20–25% of

adult ALL patients are characterized by the presence of the t(9;22) Philadelphia translocation and/or the corresponding BCR-ABL rearrangement as a disease-specific clonal marker.[1,3–6] Conventional chemotherapy induces complete morphologic remissions in 60–70% of patients but does not achieve stable long-term remissions, with few, if any exceptions.[2,6] Most patients with BCR-ABL-positive ALL who do not proceed to bone marrow or stem cell transplantation rapidly relapse and eventually succumb to their disease. Allogeneic bone marrow transplantation, accepted as the treatment of choice if an HLA-matched family donor is available, cures about 30–40% of patients.[7–9] Few, but encouraging data with 45% long-term disease-free survivors and low relapse rates are reported after transplantation from matched unrelated donors (MUD) in young patients with a median age of 25 years, supporting evidence that a graft-vs.-leukemia-effect is likely to contribute to preventing relapse.[10] However, the median age of 45 years in BCR-ABL-positive ALL patients as documented in the German multicenter adult ALL (GMALL)[2,6] and other large multicenter studies precludes a majority of patients from receiving MUD-transplants. Thus, for older patients without a matched sibling donor, autologous transplantation remains the best chance of cure. In 1992, the GMALL study group initiated a prospective autograft program including in vitro purging and sequential monitoring of residual disease by semiquantitative RT-PCR for patients with BCR-ABL$^+$ ALL without a compatible HLA-identical donor.[6,11]

## PATIENTS AND METHODS

### Patients

From 1992 through 1998, a total of 42 patients with BCR-ABL-positive ALL in morphologic complete remission and a median age of 45 years were recruited from a total of 25 referring hospitals participating in the GMALL trials 04/89 or 05/93.[2] Autologous bone marrow was harvested in 20 patients,[12,13] whereas PBSCs were collected after induction phase II (high-dose cytosine arabinoside and mitoxantrone) in 22 patients.[14] Thirty-four patients with a median age of 44 years proceeded to either single ($n$=25) or double ($n$=9) autologous transplantation as detailed in Fig. 1.

### Quantification of residual BCR-ABL-positive cells

Residual BCR-ABL-positive cells were determined using a semiquantitative limiting-log dilution RT-PCR as described in detail previously.[12,14] The level of residual BCR-ABL-positive cells before and after purging was expressed as $\log_{10}$ of the highest dilution with a positive PCR signal.

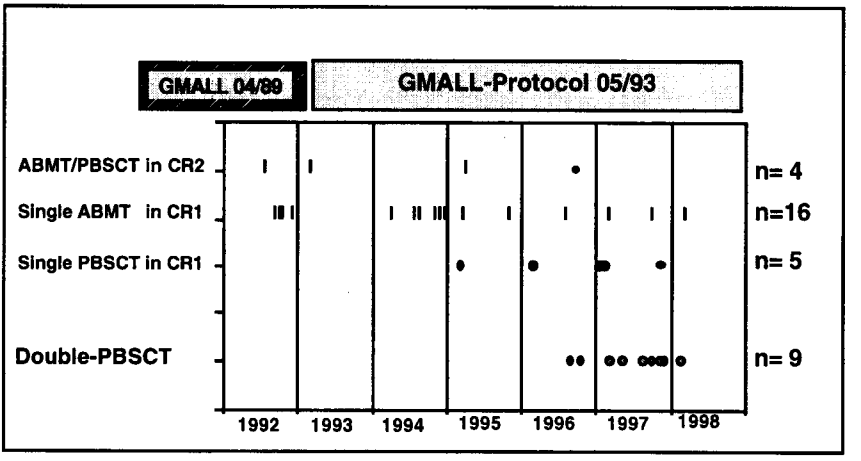


**Figure 1.** *Recruitment of BCR-ABL⁺ patients from GMALL trials for autologous BMT or PBSCT (n=34).*

### In vitro purging using immunomagnetic beads

A total of 20 autologous bone marrow grafts were harvested from BCR-ABL⁺ ALL patients in first ($n$=16) or second ($n$=4) morphologic CR. Purging was performed with anti-CD19⁺CD10 ±AB4 immunomagnetic beads (Dynabeads) and two separation cycles using the Baxter MaxSep device as described in detail previously.[12,14]

### Autologous transplantation

Twenty-five patients in first ($n$=21) or second ($n$=4) complete remission received single autologous transplants after conditioning with fractionated total body irradiation (TBI) 14.4 Gy plus 120 mg/m$^2$ cyclophosphamide ± 45 mg/m$^2$ VP-16. Of the patients autografted in CR1, seven received two courses of induction chemotherapy according to the GMALL (05/93) protocol, and 14 received three or more courses (Fig. 2). From June 1996, patients were also eligible for a pilot double autologous transplant protocol as detailed in Fig. 3. [15]

## RESULTS

### Residual BCR-ABL-positive cells in unpurged autologous grafts

Before purging, residual BCR-ABL-positive cells were detected in 19 of 20 BM grafts at median 4 (range 0–6) log and in 21 of 28 evaluable PBSC grafts at median

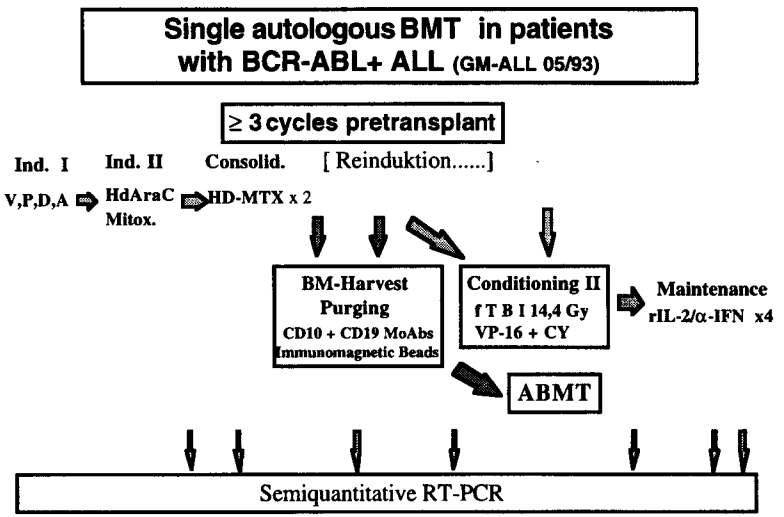


**Figure 2.** *Treatment schedule for single autologous BMT in BCR-ABL⁺ ALL.*

1 (range 0–3) log above the limit of detection ($P<0.0001$).[13] Thus unpurged BM grafts contain 3 log more residual BCR-ABL⁺ cells than unpurged PBSC grafts.

## Immunomagnetic beads purging of autologous BM and PBSC grafts

Cell recoveries were 63 and 87% ($P<0.0001$) of MNC and 74 and 95% ($P=0.065$) of CD34⁺ cells after BM and PBSC purging, respectively. Overall, the purging efficacy was superior in BM compared with PBSC grafts. Purging depleted 2.4 ± 0.2 log of RT-PCR signal from BM grafts and 1.0 ± 0.2 log from PBSC grafts. After purging, the median RT-PCR titer above the limit of detection was median 1 log (range negative titer to 4 log) in BM grafts and median 0 log (range negative titer to 3 log) in PBSC grafts.[13] BCR-ABL-negative grafts were achieved in 12 of 30 PBSC but in only one of 20 BM grafts. BM purging was superior using the triple monoclonal antibody (mAb) cocktail, which depleted 2.64 ± 0.2 log ($n=14$) compared with 1.6 ± 0.2 log ($n=5$) using the mAb cocktail not including AB4 ($P=0.02$).

## Outcome after autologous transplantation

Four patients receiving single autologous BMT in CR1 remain long-term survivors. All patients from the GMALL series autografted in CR2 relapsed after transplant. After double autologous PBSCT, there was one transplant-related death (pneumonitis after PBSCT-2) and four relapses. The follow-up is too short to make conclusions about leukemia-free survival in this group. A detailed analysis of the

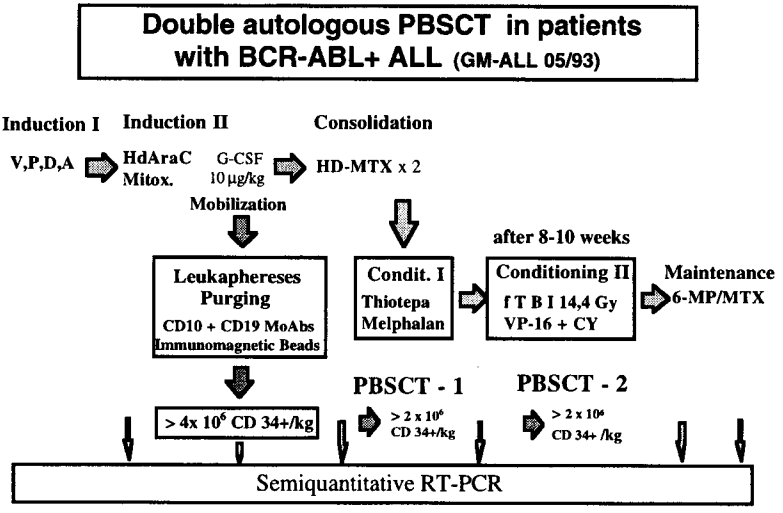


**Figure 3.** *Pilot protocol for double autologous PBSCT in BCR-ABL⁺ ALL.*

outcome after autologous transplantation in the GMALL series will be presented elsewhere.[16]

## Role of purging

Neither the pretransplant level of residual leukemia nor the RT-PCR-signal in unpurged or purged autologous grafts showed a significant impact on posttransplant relapse. Since all BCR-ABL⁺ patients autografted in the GMALL series received purged grafts, data were pooled with the available data on

**Table 1.** Patients receiving autologous transplantation in BCR-ABL⁺ ALL

| *Center* | *Author* | *Year* | *CR1* | *>CR1* | *All* |
|---|---|---|---|---|---|
| Seattle | Gehly | 1991 | 0 | 2 | 2 |
| Minneapolis | Brennan | 1991 | 4 | 7 | 11 |
| Nagoya | Miyamura | 1992 | 5 | 1 | 6 |
| Roma | Annino | 1994 | 3 | 0 | 3 |
| Houston | Preti | 1994 | 3 | 3 | 6 |
| Vienna | Mitterbauer | 1995 | 1 | 1 | 2 |
| Hamburg | Stockschläder | 1995 | 3 | 1 | 4 |
| Genova | Carella | 1995 | 0 | 5 | 5 |
| Royal Marsden | Dunlop | 1996 | 7 | 2 | 9 |
| GMALL/Frankfurt | Martin | 1998 | 28 | 6 | 34 |
| Total | | | 54 | 28 | 82 |

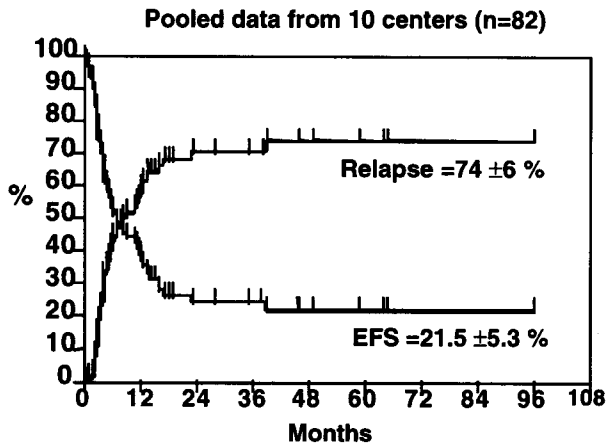


**Figure 4.** *Event-free survival and relapse after autologous BMT/PBSCT in BCR-ABL⁺ ALL (n=82). The Kaplan-Meier plots are based on pooled data from the references listed in Table 1 including the GMALL series.*[17–26]

autologous transplantation in BCR-ABL⁺ ALL from other centers as listed in Table 1.[17–26] The pooled series included 30 patients receiving unpurged grafts. There was a trend but no significant difference for better event-free survival in favor of purging. Patients receiving purged or unpurged grafts are labeled P or NP in Fig. 5, respectively.

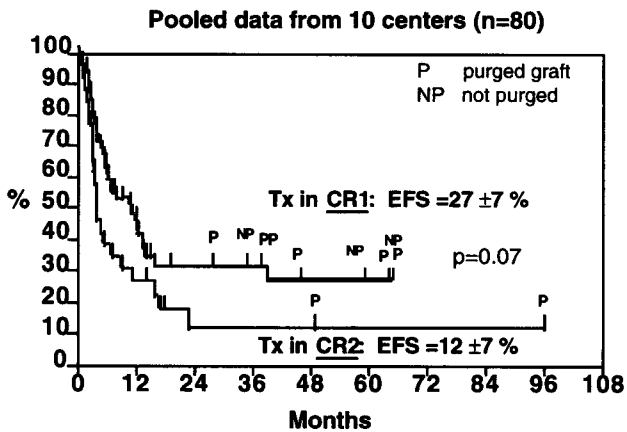


**Figure 5.** *Event-free survival after autologous BMT/PBSCT in BCR-ABL+ ALL in (n=52) or beyond (n=28) first CR. The Kaplan-Meier plots are based on pooled data from the references listed in Table 1 including the GMALL series. The remission status beforetransplant was not available in two patients from Table 1.*

## CONCLUSIONS

Molecular remission after induction and consolidation chemotherapy is rare in patients with BCR-ABL$^+$ ALL. This is consistent with the clinical experience of rapid relapse after discontinuing chemotherapy. The rationale for in vitro purging is based on the evidence of residual leukemic cells in unpurged grafts. Event-free survival after autologous transplantation in BCR-ABL-positive ALL ranges between 20 and 30%. Patients do significantly worse if autografted beyond CR1. Thus, patients without an allogeneic donor should receive an autograft in first morphologic remission. Double autologous transplants are feasible with acceptable toxicity, but longer follow-up is needed to conclude that this approach affects long-term leukemia-free survival.

## ACKNOWLEDGMENTS

We acknowledge the contribution of the dedicated hematologists participating in the GMALL trials who referred BCR-ABL$^+$ patients for autologous transplantation. This work was supported by the Deutsche Forschungsgemeinschaft (Ho 684/2-1).

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# Autologous vs. Unrelated Allogeneic Bone Marrow Transplantation (AlloBMT) for Acute Lymphoblastic Leukemia (ALL)

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## ABSTRACT

The treatment of choice in patients with high-risk acute lymphoblastic leukemia (ALL) is allogeneic bone marrow transplantation (alloBMT) from an HLA-matched sibling donor (MSD). The event-free survival after MSD BMT for ALL ranges from 30 to 65% and depends on the disease status of the patient. Relapse remains the major cause of failure of MSD BMT for ALL. The presence of acute graft-vs.-host disease (GVHD) greatly influences post-BMT relapse; in MSD BMT for first-remission ALL, the risk of relapse is decreased by a factor of 2.77 in patients with acute GVHD compared with those without GVHD. However, the graft-vs.-leukemia (GVL) benefit of acute GVHD does not always translate into improved event-free survival after MSD BMT because of GVHD-related mortality. Since most patients with high-risk ALL lack MSDs, alternative hematopoietic cell transplants have been explored. Autologous BMT (autoBMT) with marrow harvested in remission and usually treated ex vivo (e.g., with monoclonal antibodies) is generally well tolerated but is associated with high relapse rates in most series. A comparison of autoBMT and allogeneic MSD BMT for high risk ALL showed no statistically significant difference in event-free survival but substantial differences in relapse rates: 79% in autoBMT, 75% in MSD BMT with no GVHD, and only 37% in MSD BMT with GVHD. Quantitative analysis of clonogenic ALL cell content in the harvested marrow of autoBMT recipients suggests that, as with alloBMT for ALL, the failure to eradicate residual leukemia in vivo is the biggest obstacle to improving event-free survival after autoBMT for ALL. BMT from volunteer unrelated donors (UDs) is another alternative in high-risk ALL. Event-free survival after UD BMT for ALL ranges from 12 to 43%; these results are comparable to autoBMT in patients with first-remission ALL and superior to autoBMT in patients beyond first remission. In UD BMT, the major cause of failure is a high rate (50 to 70%) of transplant-related mortality. In contrast, the relapse rate after UD BMT for ALL is relatively low; after one-antigen-mismatched UD BMT, the relapse rate is 38% of that observed after MSD

BMT. Transplantation of unrelated placental/umbilical cord blood (UCB) cells is a third alternative; it is not known whether the apparently lower risk of severe GVHD after UCB cell transplantation will alter the GVL effect. Improved outcome of BMT for high-risk ALL depends on better in vivo antileukemia treatment, through either manipulation of the GVL effect of alloBMT or development of novel therapies (e.g., immunotoxins) in autoBMT.

## MATCHED SIBLING ALLOBMT VS. AUTOBMT FOR ALL

In adults, matched sibling BMT has been shown in a prospective study to be superior to autoBMT as consolidation treatment of first-remission ALL, with disease-free survival (DFS) of 68 vs. 26% ($P<0.001$) for alloBMT and autoBMT, respectively.[1] In adults beyond first remission and for refractory leukemia, matched sibling BMT is also superior to autoBMT, with relapse occurring in eight of nine autoBMT recipients vs. two of 10 alloBMT recipients.[2] In reports combining both pediatric and adult patients, matched sibling BMT again appears superior, with a 3-year DFS of 52% for alloBMT vs. 29% for autoBMT.[3]

In children with relapsed ALL, chemotherapy appears capable of rescuing a significant proportion without matched sibling BMT, but it is related to duration of first remission. A recent study from the Pediatric Oncology Group (POG 9110; $n=150$) found for first remission <24-month EFS at 4 years was 11.9% and >24-month EFS at 4 years was 42.7% with chemotherapy alone.[4] These data make it clear that all relapsed patients are not equal, at least in pediatric ALL, and require careful matching when comparing different modalities of treatment for pediatric ALL. When an attempt was made in a retrospective analysis to compare matched sibling BMT to chemotherapy alone, late relapsing children with ALL had a superior outcome with BMT (53%) vs. chemotherapy alone (32%) that was statistically significant.[5] However, even now there is controversy regarding use of matched sibling BMT for pediatric patients that experience a late relapse, due to both the uncertainty of the data based on retrospective analyses with acknowledged biases and the relatively high cost and higher morbidity/mortality associated with matched sibling BMT.

## MATCHED UNRELATED DONOR BMT VS. AUTOBMT FOR ALL

For the group of patients with ALL that appear to have a survival advantage with alloBMT over either chemotherapy or autoBMT, the majority do not have matched siblings to serve as donors. The development of large volunteer registries of adult donors around the world has made it possible to offer alloBMT to the majority of patients that would otherwise be eligible for alloBMT but lack a matched sibling donor. Based on large numbers of transplanted patients using UD, it is clear that

outcomes after UD BMT in patients with leukemias are inferior compared with matched sibling BMT, with the only exception being patients who are not in remission at the time of transplant, in which outcomes are similar (approximately 10–15% 3-year survival).[6] The inferior outcomes with UD BMT compared with matched sibling BMT are due to the two- to threefold increase in transplant-related mortality, which is up to 70% in high-risk patients. This makes it especially important to examine the outcomes of UD BMT compared with autoBMT for ALL.

Two recent, large retrospective analyses have attempted to answer the clinical question of whether to offer UD BMT vs. autoBMT for patients with high-risk ALL. One report from the European Group for Blood and Marrow Transplantation (EBMT) and International Marrow Unrelated Search and Transplant (IMUST) groups found for ALL patients matched in age, stage, and year of transplant a 2-year DFS of 39 ± 5% for UD BMT vs. 32 ± 3% for autoBMT.[7] The other report focused on 214 ALL patients treated with autoBMT at two centers compared with 337 ALL patients treated with UD BMT reported to the National Marrow Donor Program (NMDP) in a similar time period, with the majority (68%) of patients <18 years old.[8] The findings of this study were that of patients in first remission, the DFS for autoBMT was 42 vs. 32% for the UD BMT group ($P$=0.03). For patients in second remission, the DFS for autoBMT was 20 vs. 42% for the UD BMT group ($P$=0.02). For patients beyond second remission, there were no statistically significant differences in DFS. In comparing second-remission patients, which were the largest single group, relapse rates after autoBMT were higher than after UD BMT, 76 vs. 17% ($P$=0.0001), while transplant-related mortality was significantly lower, 14 vs. 48% ($P$=0.0001).

While these two studies help provide some guidance to the clinician, in reality local selection bias probably plays a significant role in transplants done to date, and will continue to play a role in the future. There are more centers that offer UD BMT than autoBMT for ALL, due to the technology necessary to perform bone marrow purging in autografts. This fact alone undoubtedly is a factor in selection bias. The fact that unrelated donor searches and activation take 3 months or longer also make for a complicated bias in that patients waiting for UD BMT may self-select and become ineligible due to disease progression. However, it is also plausible that patients are preselected for certain favorable characteristics when referred for autoBMT. Without prospective and randomized studies, many unanswered questions will remain. A crude comparison of the outcome and treatment-related mortality for the different modalities discussed is outlined in Table 1.

## MECHANISMS OF FAILURE OF ALLOBMT VS. AUTOBMT IN ALL

The causes of failure in autoBMT are overwhelmingly linked to leukemia recurrence. What role is played by purging the bone marrow graft of residual

**Table 1.** Summary of outcome for CR2 ALL (excluding children with late relapses)

| *Therapy* | *EFS* | *TRM* |
|---|---|---|
| Chemotherapy | 10% | 15% |
| AutoBMT | 20% | 15% |
| Matched sibling donor BMT | 50% | 25% |
| Unrelated donor BMT | 40% | 50% |

leukemia has remained an important question. Studies in which the leukemia contamination was quantitated by an in vitro progenitor assay done before purging as an indicator of pretransplant leukemia burden, and then done after purging, indicate that it is the burden of leukemia remaining at the time of BMT that determines relapse and not the burden of leukemia infused.[9] The availability of sensitive molecular detection methods may allow a better assessment of leukemia burden pre- and postpurging of bone marrow, and also may allow better selection of patients as candidates for autoBMT.[10] Recently, the use of peripheral blood stem cells has been identified as an interesting source for grafting which may have a lower burden of leukemia, but if in vivo leukemia burden is not eradicated, it may not lead to significant improvements in outcome. Overall, improvement in outcome with autoBMT for ALL will require improvements in "in vivo" purging.

In contrast to autoBMT, UD BMT has a relatively low relapse rate but high transplant-related mortality, both of which are related to GVHD. The existence of a GVL reaction for ALL is now unequivocal, based on lower relapse rates after UD vs. MSD BMT,[6] correlation of lower relapse rates with occurrence of GVHD,[11] and an antileukemia effect of donor leukocyte infusions for relapses of ALL post-BMT.[12] A GVL effect for first-remission ALL in matched sibling donor BMT was found to be correlated with acute GVHD but not chronic GVHD.[13] In contrast to both acute and chronic myeloid leukemias (AML and CML), there does not appear to be a GVL effect independent of GVHD. This may make for a very difficult situation in terms of improving outcomes of UD BMT for ALL, since GVHD is the largest contributor to treatment failure, and attempts to abrogate GVHD may very well lead to higher relapse rates. A hypothetical breakdown of the components of both auto- and UD BMT as they contribute to outcome in patients with high-risk ALL is listed in Table 2.

## AUTOLOGOUS GVL REACTIONS

An approach to improving autoBMT might then be to mimic a GVL effect without generating fatal GVHD. One approach is through modulation of the host immune system with agents that can induce autoimmune reactions. This has been demonstrated in autoBMT for AML with use of post-BMT cyclosporine A.[14]

**Table 2.** Hypothetical breakdown of the components of both auto- and UD BMT as they contribute to outcome in patients with high-risk ALL

| | *AutoBMT* | *UD BMT* |
|---|---|---|
| Purging | –10% | 0% |
| Chemoradiotherapy | +30% | +30% |
| GVL | 0% | +60% |
| GVHD | 0% | –50% |
| Total | 20% | 40% |

Further studies involving this approach are currently ongoing and include addition of immunostimulatory cytokines. Post-BMT interleukin (IL)-2 has been used in several studies of autoBMT for ALL but with no apparent positive effects.[1] The effects of systemic cytokines like IL-2 that activate nonspecific effectors, i.e., natural killer (NK) and lymphocyte-activated killer (LAK) cells, may have a limited role in treating drug- and/or radiation-resistant ALL based on in vitro resistance to LAK-mediated cytotoxicity (Figs. 1 and 2). In contrast, cytotoxic T lymphocytes (CTL) maintain effectiveness against radiation-resistant ALL cell lines (Fig. 3). Currently, our laboratory is actively pursuing the generation of autologous CTL from leukemia patients, with the ultimate goal of identifying antigenic peptides that could serve as targets and allow in vivo vaccination approaches as have been used for melanoma.[15]

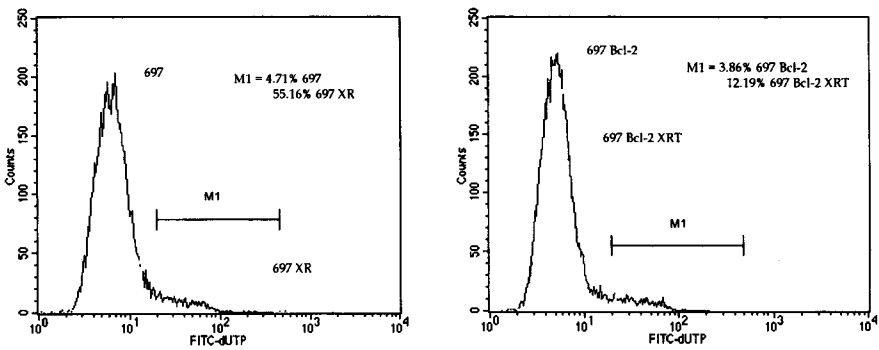


**Figure 1.** *Bcl-2 overexpression in 697 ALL inhibits radiation-induced DNA fragmentation as measured by flow cytometry. Both 697 and 697Bcl-2 were exposed to 30 Gy g-irradiation from a cesium source and then fixed overnight in 1% paraformaldehyde followed by 70% ethanol. Cells were labeled at 39-hydroxy termini of DNA breaks with fluorescein isothiocyanate (FITC)-dUTP according to manufacturer's instructions. 20,000 events were acquired and analyzed with Cell Quest software. Shown are 697 in left panel and 697Bcl-2 in right panel with percentages of FITC-positive cells both for unirradiated and 30 Gy–irradiated groups.*

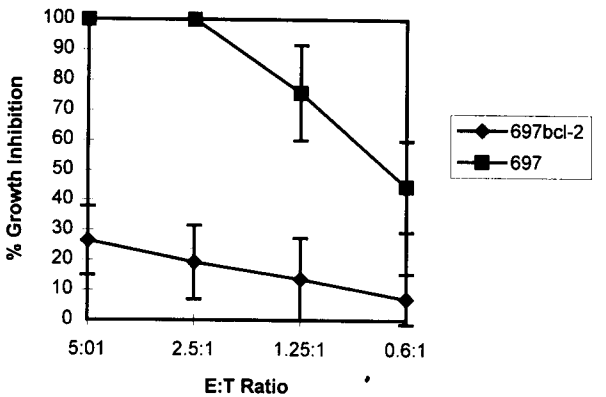


**Figure 2.** *Bcl-2 overexpression inhibits LAK cytotoxicity of 697 ALL. LAK cells were generated by incubation of random donor peripheral blood mononuclear cells at $10^6$/mL in RPMI with 10% fetal bovine serum (FBS) (CM) with addition of 5000 U recombinant human IL-2/mL for 5 days. The growth inhibition assay is performed by proliferation of target cells both with and without addition of irradiated effector cells. Targets are placed in round-bottomed 96-well plates at 25,000 per well in triplicate in CM. Effectors are irradiated (30 Gy) and plated with targets at four different effector:target ratios in triplicate. Background proliferation from irradiated effectors is calculated from parallel wells containing only effectors. Proliferation of targets only is performed with six replicates. After 4-hour coincubation, all wells are pulsed with 1 mCi [$^3$H]thymidine overnight and cpm obtained by standard liquid scintillation counting. Percentage growth inhibition is scored after subtraction of effectors alone and is based on cpm of targets alone. Shown are mean and standard deviation for five separate experiments.*

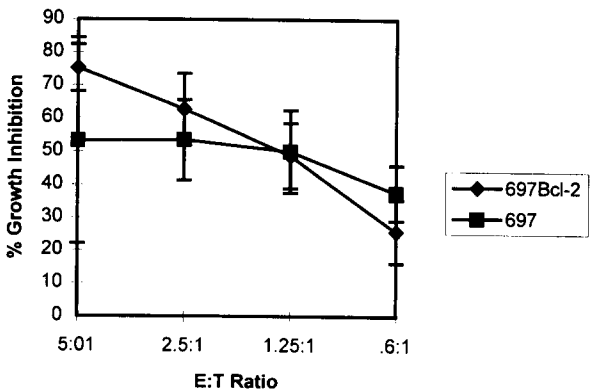


**Figure 3.** *Bcl-2 overexpression has no effect on CTL cytotoxicity of 697 ALL. CTL were generated by incubation of random donor peripheral blood mononuclear cells at $10^6$/mL in RPMI with 10% FBS (CM) with addition of 50 U recombinant human IL-2/ml for 5 days with irradiated (60 Gy) 697 or 697Bcl-2 stimulators at a 3:1 responder-to-stimulator ratio. The growth inhibition assay is performed identically to that described in Fig. 2. Shown are mean and standard deviation for four separate experiments.*

## SUMMARY

Most of the available data suggest that autoBMT and UD BMT are roughly equivalent for ALL patients in first remission, although no prospective studies have yet addressed this issue. For patients beyond first remission, the data seem to favor UD BMT, in both adults and children. This area will likely remain controversial, as both auto- and alloBMT are rapidly changing fields. Clearly, for both auto- and UD BMT, results are less than completely gratifying. In autoBMT, lack of effective in vivo purging probably overshadows effects of in vitro purging. In UD BMT, the mortality of GVHD probably overshadows much of the beneficial effects of a GVL reaction. Attempts to provide for a CTL-mediated GVL effect in the setting of an autoBMT may allow combining the benefits of both autologous and allogeneic BMT.

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# CHAPTER 3
# CML

# Donor Lymphocyte Infusions Produce Durable Molecular Remission in Patients Who Relapse After Allografting for CML in Chronic Phase: The Consequent Need to Reassess the Definition of Leukemia-Free Survival

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The options for treating patients who relapse after allogeneic hematopoietic stem cell transplantation (SCT) for chronic myeloid leukemia (CML) include a second allograft, administration of interferon-alpha, and infusion of lymphocytes from the original donor. A substantial proportion of patients will achieve molecular remission after donor lymphocyte infusions (DLI), but the durability of these remissions and therefore the contribution of DLI to management of patients who relapse is not yet established. We studied a cohort of 247 patients with CML in chronic phase who received an allogeneic SCT from an HLA-identical sibling donor ($n$=147) or a volunteer unrelated donor ($n$=100) during the period 1 January 1986 to 31 December 1995 with a minimum follow up of 27 months. During the period of observation, 74 patients relapsed, and of these, 52 received DLI. Twenty-eight patients were treated with a single ("bulk") infusion of donor lymphocytes, and 24 received donor lymphocytes on an escalating dose schedule. Twenty-seven (52%) patients achieved a complete molecular remission. No patient who achieved a molecular remission has relapsed, with a median follow-up of 21 months (range 4–77). Because conventional measures of leukemia-free survival (LFS) censor patients at the time of relapse and take no account of any subsequent remission that may prove durable, we have established a new category of LFS, termed current LFS (CLFS), which is defined as survival without molecular evidence of relapse at the time of most recent assessment. For the 247 patients in this study, the conventionally defined LFS at 5 years was 33% (95% CI 24–39%), while the CLFS was 45% (95% CI 39–52%). These data show that the molecular remissions that can be achieved in CML patients who receive DLI for relapse after allografting may continue for some years.

# Autograft Followed by Allograft Without Myeloablative Conditioning Regimen: A New Approach for Resistant Hematologic Neoplasia and Breast Cancer

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## ABSTRACT

To reduce relapse and morbidity risks of allograft in patients with refractory or relapsed hematologic malignancies or metastatic breast cancer, a trial was undertaken consisting of high-dose therapy and autotransplant followed by immunosuppressive therapy with fludarabine and cyclophosphamide (Flu-Cy protocol) and infusion of mobilized HLA-matched sibling donor stem cells. Graft-vs.-host disease (GVHD) prophylaxis consisted of cyclosporine and methotrexate. Fourteen patients with the following diagnoses entered our trial: Hodgkin's disease ($n$=4), non-Hodgkin's lymphoma ($n$=2), advanced chronic myelogenous leukemia ($n$=2) (one patient with accelerated phase Ph-negative but p190 BCR-ABL gene positive by RT-PCR and one with Ph-positive blastic phase), metastatic breast cancer ($n$=4). The two other patients had refractory anemia with excess blasts (RAEB) and received allografts without a myeloablative conditioning regimen. To date, with a median follow-up of 8 months (range 3–14) after allografting, complete chimerism (100% donor cells) was achieved in six patients and >90% in two others. Five patients did not achieve complete chimerism: two patients died of progressive Hodgkin's disease and breast cancer, one patient died in complete remission (CR) of aspergillus during progressive donor cell engraftment, and the patients with BP-CML and RAEB appear to have had autologous recovery. Two of the Hodgkin's disease patients, who were in partial remission (PR) after autografting, achieved CR after allografting. One patient is disease-free 10 months

later, and the other patient died in CR of aspergillus at 4 months. Another patient with progressive Hodgkin's disease is alive at 14 months and has chronic GVHD, and the other died of progressive disease on day 65. The patient with low-grade non-Hodgkin's lymphoma, who achieved PR after autografting, obtained a CR and is disease-free 6 months after allografting. The other patient died of progressive lymphoma 5 months after allografting. The patient with accelerated phase CML obtained hematologic remission and is now in molecular remission 8 months later. One patient with RAEB relapsed after achieving hematologic and cytogenetic remission for 3 months. Severe acute GVHD (grade >II) was the single major complication but caused no deaths. Mild acute GVHD was seen in another patient. Only one patient experienced an absolute neutrophil count below $1\times10^9$/L and in no case did platelets decrease below $20\times10^9$/L. No patients required sterile rooms or any red cell or platelet transfusions. We conclude that immunosuppressive therapy with the Flu-Cy protocol enabled the engraftment of HLA-matched sibling donor stem cells without procedure-related deaths. Moreover, we have demonstrated that this combined procedure can be pursued safely in a seriously ill population, and some can achieve complete remission. This procedure is likely not curative, but is a fascinating step along the path to curing these diseases.

## INTRODUCTION

To circumvent the inherent problems of toxicity and treatment-related deaths associated with allografting, it was recently shown that engraftment of donor hematopoietic stem cells can be achieved after immunosuppressive therapy combined with myelosuppressive but nonmyeloablative therapy.[1,2] The observations that nonmyeloablative regimens based on fludarabine have resulted in engraftment of allogeneic cells in patients with hematologic malignancies, raises the possibility that such conditioning might be useful in achieving a graft-vs.-tumor effect. In this pilot study, we treated nine patients with resistant malignancies who received high-dose therapy and autologous stem cell transplantation as tumor debulky therapy followed by immunosuppressive therapy and infusion of HLA-matched sibling donor stem cells in an attempt to induce an immune-mediated antitumor effect.

## PATIENTS AND METHODS

The patients were registered in the Hematology and ABMT Unit, Department of Hematology, San Martino Hospital in Genoa. This pilot study was approved by the Ethics Committee of San Martino Hospital, and informed consent was obtained from patients and donors. Fourteen patients were treated, ages 22 to 57 years (median age 40 years). Four had primarily refractory ($n$=2) or relapsed ($n$=2) Hodgkin's disease and two had primarily refractory non-Hodgkin's lymphoma

(one low-grade and one high-grade). These patients were treated with first- and second-line therapies without success. Two patients had chronic myeloid leukemia (CML), one in accelerated phase (this patient did not have evidence of the Philadelphia chromosome at diagnosis, but the p190 BCR-ABL gene was found by reverse transcriptase polymerase chain reaction [RT-PCR]) and one in myeloblastic transformation with 70% marrow blasts. Both showed cytogenetic/molecular resistance to interferon alpha (5 mU/m$^2$/d). Four patients had metastatic breast cancer to bone and/or liver. All patients were previously treated with first-line therapy (adriamycin-containing regimens) and second-line therapy ± tamoxifen. All were in an advanced phase of disease before our two-step transplant approach. Two patients had RAEB, one with t(1;3) (p36;q21).

In the first stage of the protocol, autologous peripheral blood stem cells were mobilized from 12 patients, in 10 after treatment with cyclophosphamide at 3–4 g/m$^2$ and granulocyte colony-stimulating factor (G-CSF) (5 µg/kg/d) and in the CML patients with ICE (blastic phase) or mini-ICE (accelerated phase) protocols.[3,4] All but RAEB patients went on to the next phase of the protocol within 28 days (range 15–270). In preparation for autotransplant, patients underwent high-dose chemotherapy on protocols appropriate for the underlying disease. The patients with CML received high-dose busulfan 3 mg/kg/d × 4 days (accelerated phase) or high-dose mitoxantrone (20 mg/m$^2$/d × 3 days) with cytosine arabinoside (1000 mg/m$^2$/d × 3 days) (blastic phase). Patients with Hodgkin's disease and non-Hodgkin's lymphoma were treated with the BEAM protocol[5]: carmustine 300 mg/m$^2$ intravenously on day 1, etoposide 200 mg/m$^2$ intravenously on days 2–5, cytosine arabinoside 200 mg/m$^2$ b.i.d. on days 2–5, and melphalan 140 mg/m$^2$ intravenously on day 6. Breast cancer patients were treated with thiotepa, paraplatin, and cyclophosphamide (two patients), mitoxantrone, taxotere, and paraplatin (1 patient), and thiotepa and paraplatin (1 patient). Three patients with Hodgkin's disease, both patients with non-Hodgkin's lymphoma and two patients with breast cancer achieved PR after autografting. The patients with CML achieved a second chronic phase, but all metaphases remained Philadelphia chromosome–positive (blastic phase) and BCR-ABL–positive (accelerated phase). The median time from autografting to allografting was 38 days (range 30–96). Patients received fludarabine (30 mg/m$^2$/d on days 1–3) with cyclophosphamide (300 mg/m$^2$/d on days 1–3). GVHD prophylaxis consisted of cyclosporine beginning the day before donor stem cell infusion at 1 mg/kg/d by continuous infusion and methotrexate 10 mg/m$^2$ on days 3 and 5; cyclosporine was continued by intravenous infusion for 12–29 days (median, 16 days), thereafter by the oral route. Donors were treated with G-CSF at 10 mg/kg b.i.d. for 2 to 4 days and then underwent leukaphereses of stem cells. A median of $3\times10^6$/kg (range 1.3–7.8) donor CD34$^+$ cells were obtained and infused fresh into the patient 48 hours after the conclusion of Flu-Cy therapy. Patient blood samples were serially studied for chimerism.

### Evaluation of chimerism

Cytogenetics and DNA polymorphisms by fluorescence-based technology of multiplexed PCR products (STR) from bone marrow cells were used as a marker for chimerism. Allogeneic stem cells were monitored with this technique, first by the multiplex reaction and then by detecting donor/recipient cell population ratios at 10-day intervals the first month and 15-day intervals in the second and third month after allografting by evaluating peak areas in singleplexed PCR products of each informative marker.

## RESULTS

The Flu-Cy protocol was well tolerated, with no severe procedure-related toxicity. No patient required platelet or red cell transfusions. Patients were discharged from the hospital 16 to 39 days (median 19) after donor stem cell infusion. There was evidence of >90% donor cell engraftment in eight patients. Severe acute GVHD (>grade II) was observed in two patients (Hodgkin's disease) and mild acute GVHD in one patient (accelerated phase CML). In another patient, there was a suggestion of grade I acute GVHD of the skin (erythema) and diarrhea (500 mL/day) which disappeared after therapy with octreotide, corticosteroids, and oral cyclosporine.

### Disease response

Two of the Hodgkin's disease patients, who were in PR after autografting, achieved CR after allografting. One patient is disease-free 10 months later, while the other died in CR of aspergillus at 4 months. Another patient with Hodgkin's disease is alive after 12 months but has progressive disease in the liver, and the last patient died of progressive Hodgkin's disease on day 65. The patient with low-grade non-Hodgkin's lymphoma, who achieved PR after autografting, is disease-free 6 months after allografting. The other patient died of lymphoma 5 months after allografting. The blastic phase CML patient is in chronic phase 11 months after allografting but requires hydroxyurea. In contrast, the patient with accelerated phase, who obtained a second chronic phase after autografting, achieved complete disappearance of the BCR-ABL hybrid transcript and is now in complete hematologic and molecular remission with 100% donor cells in the marrow at day 240. The patient with RAEB and the t(1;3) abnormality, who received only an allograft, obtained complete hematologic and cytogenetic remission with 100% donor cells for 3 months, and then relapsed with 50% t(1;3) cells and 20% donor cells. The other patient with RAEB had autologous recovery. To date, nine patients are alive between 3 and 14 months (median 8 months).

## DISCUSSION

Myeloablative chemoradiotherapy regimens, generally considered a mandatory first step in preparation for allografting, are associated with substantial toxicity and mortality, mainly in older patients. Recently, it was demonstrated that fludarabine, like other purine analogs, has substantial immunosuppressive activity, inducing long-lasting T-cell lymphopenia when used in the treatment of patients with lymphoproliferative disorders and when administered to patients with chronic lymphocytic leukemia as part of their primary therapy before allografting.[6] Fludarabine may also modulate the host immune system, thereby reducing the severity of the GVHD[7,8]; moreover, transfusion-associated GVHD appears to be more frequent in fludarabine-treated patients, because of the profound $CD4^+$ and $CD8^+$ T cell depletion induced by the drug.[9]

This drug in combination with other myelosuppressive drugs was recently shown to enable the engraftment of HLA-matched sibling donor stem cells.[1,2] These teams used nonmyeloablative but myelosuppressive drugs in their regimens. Because of this, severe hepatic toxicity was seen combined with neutropenia,[2] or neutropenia alone was documented.[1] Five patients died of infections and multiorgan failure in Houston and four died of severe acute GVHD in Jerusalem. In addition, in no case was cytogenetic and/or molecular evidence of remission documented. In contrast, the Flu-Cy protocol employed by our team is free of myelosuppressive drugs but is sufficiently immunosuppressive to allow the engraftment of donor cells without potential side effects. We found no hepatic toxicity, and only one patient had neutropenia lower than $1 \times 10^9$/L. To date, with the exception of the patient with blastic phase CML and one with RAEB who appears to have had autologous recovery, >90% donor cells was demonstrated in eight patients.

The second objective of our pilot study was to determine if autologous and allogeneic transplants can be combined to harness the reduction of tumor burden following autografting and the immune-mediated effects on minimal residual disease after allografting without high overall toxicity and/or procedure-related deaths. We have demonstrated that this approach can be pursued safely in a seriously ill population. There were no procedure-related deaths, and patients did not require a sterile room or any red cell or platelet transfusions. They did not require hyperalimentation, and none had mucositis. The patients were discharged from the hospital at a median of 19 days, and they were followed as outpatients. Two patients showed >grade II acute GVHD, but neither died. Seven of 12 patients treated by autografting followed by allografting are alive, and four achieved complete remission.

Of particular interest are the results achieved in the patients with RAEB and accelerated phase CML. In the first case, the patient was pretreated with high-dose

erythropoietin followed by corticosteroids and chemotherapy without success. She received weekly red cell transfusions to maintain hemoglobin at 7–8 g/dL. All metaphases contained t(1,3) (p36;q21). On day 63 after allografting, the karyotype showed only 46 XY without evidence of the 1; 3 translocation. Reticulocytosis of 12% was documented on day 40 and was followed by an increase in hemoglobin to 12.6 by day 63. No sign of acute GVHD was observed.

The second patient had CML diagnosed in 1996 without evidence of Ph chromosome, but the p190 BCR-ABL gene was detected by RT-PCR. He was treated with hydroxyurea and interferon-alpha (5 $mU/m^2/d$) for 6 months, but the RT-PCR remained positive and the white blood cell (WBC) count and platelets increased. In October 1997, the disease was considered to be in accelerated phase, WBC $105\times10^9$/L and platelets $1200\times10^9$/L. He was treated with the mini-ICE protocol[4] and, while recovering from aplasia, underwent leukaphereses which yielded only PCR-positive cells with BCR-ABL/ABL ratio of 0.1. The patient received an autotransplant but no change in BCR-ABL/ABL ratio was seen, and the white count and platelets remained high. He had an HLA-matched brother age 69 years; after he received the Flu-Cy protocol, mobilized donor hematopoietic stem cells were infused. Granulocytes and platelets never decreased below $1\times10^9$/L and $20\times10^9$/L, respectively. He was discharged on day 14 and followed as an outpatient. Complete chimerism was achieved on day 108 (100% donor cells) with BCR-ABL/ABL ratio 0.0008. On day 240, complete chimerism with BCR-ABL/ABL ratio <0.0001 persists.

In conclusion, the long-term benefit of this treatment has yet to be determined. Considering that our patients were all at high risk, we think that this sequential procedure represents a new approach for a large variety of clinical situations in which allografting is indicated.

## ACKNOWLEDGMENTS

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# Autografting for Chronic Myeloid Leukemia in Chronic Phase: EBMT Retrospective Analysis and Proposals for a Prospective Study

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In the late 1970s, selected patients with CML in transformation were treated by high-dose chemotherapy and autografted with marrow and later with blood-derived stem cells. The majority of patients were restored to chronic phase hematopoiesis, but there was no convincing evidence that their survival was prolonged. In the early 1980s, various groups performed autografts for patients in chronic phase using unmanipulated marrow or blood stem cells. The collected data for EBMT collaborating centers has been analyzed on a number of occasions. The toxicity of the procedure was low. Patients who achieved some degree of Ph-negativity survived longer than those who recovered exclusively Ph-positive hematopoiesis. Some patients achieved durable Ph-negativity. Again, there was no formal proof that survival was prolonged in comparison with conventional treatment with cytotoxic drugs or interferon-alpha. Against this background, the EBMT has designed and activated a prospective study whereby patients at diagnosis will be randomly allocated to receive either treatment with interferon-alpha (± cytarabine) or an autograft with stem cells collected at diagnosis or after mobilization with cytotoxic drugs and G-CSF. This study addresses the issue of whether the addition of an autograft procedure early after diagnosis contributes to prolongation of survival for patients not eligible for an allograft procedure.

# The Case for Manipulation of CML Autografts

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It is established that in many patients with chronic myeloid leukemia (CML) there persists a functional population of Philadelphia chromosome (Ph)-negative stem cells that are normal. Quantification of this population suggests that, from a therapeutic standpoint, these cells represent a useful reservoir. On the other hand, past experience with unirradiated granulocyte transfusions from donors with CML, supported by recent studies with genetically marked cells, has provided evidence that the CML clone can be transplanted. Taken together, these findings provide a rationale for the selective isolation of Ph-negative stem cells for use in conjunction with autograft-based protocols. A number of techniques for the manipulation of CML autografts have been developed and are under clinical evaluation. The results from these studies indicate that at least in some cases it may be possible to restore Ph-negative hematopoiesis for significant periods of time postautograft. Although the relative contribution of the autograft manipulation to this outcome is not clear, results to date are sufficiently encouraging to suggest that this approach may be beneficial. The Vancouver experience with cultured marrow autografts provides an example of the progress to date. Prospective, randomized clinical trials are now in progress to determine whether or not intensive therapy with autografting offers significant survival advantage to patients with CML. It will take a number of years before the results of these trials are known, and in the meantime it would seem reasonable for specialized centers to try to improve the methodology on the assumption that this treatment is superior to standard therapy. In this regard, the application of gene transfer technology to modify the autografts (i.e., gene therapy) holds promise as a therapeutic maneuver.

# Preclinical Evaluation of an Inhibitor of the ABL Tyrosine Kinase as a Therapeutic Agent for Chronic Myelogenous Leukemia

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p210BCR-ABL, present in virtually all cases of chronic myelogenous leukemia (CML) is derived from a hybrid gene created by the Philadelphia chromosome translocation. This hybrid gene has been shown to cause a CML-like syndrome in mice and is capable of transforming immature hematopoietic cells in vitro. The hybrid BCR-ABL protein has elevated tyrosine kinase activity compared with c-ABL, and the tyrosine kinase activity of ABL is required for the transforming function of the BCR-ABL fusion protein. Using the known structure of the ATP binding site of protein kinases, a series of potentially inhibitory compounds were synthesized, and CGP 57148 was found to be a potent and specific inhibitor of the ABL protein tyrosine kinase. Using factor-dependent myeloid cells (32D and MO7) and derivatives expressing p210BCR-ABL, we demonstrated specific killing of BCR-ABL-expressing cells by CGP 57148. Similar cytotoxicity has been observed using a variety of Philadelphia chromosome–positive cell lines and cells expressing the p185BCR-ABL protein associated with acute lymphoblastic leukemia. In vivo, antitumor activity of the compound has been validated in syngeneic mice inoculated with BCR-ABL-expressing cells. In colony-forming assays of peripheral blood or bone marrow from patients with CML, there was a 60–80% decrease in the number of colonies formed in the presence of CGP 57148 but minimal inhibition of colony formation by hematopoietic cells from non-CML patients or a BCR-ABL-negative CML patient. This compound may be useful in the treatment of CML and other BCR-ABL-positive leukemias. Phase I clinical trials in CML patients resistant to interferon-alpha are underway.

# Toward a Cure by Drug Treatment?

# The German CML Study Group Experience

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## ABSTRACT

It is now generally accepted that interferon (IFN) α and, to a lesser extent, hydroxyurea (HU) prolong survival of patients with chronic myeloid leukemia (CML). Intensification of drug treatment appears to further prolong survival, provided drugs are used with sufficiently low toxicity to allow the necessary dose intensification. Examples are the longer survival times after addition of cytosine arabinoside (Ara-C) to IFN-based regimens, or after myeloablative chemotherapy for allogeneic bone marrow transplantation (alloBMT) and relapse. Both groups of patients show significantly better survival than control groups. The availability of high-dose chemotherapy and autografting technologies offer the potential to further intensify treatment and prolong survival. The German CML Study Group therefore decided to study the effect of high-dose chemotherapy with autografting followed by IFN maintenance therapy on survival in a randomized study (CML Study III A). Since normal hematopoietic progenitor cells are predominantly found at diagnosis and in early chronic phase, and since treatment outcome in CML seems better the earlier treatment is started, it was decided to study high-dose chemotherapy with autografting as first-line therapy up-front and to use stem cells that were mobilized within 3 months after diagnosis. The preferred mobilization treatment consists of

idarubicin, Ara-C, and etoposide (mini-ICE). CML Study III A was activated in July 1997, and by December 1998, 138 patients were recruited. An earlier arm of the study (CML Study III) which was activated in January 1995, analyzed treatment intensification with intermediate-dose Ara-C and idarubicin. About 600 patients have been recruited for CML Study III. Both studies define suitability for, and consent to, allogeneic BMT before any treatment decision and compare BMT and drug treatment in a prospective randomized approach.

## INTRODUCTION

There is now general agreement that IFN, and to a lesser extent HU, can prolong survival in CML. Figure 1 shows a recent update of the randomized comparison of busulfan and hydroxyurea.[1] The evidence for a prolongation of survival by IFN stems mainly from four randomized studies as shown in Table 1.[2–5] Long-term follow-ups of the German and the Italian randomized studies[6] and several observational studies[7,8] have shown that some patients, particularly of the low-risk group, have long median survivals of 8–9 years or more. Table 2[6,9] shows a summary of the results of the German CML Study I in comparison to a recent update of the Italian study. IFN is particularly effective in early CML and in good-risk patients. The visibility of the survival advantage by IFN therefore depends to a considerable extent on the patient's risk group composition.[10] In low-risk patients, the survival

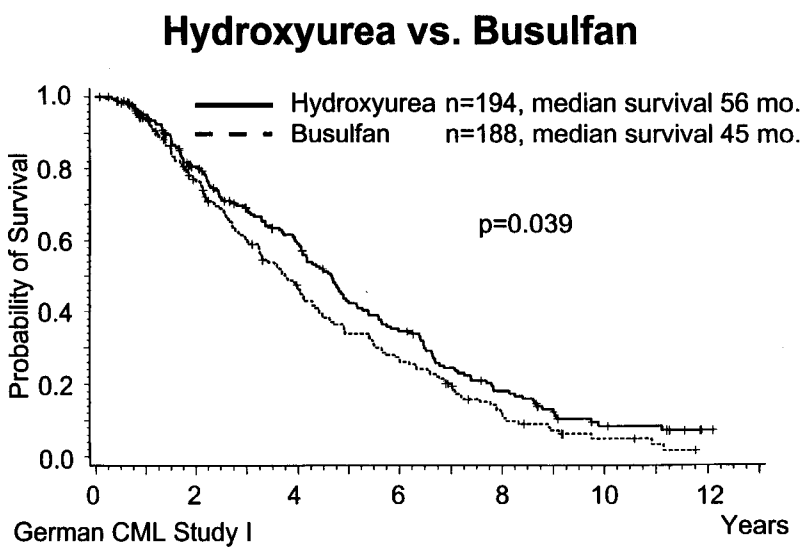


**Figure 1.** *Long term follow-up of the randomized comparison of hydroxyurea vs. busulfan in CML as of 28 February 1998 after about 94% of patients have reached endpoints, German CML Study Group, CML Study I.*[1]

**Table 1.** Prolongation of survival by IFN on the basis of randomized studies

| | *Median survival (months)* | | | *Significant difference?* | |
|---|---|---|---|---|---|
| | *IFN* | *HU* | *Bu* | *IFN vs. HU* | *IFN vs. Bu* |
| Italian group, 1994[2] | 72 | 52 | ND | Yes | ND |
| Hehlmann et al., 1994[3] | 66 | 56 | 45 | No | Yes |
| Allan et al., 1995[4] | 61 | 41 | 41 | Yes | Yes |
| Ohnishi et al., 1995[5] | 71 | ND | 52 | ND | Yes |

*ND, not done.*

advantage of IFN-treated over HU-treated patients of about 3 years is almost twice that observed in the total patient group (Table 2).

The induction of complete and durable cytogenetic remissions by IFN in a minority of patients,[7,8] in some instances lasting many years, has suggested that IFN might cure these patients. The long survival times of IFN-treated low-risk patients is supportive of such expectations. Although molecular studies have demonstrated residual leukemic cells in almost all patients with IFN-induced complete cytogenetic remissions,[11] in contrast to successfully transplanted patients,[12] patients with low BCR-ABL transcript numbers show a high probability of continued relapse-free survival, which might indicate operational cure.[8] By using very sensitive polymerase chain reaction (PCR) methods, low BCR/ABL transcript levels have been detected in a significant proportion of normal blood donors.[13,14] Figure 2 illustrates the quantitative differences between transcript levels after treatment with IFN or BMT and in healthy subjects. The sensitivity of the assay systems is of utmost importance for interpretation.

In spite of these optimistic results, the great majority of CML patients cannot be cured by drug treatment and require allogeneic BMT or new innovative therapies. Treatment intensification, e.g., with intensified chemotherapy or high-dose chemotherapy followed by autografting,[15–18] are promising options for these patients. The purpose of this report is to summarize rationale, outline, and state of recruitment of studies of the German CML Study Group that address intensive

**Table 2.** IFN-α in CML: long-term survival

| | | *Median survival (years)* | |
|---|---|---|---|
| | *Treatment* | *German study*[9] | *Italian study*[6] |
| All | IFN-α | 5.2 | 6.3 |
| | CHT | 4.7 (HU), 3.8 (Bu) | 4.3 |
| Low-risk | IFN-α | 9.5 | 8.7 |
| | CHT | 6.5 (HU), 6.0 (Bu) | 5.3 |

*Median observation time for the German study was 9 years; for the Italian study, 10 years.*

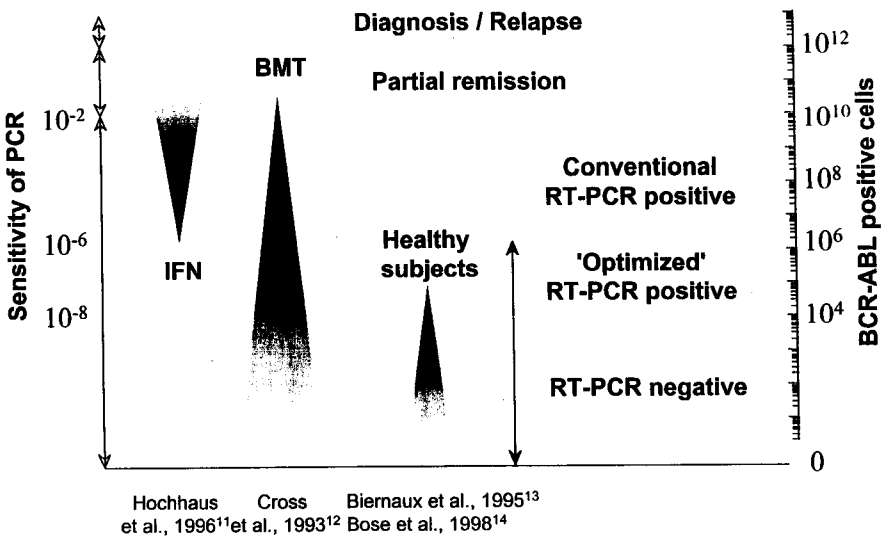


**Figure 2.** *Detection of BCR-ABL transcripts by RT-PCR in cytogenetic IFN responders, successfully transplanted patients and healthy subjects. The different detection levels are shown in relation to the sensitivity of the assays.*

treatment approaches with and without subsequent autografting and prospective comparison with allogeneic BMT.

## PATIENTS AND METHODS

The German CML Study Group started randomized trials in 1983 and to date has recruited more than 1850 CML patients for three consecutive randomized studies. The first study, CML Study I, recruited patients from 1983 to 1991 and compared survival after IFN, HU, and busulfan in a three-arm randomized trial. The results have been published and show that IFN and HU were both superior to busulfan, but that IFN was superior to HU only for low-risk patients.[1,3,9] The second study (CML Study II) recruited patients from 1991 to 1994 and compares the effects on survival of the combination of IFN and HU vs. HU monotherapy.[19] The study is up for final evaluation in 1999. In 1995, the third study (CML Study III) was started which analyzes the following:

1. the long-term impact of allogeneic bone marrow transplantation vs. the best available drug treatment on survival and
2. the survival outcome after intensification of treatment with idarubicin and intermediate-dose Ara-C vs. IFN/HU/low-dose Ara-C standard therapy.

Seven hundred fifty patients are to be recruited within 6 years.

**Table 3.** German CML studies as of December 1998

| | | |
|---|---|---|
| CML Study I: IFN vs. HU; IFN vs. busulfan | 1983–1991 | 701 patients |
| CML Study II: IFN + HU vs. HU monotherapy | 1991–1994 | 426 patients |
| CML Study III | Since 1995 | 598+ patients |
| 1. AlloBMT vs. IFN | | |
| 2. IFN vs. intensive CHT | | |
| CML Study III A: IFN vs. high-dose CHT with autografting | Since July 1997 | 138+ patients |
| Total | 1983–1998 | ~1900 patients |

In 1997, a third arm was started (CML Study III A) that analyzes the impact of high-dose chemotherapy followed by autografting and IFN maintenance on survival in comparison to the two arms with IFN standard therapy and with intensive treatment. An estimated 1000 patients are to be recruited and 160 autografted to reach conclusive results. The three studies and the patient recruitment by December 1998 are shown in Table 3.

## RESULTS

### Treatment intensification (CML Study III)

One therapeutic concept that underlies several ongoing randomized studies is based on the consideration that the reduction of clonal, genetically unstable cells should also reduce the rate of secondary genetic changes and thereby postpone blast crisis. According to this concept, the degree of reduction of tumor burden directly correlates with the prolongation of the chronic phase or survival. Several lines of evidence suggest that treatment intensification with further reduction of tumor burden correlates with survival in CML[20–22] (Table 4). In particular, addition of low-dose Ara-C to IFN significantly increases cytogenetic remission rates and apparently prolongs survival, as was shown by Guilhot in 1997[23] and more recently by Tura.[24]

In our group, treatment intensification is accomplished by the addition of intermediate-dose Ara-C (2× 100 mg/m$^2$/d for 5 days) and idarubicin (10 mg/m$^2$ on days 3 and 4) in those CML patients who are not candidates for allogeneic BMT and who have not achieved a cytogenetic IFN response. The study plan of CML Study III is shown in Fig. 3.

Of 21 evaluable patients, thus far, 14 (67%) show a cytogenetic response, mostly complete or partial ($n$=12), which is a very high rate considering the older age of this patient group and the lack of cytogenetic responsiveness to IFN. Final evaluation is expected in 2003.

**Table 4.** Correlation of intensity of treatment, reduction of tumor load and survival

- Survival of relapses after allo-BMT (Zhang et al., 1997[22])
- Interferon-α intensified by Ara C (Guilhot et al., 1997[23]; Tura, 1998[24])
- High dose CHT and autografting (McGlave et al., 1994[18]; Carella et al., 1996[25]; Goldman et al., 1996[26])
- Intensive chemotherapy, no maintenance (Cunningham et al., 1979[27]; Sharp et al., 1979[28]; Kantarjian et al., 1987[21])
- High-dose Hydroxyurea (Kolitz et al., 1992[29])

## High-dose chemotherapy followed by autografting (CML Study III A)

This approach represents a further intensification of treatment. It makes use of the observation that normal stem cells are present in CML,[25,26] particularly in the early phase of the disease, and can be mobilized by chemotherapy and growth factors.[17] First observations of normal progenitor cells in CML date back to the early 1970s and 1980s when cytogenetic mosaicism was observed after busulfan treatment and Philadelphia chromosome (Ph)-negative progenitor cells were detected in long-term culture (Table 5).

Favorable survival results after autografting have been observed in several studies (Table 6). The largest series is that by McGlave and coworkers,[18] which was updated in 1997. The update continues to show a 5-year survival of patients treated in chronic phase of more than 50%.[27] The more general feasibility of high-

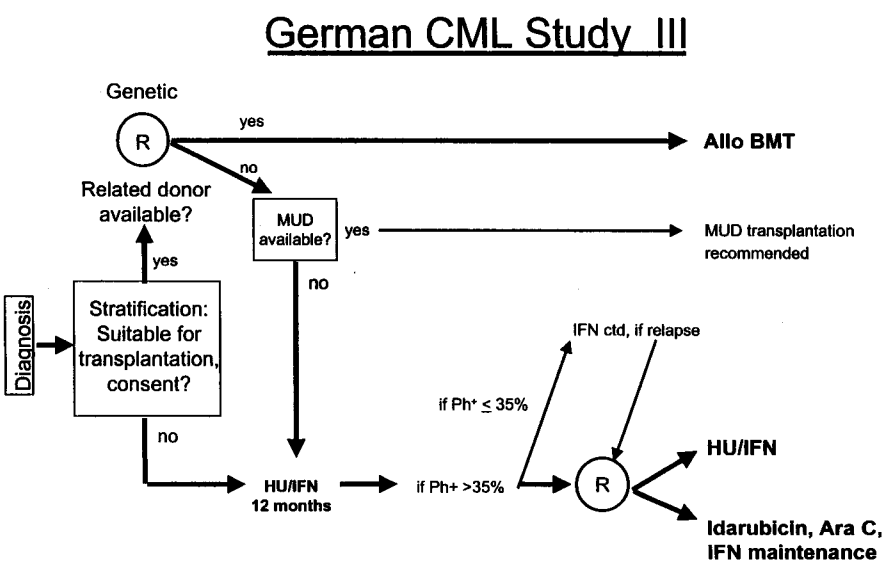


**Figure 3.** *Study plan of CML Study III.*

**Table 5.** Normal stem cells in CML

- Cytogenetic mosaics after busulfan treatment (Finney et al., 1972[30]; etc.)
- Ph-negative precursors in long term culture (Coulombel et al., 1983[31]; Verfaillie et al., 1992[32]; Barnett et al, 1994[33])
- Partial and complete cytogenetic remissions after intensive CHT (Cunningham et al., 1979[27]; Sharp et al., 1979[28]; Goto et al., 1982[34])
- Cytogenetic remissions after IFN-α (Talpaz et al., 1986[35]; Kantarjian et al., 1991[36]; and others)
- Ph-negativity after autografting with and without mobilization (Butturini et al., 1990[37]; Carella et al., 1993[17]; Simonsson et al., 1994[38])

dose chemotherapy and autografting has been shown,[28] and several randomized studies use this new intensive approach. The study scheme of CML Study III A is shown in Fig. 4. It involves one stratification and two randomization steps:

1. Determination of suitability for, and consent to, allogeneic BMT.
2. Genetic randomization according to donor availability between BMT vs. no BMT.
3. Randomization of IFN-based standard therapy vs. high-dose chemotherapy followed by autografting for non-BMT patients.

By December 1998, 138 patients had been recruited for CML Study III A, 34 were included in the second randomization step, and 16 were randomized for high-dose chemotherapy and autografting. Since treatment in CML appears to be the more effective the earlier during the course of the disease it is carried out (Table 7), high-dose chemotherapy and autografting are applied up-front as soon as it is clear that no donor is available and stem cells have been collected. The preferred mobilization treatment consists of idarubicin, Ara-C, and etoposide (mini-ICE).[29]

## How do BMT and drug treatment compare with regard to survival?

Until recently, the only potentially curative treatment option in CML was allogeneic BMT. Since IFN can also change the natural course of CML and prolong survival, the risks and limitations of BMT have to be critically considered. Transplantation-related early morbidity and mortality are serious problems. BMT-

**Table 6.** Favorable survival results with autografting

- Unmanipulated marrow (Goldman et al., 1981[16]; Hoyle et al., 1994[39])
- Ph-negative marrow induced with IFN and intensive CHT (Simonsson et al., 1993[15])
- Ph-negative peripheral stem cells mobilized with CHT (Carella et al., 1993[17])
- EBMT experience (Reiffers et al., 1994[40])
- 8-center study (McGlave et al., 1994[18]; update: Bhatia et al., 1997[41])

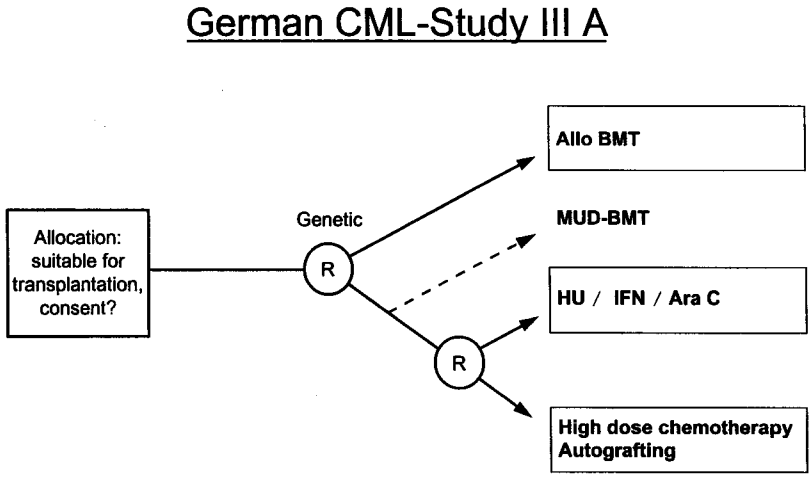


**Figure 4.** *Study scheme of CML Study III A.*

related mortality considerably exceeds mortality in IFN-treated patients during the first years after diagnosis. Only in the long term will BMT patients have a survival advantage. No studies exist thus far that prospectively quantify risks and chances of treatment by BMT vs. IFN. A simulation of possible outcomes was undertaken by the IBMTR and the German CML Study Group.[30] The results showed that a survival advantage for most BMT patients became significant after about 4–8 years. Only for low-risk IFN- or HU-treated patients a significant survival advantage of BMT was not observable by year 8.

A prospective controlled comparison therefore was started in 1995 as part of CML Studies III and III A. About 725 patients have been recruited for this comparison within the two studies by now, and about 150 have been transplanted. After a follow-up of about 2.5 years, IFN has a survival advantage, but it can be

**Table 7.** Superiority of early therapy in CML

- Allogeneic BMT, survival advantage after early BMT (Clift et al., 1993[43]; Goldman et al., 1993[44]; Clift and Storb, 1996[45])
- IFN-α superior in early CML (Alimena et al., 1988[46]; Talpaz et al., 1988[47]; Hasford et al., 1996[10])
- Mobilization of Ph-negative progenitor cells in early chronic phase (Carella et al., 1996[48])
- Chemotherapy, cytogenetic responses in (early) chronic phase (Cunningham et al., 1979[27]; Sharp et al., 1979[28])
- Intensified hydroxyurea treatment, cytogenetic responses in early chronic phase (Kolitz et al., 1992[29]; Johnson et al., 1996[49]; Coutinho et al., 1996[50])

**Table 8.** Survival of CML after BMT and IFN

| | *Proportion surviving* | | |
|---|---|---|---|
| | *3 years* | *5 years* | *10 years* |
| BMT (early) | 66–75% | 50–75% | 50–65% |
| IFN | | | |
| Low-risk | 95% | 75% | 45% |
| Intermediate- and high-risk | 80% | 50% | 20% |

*Data from Hansen et al.[52]; Tura et al.[6]; Appelbaum et al.[53]; van Rhee et al.[54]; German CML Study Group.[9,50]*

expected that the survival curves of IFN-treated and transplanted patients will cross soon. The observation period is still too short for a risk-stratified analysis.

## DISCUSSION

In summary, a considerable advance has been made with survival in CML after both BMT and drug treatment. Table 8 gives an overview of the survival rates after 3, 5, and 10 years based on published reports.[6,9,31,32] The perspectives for the future include new experimental strategies such as high-dose chemotherapy and autografting with early stem cell harvest as outlined above, evaluation of new active drugs, and the optimization of allogeneic BMT procedures. The German CML Studies III and III A are expected to provide contributions to some of these perspectives. Outcome of CML Study III A will depend on the feasibility of the study protocol in hematologic practice, on sufficient numbers of recruited and autotransplanted patients within a reasonable time period, and on availability and success of alternative treatment approaches. If recruitment proceeds as planned, the required patient number would be reached in 2003, and initial results based on surrogate markers (hematologic and cytogenetic responses) could be available in 2004.

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# The Tyrphostin AG957 Inhibits the In Vitro Growth of CD34+ Chronic Myelogenous Leukemia Progenitor Cells

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## ABSTRACT

The hallmark of chronic myelogenous leukemia (CML) is the Philadelphia (Ph) chromosome that fuses genetic sequences of the BCR gene with c-ABL sequences. The resulting BCR-ABL fusion proteins have a dysregulated protein tyrosine kinase (PTK) activity. Using Ph-positive cell lines, it has been shown that AG957, a member of the tyrphostin compounds, exerts a selective inhibition of $p210^{BCR\text{-}ABL}$ tyrosine phosphorylation. We report here that preincubation of CML or normal $CD34^+$ cells with a graded concentration of AG957 (1–100 μM) resulted in a statistically significant, dose-dependent suppression of colony growth from multipotent, erythroid, granulocyte-macrophage progenitors (CFU-Mix, BFU-E, CFU-GM) as well as the more primitive long-term culture-initiating cells (LTC-IC). AG957 doses causing 50% inhibition ($ID_{50}$) of CML and normal progenitors were significantly different for CFU-Mix (12 vs. 63 μM, $P \leq 0.008$), BFU-E (29 vs. 78 μM, $P \leq 0.004$), and CFU-GM (36 vs. 67 μM, $P \leq 0.02$). AG957 treatment resulted in significantly higher percentages of apoptotic cells (30 vs. 9%) in the BCR-ABL-transfected 32DLG7 cells compared with 32D-T2/93 cells (BCR-ABL-negative). Analysis of BCR-ABL mRNA on single progenitors by reverse transcription polymerase chain reaction (RT-PCR) revealed that preincubation with 50 μM AG957 significantly reduced the mean (± standard deviation [SD]) percentage of BCR-ABL positive progenitors (92 ± 11 vs. 32 ± 5%, $P \leq 0.001$). We conclude that *1*) AG957 inhibits in a dose-dependent manner CML CD34-derived colony formation by both primitive LTC-IC and committed CFU-Mix, BFU-E, and CFU-GM; *2*) this growth inhibition is associated with the selection of a substantial amount of BCR-ABL-negative progenitors.

## INTRODUCTION

CML is associated with a specific chromosomal abnormality known as the Philadelphia chromosome, which results from a reciprocal translocation between chromosomes 9 and 22 and fuses genetic sequences of the BCR gene on chromosome 22 with c-ABL sequences translocated from chromosome 9.[1,2] The resulting BCR-ABL gene generates fusion proteins with dysregulated PTK activity[3] and transforming activity for hematopoietic cells.[4]

Although considered with growing interest in CML, autologous stem cell transplantation (autoSCT) requires improvement either by purging of the leukemic stem cells or selection of the nonleukemic stem cells.[5] Selection strategies have focused either on in vivo[6,7] or in vitro[8–15] purging. Targeting the tyrosine kinase activity of BCR-ABL has been proposed as an attractive therapeutic strategy due to the potential for malignant transformation of this kinase activity.[16]

Inhibition of the BCR-ABL PTK activity has been obtained with nonselective compounds, such as genistein, as well as selective compounds, such as herbimycin A.[17,18] Recently, a selective inhibition of the BCR-ABL tyrosine kinase activity has been demonstrated with the 2-phenylaminopyrimidine derivative CGP57148B.[19,20] Tyrphostins represent an additional family of PTK inhibitors acting as competitive inhibitors of protein substrate and/or ATP binding.[21]

It was therefore the aim of the present study to investigate the differential effects of graded concentration of AG957 on the in vitro growth of CML and normal hematopoietic progenitors. The capability of AG957 to select in vitro for BCR-ABL-negative progenitors was analyzed by detecting the BCR-ABL mRNA on single progenitors by RT-PCR. We also studied the apoptotic effect of AG957 on a BCR-ABL-positive and -negative cell line.

## MATERIALS AND METHODS

### Patients

Eight patients (median age 46 years; range 38–61) with a diagnosis of Ph-positive CML in chronic phase were studied. Five patients were studied at diagnosis and before any treatment, whereas three patients had received hydroxyurea and/or IFN-α therapy for 4 to 16 months before being studied. All patients were 100% Ph-positive on direct cytogenetic analysis.

### Cell separation procedures

CML marrow cells were obtained by aspiration from the posterior iliac crest. Normal cells were obtained from healthy donors undergoing peripheral blood progenitor cell

mobilization. All patients and normal individuals provided informed consent for these studies. Mononuclear cells (MNCs) were separated by centrifugation on a Ficoll-Hypaque gradient (density 1.077 g/ml). $CD34^+$ cells were enriched according to a magnetic cell sorting methodology (MACS; Miltenyi Biotec, Germany).[22] Purity of CML and normal $CD34^+$ cell fractions ranged from 63 to 97% and 75 to 86%, respectively.

### Cell lines

32D-T2/93 (BCR-ABL-negative) and 32DLG7 (BCR-ABL-positive) cell lines[23] (kindly provided by Dr. A. Santucci, Hematology Department, Bologna, Italy) were used to investigate the apoptosis-inducing effect of AG957. Both cell lines were cultured in RPMI-1640 supplemented with fetal bovine serum (FBS) (10%, vol/vol) and L-glutamine (2 mM). Culture medium for 32D-T2/93 cells was also supplemented (10% vol/vol) with a conditioned medium of the WEHI-3 cell line as source of murine interleukin (IL)-3 .

### 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.

### LTC-IC assay

The long-term culture-initiating cell (LTC-IC) assay was performed according to Sutherland et al.[24]

### Cytogenetic analysis

Cytogenetic analysis and standard GTG- or QFQ-banding techniques were performed according to standard methods.[25]

### Detection of BCR-ABL mRNA in individual progenitors

Colonies were individually removed under an inverted microscope and processed for BCR-ABL mRNA detection according to a previously described procedure.

### *DNA fragmentation*

To investigate the capability of AG957 to trigger apoptosis, the BCR-ABL-transfected 32DLG7 cell line and its parental clone 32D-T2/93 were used. Nuclear

DNA fragmentation was detected by terminal deoxynucleotidyl transferase (TdT) assay.[26]

### AG957 treatment

AG957 was diluted in dimethylsulfoxide to prepare 1000-fold concentrated solutions that were used at a final concentration of 0.1% (vol/vol) to obtain the appropriate concentrations in culture. To evaluate the effect of AG957, $CD34^+$ cells ($1\times10^5$/mL) were exposed (30 minutes, 37°C, 5% $CO_2$) to either control medium (Iscove's modified Dulbecco's medium [IMDM], 10% FBS) or medium containing AG957 (1–100 μM). At the end of the incubation period, the cells were washed three times and cultured to quantitate CFU-Mix, BFU-E, CFU-GM, and LTC-IC. For each experiment, appropriate controls with vehicle alone (DMSO 1 μL/dish) were set up.

### 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, Calabasas, CA) run on a Macintosh 6300 personal computer (Apple, Cupertino, CA). The Student *t* test for paired or unpaired data (two-tail) or the Wilcoxon signed-rank test was used where appropriate to test the probability of significant differences between samples. AG957 concentrations resulting in 50% inhibition ($ID_{50}$) of colony formation were calculated for each experiment by extrapolating from a least square linear regression line relating AG957 concentration to the percentage of colony growth inhibition.

## RESULTS

### Effect of AG957 on CML and normal progenitors

As shown in Fig. 1, preincubation of CML $CD34^+$ cells ($n$=8) with AG957 (1–100 μM) resulted in a statistically significant ($P\leq0.04$ at least), dose-dependent suppression of colony growth from multipotent (Fig. 1A), erythroid (Fig. 1B), and granulocyte-macrophage (Fig. 1C) progenitors. Regression analysis showed that inhibition was linearly related to AG957 concentrations. A statistically significant and dose-dependent suppression of LTC-IC growth was also observed ($ID_{50}$ of LTC-IC growth was 43 ± 8 μM).

Preincubation of normal ($n$=4) $CD34^+$ cells with AG957 resulted in a dose-dependent reduction of colony formation (Fig. 1A–C), with AG957 toxicity being

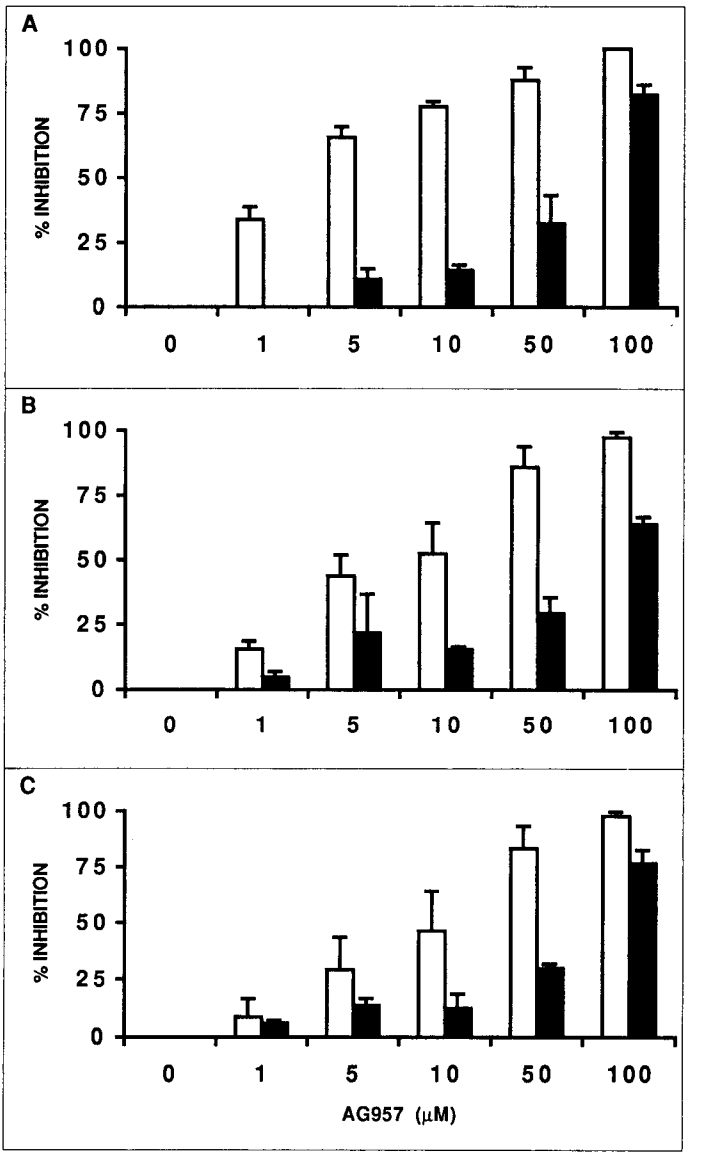


**Figure 1.** *Effect of AG957 on CML (q) and normal (n) CD34⁺-derived CFU-Mix (A), BFU-E (B), and CFU-GM (C). Each data point represents the mean (± SEM) percentage of inhibition from separate experiments using eight CML and four normal samples. When compared with control cultures (Wilcoxon signed-rank test), the inhibitory effect of AG957 on CML progenitors was statistically significant at the dose of 1 μM for CFU-Mix (P≤0.04) and BFU-E (P≤0.01) and at 5 μM for CFU-GM (P≤0.04). The inhibitory effect of AG957 on normal progenitors was statistically significant at the dose of 10 μM for CFU-Mix (P≤0.02) and BFU-E (P≤0.04) and at 50 μM for CFU-GM (P≤0.03).*

particularly evident when AG957 doses ≥50 μM were used. AG957 doses causing 50% inhibition of CML and normal progenitors were significantly different for CFU-Mix (12 vs. 63 μM, $P \leq 0.008$), BFU-E (29 vs. 78 μM, $P \leq 0.004$), and CFU-GM (36 vs. 67 μM, $P \leq 0.02$).

### DNA fragmentation

To investigate whether apoptosis was involved in AG957-induced inhibition of progenitor cell growth, two cell lines, namely 32D-T2/93 and 32DLG7, were treated with AG957 (0–100 μM, 12 hours), and apoptosis was analyzed by the TdT assay. On AG957 exposure, 9% of 32D-T2/93 cells (BCR-ABL-negative) and 30% of BCR-ABL-transfected 32DLG7 cells were in a progressive stage of apoptosis.

### BCR-ABL mRNA expression in single colonies

CML colonies were individually harvested and analyzed by RT-PCR for the expression of hybrid BCR-ABL mRNA. At the time of the study, all patients were 100% Ph-positive by standard cytogenetics. Preincubation with 1–10 μM of AG957 failed to reveal any antileukemic affect. In four of eight patients, AG957 at 50 μM significantly reduced the mean (± SD) percentage of BCR-ABL-positive progenitors (92 ± 11 vs. 32 ± 5%, $P \leq 0.001$).

## DISCUSSION

In the present study, we demonstrate that the PTK inhibitor AG957, a member of the tyrphostin family, inhibits in a dose-dependent manner, CML CD34-derived colony formation by primitive LTC-IC as well as committed CFU-Mix, BFU-E, and CFU-GM. The inhibitory effect of AG957 on CD34-derived colonies indicates that the antiproliferative action of AG957 does not involve accessory cells.

Analysis of AG957-induced apoptosis as well as comparison of $ID_{50}$ values for CML and normal CFU-Mix, BFU-E, CFU-GM reveal that BCR-ABL-positive clonogenic cells are more sensitive than BCR-ABL-negative clonogenic cells to the suppressive effect of AG957. However, concentrations of AG957 ≥50 μM are associated with a significant toxicity on normal progenitor cell growth. This loss of selectivity may be intrinsic to the mechanism of action of tyrphostins that are competitive inhibitors not only of protein substrate but also of ATP binding.

In four of eight patients, AG957 at 50 μM induced a substantial depletion of leukemic colonies. The failure of AG957 to select nonleukemic colonies, which was observed in half of the patients, may be explained by the rarity of BCR-ABL-negative progenitors which could be underscored when a limited number of colonies (on average, 20 to 30) was analyzed.

The in vitro selection for normal hematopoietic stem and progenitor cells from within CML marrow and the potential for using these cells as leukemia-free autografts has been the topic of increasing discussion. Data reported herein demonstrate the possibility to select nonclonal CML progenitors by means of a simple incubation with the PTK inhibitor AG957, thus suggesting that it may be feasible to select a population of benign progenitors from CML marrow which could be used to autograft patients without suitable allogeneic bone marrow donors.

## ACKNOWLEDGMENTS

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# CHAPTER 4
# LYMPHOMA

# Progressive Hodgkin's Lymphoma Following High-Dose Chemotherapy

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High dose chemotherapy (HDCT) with autologous hematopoietic support is an established treatment modality for relapsing and refractory Hodgkin's lymphoma. In a series of 93 patients at two centers, 37 have progressed at a median of 7 months following HDCT. Further treatment produced a "response" in 81% whose median survival was 13 months. No response was seen in 19%, and in this group median survival was 4 months. The therapies employed included local radiotherapy, single-agent chemotherapy (usually vinblastine or lomustine), or multiagent chemotherapy. Patients managed using a single sequential approach had a median survival of 8 months, with vinblastine and local radiotherapy being most reliable in producing a symptomatic response. Patients managed using multidrug chemotherapy had a median survival of 13.5 months with a low-dose continuous regimen comprising lomustine, chlorambucil, daily s.c. bleomycin, vinblastine, and methotrexate employed on an 8-weekly schedule. The regimen was well tolerated and appears efficacious. These results indicate the heterogeneity of management of relapse after HDCT at two major centers and present some argument for a more uniform approach.

# Late Non-Relapse Mortality After Autologous Stem Cell Transplantation in Patients with Hodgkin's Disease

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## ABSTRACT

As patients with Hodgkin's disease have been followed for longer periods of time after autologous stem cell transplantation (autoSCT), the importance of late nonrelapse mortality (NRM) is increasingly recognized. The initial 100 Hodgkin's disease patients treated in Vancouver with regimens containing high-dose cyclophosphamide, carmustine (BCNU) and etoposide (CBV) have now been followed for a median of 8 years (range 5.8–12.8). The probability of progression-free survival is 50% (95% confidence interval [CI] 39–60%). Eight nonrelapse deaths occurred "early" (≤day 100) and 9 occurred "late" (>day 100) after autoSCT, for a probability of overall NRM of 19% (95% CI 12–30%). Specific causes of late NRM included progressive pulmonary fibrosis (four patients), bacterial pneumonia (one patient), motor vehicle accident (one patient), and fatal secondary malignancies consisting of solid tumors (two patients) and myelodysplastic syndrome/acute myelogenous leukemia (MDS/AML) (one patient). Two additional patients developed secondary malignancies; one is alive after treatment for lymphoma, whereas a second MDS/AML patient succumbed due to recurrent Hodgkin's disease. These causes of late NRM are similar to those described in other series with relatively long follow-up data. Efforts are ongoing to define risk factors for these complications so as to develop preventative strategies.

## INTRODUCTION

Dose-intensive therapy and autoSCT produce durable progression-free survival (PFS) in a significant proportion of patients with Hodgkin's disease. Prolonged

survival is observed in 30–50% of patients with relapsed or refractory disease[1,2] and in 80–100% of those with putative high-risk features transplanted in an initial complete or partial remission.[3,4] Numerous reports have demonstrated that the main problems with autoSCT are disease recurrence and, to a much lesser extent, fatal nonrelapse mortality occurring relatively early after the procedure, i.e., ≤day 100. As patients have been followed for longer time periods, however, the importance of late complications and NRM is emerging.

The long-term single-institution results from Vancouver illustrate the impact of late NRM on the outcome of autoSCT in patients with Hodgkin's disease. The initial 100 patients in this center were autografted between 1985 and 1992, and the median follow-up is now 8.0 years (range 5.8–12.8).

## MATERIALS AND METHODS

### Patient characteristics

Patients were required to have recurrent or refractory Hodgkin's disease after chemotherapy, proven by biopsy or unequivocal radiologic progression. Additional eligibility criteria included age <60 years and major organ function >75% of normal. Patients were also required to have either a bone marrow biopsy negative for tumor prior to bone marrow harvest or an adequate peripheral blood stem cell (PBSC) collection. Patient characteristics are shown in Table 1. Most patients had received regimens such as MOPP/ABVD (mechlorethamine, vincristine, procarbazine, prednisone/doxorubicin, bleomycin, vinblastine, daccarbazine) regimens as

**Table 1.** Characteristics of 100 patients with progressive Hodgkin's disease treated with autologous transplantation

| | |
|---|---|
| Age | 28 (16–52) |
| Sex (M/F) | 58/42 |
| Initial chemotherapy | |
| MOPP/ABVD or variants | 73 |
| Other | 27 |
| Prior radiotherapy | 40 |
| Prior regimens | |
| At protocol entry | 1 (1–7) |
| At transplant | 2 (2–8) |
| Disease status | |
| First relapse | 63 |
| Induction failure | 63 |
| Advanced disease | 14 |

*Progression-free survival of 100 Hodgkin's disease patients undergoing autoSCT.*

**Table 2.** Treatment features of 100 patients undergoing autologous transplantation for Hodgkin's disease

| | |
|---|---|
| Conventional cytoreduction | |
| None | 12 |
| CT | 39 |
| CT + RT | 38 |
| RT | 11 |
| Conditioning | |
| CBV | 29 |
| CBVP | 71 |
| Stem cell source | |
| Marrow | 91 |
| Blood ± marrow | 9 |
| Posttransplant growth factors | 24 |

*CT, chemotherapy with MVPP; RT, involved field radiotherapy.*

initial therapy, and the majority of patients were in first relapse after combination chemotherapy at the time of entry into transplant protocols.

After protocol entry, 77 patients received brief conventional cytoreduction therapy with two cycles of MVPP (mechlorethamine, vinblastine, prednisone, procarbazine), with or without involved-field radiotherapy. Nine received involved-field radiotherapy alone before conditioning, whereas two were given radiotherapy after, rather than before, transplant. The median number of regimens before conditioning was 2 (range 2–7). Patients were not formally restaged after conventional cytoreduction; all patients receiving MVPP and/or radiotherapy proceeded to autoSCT.

These patients were conditioned with high-dose cyclophosphamide (7.2 g/m$^2$), BCNU (500–600 mg/m$^2$), and etoposide (2.4 g/m$^2$) with or without conventional-dose cisplatin (150 mg/m$^2$) (CBV or CBVP).[1,2] Steady-state bone marrow was the source of stem cells in the majority of patients, while post-autoSCT growth factors were available only for patients treated after 1990. Treatment features are summarized in Table 2.

All patients were hospitalized in single rooms with high-efficiency particulate air filtration. Blood products were irradiated and given to keep hemoglobin >90 g/L and platelet count $>20\times10^9$/L. Antibiotics, antiviral drugs, and antifungal agents were given as needed.

## RESULTS

At a median follow-up period of 8.0 years, PFS is 50% (95% CI 39–60%) (Fig. 1). The probability of relapse is 38% (95% CI 28–51%) (Fig. 2), and the risk of

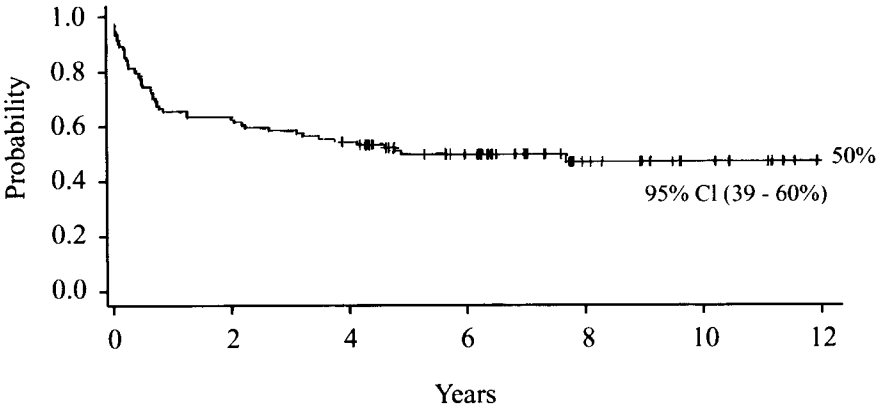


**Figure 1.** *Progression-free survival after autoSCT in 100 Hodgkin's disease patients.*

overall NRM is 19% (95% CI 12–30%) (Fig. 3). The specific causes of NRM are listed in Table 3. Eight early deaths (≤100 days) occurred: sepsis in one patient, regimen-related toxicity in five patients (two cardiac, two lung, one central nervous system), and miscellaneous causes in two patients (pulmonary hypertension in one and marantic endocarditis in one). NRM occurred later (>100 days) in nine patients. One patient succumbed to bacterial pneumonia 8 months after autoSCT, and another died in an automobile accident 52 months after autoSCT. Four died of progressive pulmonary fibrosis 4.5, 6, 24, and 24 months after autoSCT. Secondary malignancies occurred in five patients, at a median of 120 months (range 84–177) after diagnosis and 49 months (range 34–70) after autoSCT; the cumulative incidence of secondary malignancies was 9%.[5] Tumor types included glioblastoma multiforme in one patient

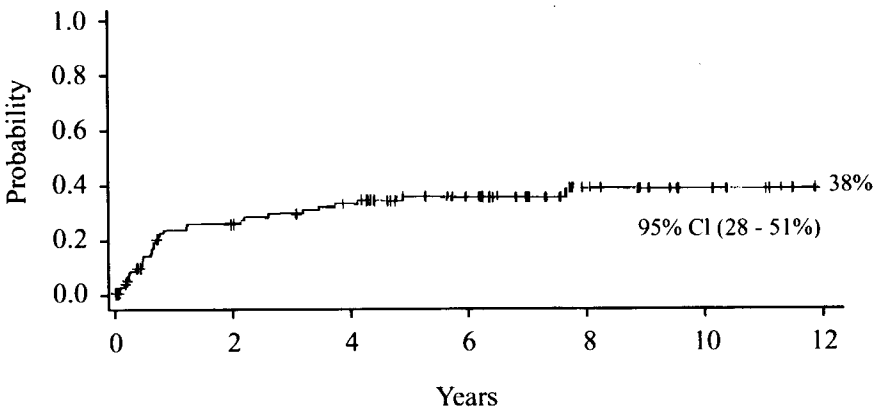


**Figure 2.** *Relapse rate after autoSCT in 100 Hodgkin's disease patients.*

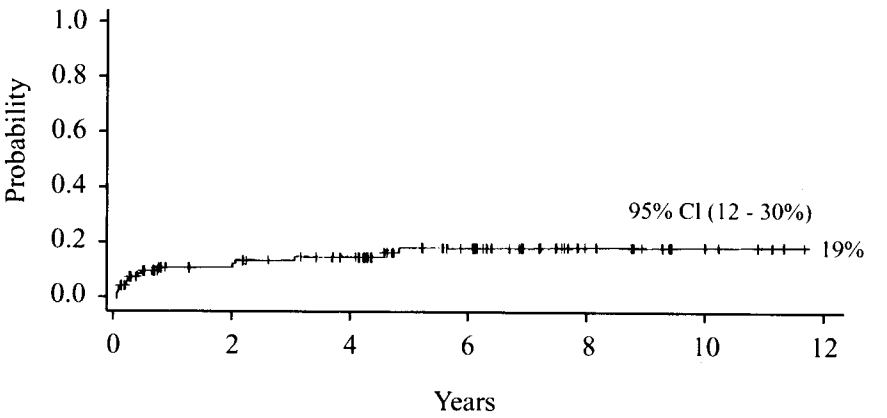


**Figure 3.** *Nonrelapse mortality rate after autoSCT in 100 Hodgkin's disease patients.*

at 34 months, colon cancer in one patient at 36 months, MDS/AML in two patients at 49 and 50 months, and lymphoma in one patient at 70 months after autoSCT. Secondary malignancies were the direct cause of death in three individuals with glioblastoma multiforme, colon cancer, and MDS/AML at a median of 52 months (range 37–59) after autoSCT; one other patient with MDS/AML died of recurrent Hodgkin's disease 58 months after transplant. The patient with lymphoma is alive and well after further chemotherapy 80 months after autoSCT.

## DISCUSSION

In this series of Hodgkin's disease patients treated with autoSCT, late deaths occurred predominantly due to pulmonary toxicity, infection, and secondary malignancies. These causes are similar to those reported by other centers with prolonged follow-up data.[6–9]

Among Hodgkin's disease patients undergoing autoSCT, pulmonary toxicity is most commonly reported in patients who have received BCNU-containing conditioning regimens, particularly when doses >450 mg/m$^2$ are used.[10] Among patients treated in Vancouver with CBV-based regimens containing BCNU doses of 500 or 600 mg/m$^2$, the incidence of all grades of lung toxicity has been 20%. Although the median onset was 57 days after autoSCT, the diagnosis has been made as late as 11 months after the procedure. BCNU-related pulmonary toxicity may also have an insidious clinical onset, and, in our hands, corticosteroid therapy immediately at the onset of even mild symptoms was required to reverse the syndrome (D.E.R., unpublished data). Fatal progressive pulmonary fibrosis occurred in four patients early in our experience; steroid therapy had not been instituted until the process was well established in all four.

**Table 3.** Causes of nonrelapse mortality after autologous transplantation in 100 patients with Hodgkin's disease

| | |
|---|---|
| Early (<day 100) ($n$=8) | |
| Sepsis | 1 |
| Regimen-related toxicity | 5 |
| Miscellaneous | 2 |
| Late (>day 100) ($n$=9) | |
| Pulmonary fibrosis | 4 |
| Secondary malignancy | 3 |
| Infection | 1 |
| Motor vehicle accident | 1 |

Prior exposure to nitrosoureas was the most important risk for the development of lung toxicity after autoSCT ($P$=0.001) in our series. Although prior chest irradiation has been associated with an increased risk in some studies,[11,12] our analysis did not find a significant association between radiotherapy and pulmonary toxicity ($P$=0.68) (D.E.R., unpublished data).

Few details are available regarding late fatal infections after autoSCT. However, a survey of transplant series indicates that the raw incidence may be as high as 3% in patients with Hodgkin's disease. Bacterial, viral, and fungal organisms have all been implicated.[6–9] The relative contributions of the known T cell defect in this disease[13] and of posttransplant immunodeficiency[14] are unknown at present.

Secondary malignancies represent one of the most ominous complications seen after autoSCT. Gisselbrecht et al. in France have recently reported the findings of a large registry analysis of Hodgkin's disease patients. At a median follow-up of 23 months, the cumulative incidence of secondary cancers after autoSCT was 8.9%,[15] comparable to our single-institution results in patients with longer follow-up. The incidence was similar for MDS/AML (4.3%; 95% CI 1.9–9.3%) and for solid tumors (3.7%; 95% CI 1.8–7.3%).[15]

Several other studies confirm that the cumulative incidence of secondary MDS/AML for Hodgkin's disease after autoSCT is, in general, from 5 to 10% at 5 to 10 years after transplant.[15–18] Secondary MDS/AML after autoSCT is typically seen 3–6 years after diagnosis and 0.8–4 years after autoSCT;[15–18] the interval from diagnosis is similar to that reported for MDS/AML after conventional therapy.[19] The French study included a matched analysis that compared the risk of MDS/AML after autoSCT vs. conventional therapy. The incidence was not significantly different in the two treatment groups.[15]

A number of risk factors for the development of MDS/AML after conventional therapy have been reported, including heavy alkylator use, combined modality therapy, and, in some studies, splenectomy.[19,20] Possible risk factors for MDS/AML

after autoSCT have been examined in five series (D. Milligan for the European Group for Blood and Marrow Transplantation [EBMT], personal communication).[15–17,21] Of note, two of these studies included non-Hodgkin's lymphoma patients,[17,21] and the variables examined were not the same in each series. Age >35–40 years was a risk factor in the EBMT and Minnesota analyses (D. Milligan, personal communication).[21] Splenectomy[15] and female sex (D. Milligan , personal communication) were also associated with a higher risk in single series. The risk was higher with the use of stem cells collected from the blood, rather than bone marrow, in three of these studies. Specifically, the relative risk was 5.8 ($P$=0.01) in the Minnesota study,[21] 3.73 ($P$=0.077) in the French registry study,[15] and 3.57 ($P$=0.07) in the City of Hope study.[17] The reason for this observation is currently not known, although patient selection may play a role. More information will be forthcoming from an Autologous Bone Marrow Transplant Registry-National Cancer Institute (ABMTR/NIH)study that evaluates post-autoSCT secondary MDS/AML in more than 2500 patients with either non-Hodgkin's lymphoma or Hodgkin's disease (C. Metayer for the NIH and ABMTR, personal communication).

Many types of solid tumors, as well as non-Hodgkin's lymphoma, have also been reported after autoSCT. The types of tumors we and others have observed overlap with those seen after conventional therapy[22–24] and include soft tissue sarcomas as well as breast, lung, gastrointestinal, and other malignancies. In contrast to MDS/AML, the French matched-comparison analysis showed that solid tumors were more common after autoSCT than after conventional therapy.[15] Although radiotherapy is a known risk factor for solid tumors after conventional therapy,[22–24] risk factors for this complication after autoSCT have not yet been identified.[15]

In conclusion, late NRM has emerged as a worrisome cause of treatment failure after autoSCT. The risk of fatal pulmonary fibrosis can likely be minimized by using lower BCNU doses in the conditioning regimen and by prompt initiation of steroids if symptoms develop. The risk of MDS/AML after autoSCT appears similar to that seen after conventional therapy, and the available clinical data suggest that marrow damage from prior cytotoxic therapy is likely the main factor for the development of this complication, although a role for other factors cannot be completely excluded. Further investigation of a possible increased risk of MDS/AML with the use of blood stem cell transplants is needed. Finally, the risk of secondary solid tumors appears to be higher after autoSCT compared with conventional therapy in Hodgkin's disease, and new strategies are required to minimize this problem.

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# CD34+ Selection of Hematopoietic Blood Cell Collections and Autotransplantation in Lymphoma: Overnight Storage of Product at 4°C Does Not Affect Outcome

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## ABSTRACT

We previously reported that storing mobilized peripheral blood progenitor cell (PBPC) collections overnight before CD34+ selection using the CellPro CEPRATE SC Stem Cell Concentration System may be associated with delayed platelet count recovery after high-dose chemotherapy and reinfusion (Koc ON, Gerson SL, Phillips GL, Cooper BW, Kutteh L, Van Zant G, Reece DE, Fox RM, Schupp JE, Tainer N, Lazarus HM: Autologous CD34+ cell transplantation for patients with advanced lymphoma: Effects of overnight storage on peripheral blood progenitor cell enrichment and engraftment. *Bone Marrow Transplant* 21:337–343, 1998). To investigate this issue, we undertook a randomized, three-center trial comparing overnight storage prior to CD34 selection (at 4°C) vs. immediate CD34 selection and cryopreservation of PBPC collections in 68 lymphoma patients undergoing autologous transplant. PBPCs were mobilized with cyclophosphamide 4 g/m$^2$ intravenously, prednisone 2 mg/kg/d orally for 4 days, and granulocyte colony-stimulating factor (G-CSF) 10 µg/kg/d subcutaneously. Thirty-four patients were randomized to overnight storage while 34 patients were assigned to immediate processing. Fifteen patients were excluded from the primary analysis of time to platelet recovery: three had tumor progression before autotransplant and 12 received unselected cells alone or in combination with selected cells since $<2\times10^6$ CD34+ cells/kg recipient weight were available for autotransplant. PBPCs from 23 patients were stored overnight, and PBPCs from 30 patients underwent immediate CD34 selection and cryopreservation. Groups did not differ with regard to

age, sex, diagnosis, or performance status. Neutrophil recovery >500/μL occurred a median of 11 days (range 9–16) in the overnight storage group compared with 10.5 days (range 9–21) in the immediate processing group ($P$=0.421). Similarly, platelet transfusion independence did not differ significantly between the two groups: 13 (range 7–43) days in the overnight storage group vs. 13.5 (range 8–53) days in those assigned to immediate processing ($P$=0.933). Storage of PBPC overnight at 4°C did not affect the number of $CD34^+$ cells available for selection, the number of leukapheresis procedures necessary to reach $2\times10^6$ $CD34^+$ cells/kg, or the percentage of $CD34^+$ cells in the enriched product after collection. Storage of PBPCs overnight at 4°C may allow the combining of multiple-day collections, thereby decreasing costs associated with $CD34^+$ cell selection.

## INTRODUCTION

Autologous hematopoietic progenitor cell transplantation is used increasingly as a form of anticancer therapy.[1] In recent years, hematopoietic progenitor cells have been collected more often from blood than bone marrow, since hematopoietic reconstitution appears faster.[2] Although one study reported that in lymphoma patients there was less than a 1 log difference between blood and marrow sources for number of tumor cells contaminating the graft, several publications note that blood stem cell grafts contain significantly fewer malignant cells than bone marrow.[3–7] Transplantation of these neoplastic cells carries the risk of infusing sufficient numbers of clonogenic cells to cause relapse.[5,6,8,9]

Many techniques have been developed to eliminate unwanted malignant cells in the autologous graft, but such techniques often are associated with substantial loss of hematopoietic stem and committed progenitor cells, leading to delayed bone marrow recovery.[10,11] Alternative approaches include the selection of $CD34^+$ cells, which has been reported to reduce the number of contaminating tumor cells by 1–3 logs in autografts of patients with non-Hodgkin's lymphoma,[12] multiple myeloma,[13] and breast cancer,[14] while still providing for an engraftment rate comparable to unselected autografts.[15,16] Since lymphoma cells do not express the CD34 surface marker, CD34 selection of autografts may provide a feasible method to improve the outcome of relapsed lymphoma patients.[17]

We previously investigated the use of a CD34 immunoaffinity column (CEPRATE SC; CellPro, Bothell, WA) for positive selection of $CD34^+$ cells from the peripheral blood of patients with advanced lymphoma.[18] Median times to neutrophil and platelet engraftment were rapid when the leukapheresis was processed on the same day it was collected, at 11 and 13 days, respectively. In an effort to optimize usage of the immunoaffinity columns, leukapheresis products for 11 patients were stored overnight at 4°C before processing. Although overnight storage did not affect neutrophil engraftment (median 11 days, range 11–14), platelet engraftment was

significantly delayed (median 28.5 days, range 12–39, $P$=0.02). This effect was observed even in patients who received >2.0×$10^6$ $CD34^+$ cells/kg.

Since CD34 selection may be a beneficial technique, especially if costs can be minimized, we concluded that the effects of overnight storage should be further evaluated.[19] We designed a randomized study to investigate whether overnight storage has an effect on the $CD34^+$ immunoaffinity procedure and platelet engraftment.

## MATERIALS AND METHODS

### Study population

Patients (14–65 years) with relapsed, primary refractory (induction-failure), or high-risk non-Hodgkin's lymphoma or Hodgkin's disease and who were eligible for high-dose cytotoxic therapy were considered for entry to this multicenter, phase III study.[20] The study was conducted under an Investigational Device Exemption from the Food and Drug Administration (FDA) and approved by the Institutional Review Boards at the University Hospitals of Cleveland, Cleveland, OH; Hackensack Medical Center, Hackensack, NJ; and University of North Carolina, Chapel Hill, NC. All patients gave written informed consent to participate.

Patients were required to have an Eastern Cooperative Oncology Group (ECOG) status of 0 or 1 and were required to have adequate visceral organ function including left ventricular ejection fraction at least 45% of predicted, no uncontrolled hypertension, forced expiratory volume in 1 second ($FEV_1$) and DLCO >50% of predicted, actual or calculated creatinine clearance >60 mL/min, SGOT and SGPT less than three times normal, and no severe endocrine or neurologic disorders. At the start of mobilization therapy, a blood neutrophil count >1200/μL and platelet count >100,000/μL were required.

Patients were excluded if they had more than two prior chemotherapy regimens; prior radiation in excess of defined amounts (1500–4000 cGy depending on site); cumulative exposure to carmustine >200 mg/$m^2$, bleomycin >100 units/$m^2$, or doxorubicin >550 mg/$mg^2$; evidence of active infection; or a history of another malignant disease within the past 5 years. Patients were not excluded for evidence of tumor on routine histologic staining of bilateral paraffin-embedded posterior iliac crest bone marrow biopsies.

Sixty-nine patients were registered between November 1996 and January 1998. Of these patients, 68 proceeded with mobilization and leukaphereses; one patient died before mobilization. Fifteen of the 68 patients were excluded from the analysis: three were not transplanted (one was found before transplant to have progressive disease and two died before transplant), and 12 patients later underwent autotransplant using unselected cells alone or a combination of selected and unselected cells since they had <2.0×$10^6$ $CD34^+$ cells/kg.

## Mobilization and collection of PBPCs

Patients began PBPC mobilization after registration, using a regimen reported previously.[18] The mobilization regimen consisted of cyclophosphamide (4.0 g/m$^2$) intravenously over 3–6 hours on the first day of mobilization along with 3.0 g/m$^2$ mesna within the cyclophosphamide dosing bag, then 500 mg every 3 hours by mouth or by vein for eight doses. Prednisone (2 mg/kg/d) was given orally for the first 4 days of mobilization. G-CSF (Amgen, Thousand Oaks, CA) (10 μg/kg/d) was given subcutaneously beginning between 36 and 48 hours after the completion of cyclophosphamide therapy and continuing until the last day of leukapheresis or until the white blood cell (WBC) count was ≥75,000/μL.

On return of blood neutrophils >1000/μL and platelet count >30,000/μL (usually 12–15 days after cyclophosphamide therapy), PBPCs were collected using standard leukapheresis procedures (COBE SPECTRA, Lakewood, CO, or a similar machine). The target for the collection was to process 10–15 liters of whole blood (or three patient volumes). Leukapheresis continued for a maximum of 4 days until a combined minimum collection of $\geq 10.0 \times 10^8$ mononuclear cells/kg was achieved. Processing of the collection using the CEPRATE SC System was to yield a combined minimum of $2.0 \times 10^6$ total CD34$^+$ enriched cells/kg patient weight. If either of these minimum numbers was not reached in four or fewer leukaphereses, additional leukaphereses or alternative treatments were to be determined by the site investigator and the principal investigator.

## Overnight storage

To evaluate the effect of overnight storage, patients were randomly assigned to the overnight storage or immediate-processing group at the initiation of the leukapheresis procedures. Leukapheresis products obtained from patients in the overnight storage group were maintained overnight at 4°C, while leukapheresis products procured from patients in the immediate group were processed using the CEPRATE SC System the same day. All subsequent collections for each patient were processed in the same fashion. None of the leukapheresis products were combined with the next leukapheresis product for CD34 selection.

## Positive selection of CD34$^+$ cells from mobilized PBPCs

Harvested mononuclear cells from each leukapheresis collection were prepared and passaged over the immunoaffinity column device (CEPRATE SC System) as directed by the manufacturer (CellPro, Bothell, WA). Adsorbed CD34$^+$ cells were resuspended at $2 \times 10^7$ cells/mL and frozen using a controlled-rate liquid nitrogen freezer in the presence of 7.5% (final concentration) dimethylsulfoxide (DMSO)

(Sigma, St Louis, MO). Aliquots of the unprocessed PBPCs enriched for $CD34^+$ cells and nontarget (unbound) cells were analyzed for progenitor cell content by clonogenic assays and for $CD34^+$ surface markers using flow cytometry.

## Flow cytometry

Each site used institutional protocols for flow cytometry for enumerating $CD34^+$ cell number.

## High-dose chemotherapy and stem cell support

The protocol required administration of a high-dose BCNU (carmustine)-containing chemotherapy regimen. It was suggested that the preparative regimen described by Lazarus et al. be used.[21] Each site used a different regimen[21–23]; however, all patients at each site received the same regimen. Involved-field radiation therapy was given to active/previously bulky tumors (>5 cm) as 1500 to 2000 cGy in 200-cGy fractions in selected patients.

All days referred to herein were calculated from the day of $CD34^+$ cell infusion (day 0). Starting the first day after PBPC infusion (day 1), G-CSF was administered subcutaneously in single or divided injections at a dose of 10 μg/kg/d until the patient's blood absolute neutrophil count was ≥1000/μL for three consecutive days. Platelet engraftment was defined as the first of seven consecutive days that platelet count was ≥20,000/μL without transfusion support for low counts and no platelet count <20,000/μL for six days after that day.

## Supportive care

All patients received multilumen, indwelling central venous pheresis catheters and were cared for in single hospital rooms. Antibiotics were given prophylactically or empirically for fever and neutropenia according to the guidelines for each participating institution, and all patients were supported with irradiated blood components. Irradiated, packed red blood cell (RBC) transfusions were given in an attempt to keep the hematocrit >25%, and irradiated platelet transfusions were given for platelet counts less than 10,000–20,000/μL or bleeding complications. Cytomegalovirus (CMV)-negative blood products were given to CMV-seronegative patients.

## Statistical methods

Unless otherwise stated, the statistical analyses presented are for the patients who received an autograft of CD34 selected cells only.

Missing data were not estimated or carried forward in any statistical analyses. The analysis of the time to platelet engraftment used a one-sided test. For the additional end points, the between-group comparisons used two-tailed test procedures. A significance level of 0.05 ($\alpha$=0.05) was used for all analyses. The null hypothesis of interest for all analyses was that there was no difference between the study groups. The study groups were compared with respect to age and the processing data using two-sample *t* tests. The Cox proportional hazards regression model was used to analyze the engraftment data. Fisher's exact test was used to analyze sex and performance status.

Analyses based on the $\chi^2$ test for homogeneity of proportions were performed only if the counts in all cells of the frequency table represented at least 5% of the patients included in the analysis. If any of the counts were lower than this value, Fisher's exact test was used.

## RESULTS

### Patient characteristics

The clinical characteristics of the study patients who were included in the analyses are summarized in Table 1. There were no significant differences between the study groups in terms of sex, age, or ECOG performance status.

### Characterization of peripheral blood CD34+ cells and results of positive selection

Seventy-four leukapheresis products (collected from 30 patients) processed the same day as the leukapheresis procedure were compared with 62 leukapheresis

**Table 1.** Patient characteristics

| | *Immediate processing (n=30)* | *Overnight storage (n=23)* | *P value* |
|---|---|---|---|
| Sex | | | 1.000 |
| Male | 15 (50%) | 12 (52%) | |
| Female | 15 (50%) | 11(48%) | |
| Age (years) | | | 0.680 |
| Median | 50 | 44 | |
| Range | 22–65 | 18–66* | |
| ECOG rating | | | 1.00 |
| 0–1 | (n=28) 24 (86%)† | 19 (83%) | |
| 2 | 4 (14%) | 4 (17%) | |

**One patient was age 65 years at study registration, but was age 66 years at time of transplant.*
*†n=28; two patients were evaluated using Karnofsky performance test and had 90% ratings.*

**Table 2.** Effect of overnight storage on PBPCs before and after $CD34^+$ selection

| | *Mean ± SD or median (range)* | | |
|---|---|---|---|
| | *Immediate processing* | *Overnight storage* | *P value* |
| Before $CD34^+$ cell selection | | | |
| Nucleated cells $\times 10^8$/kg | 7.8 (3.3–19.3) | 6.7 (3.0–22.1) | 0.659 |
| % $CD34^+$ cells | 2.0 ± 2.2 | 2.1 ± 2.6 | 0.815 |
| $CD34^+$ cells $\times 10^6$/kg | 10.9 (1.5–136.1) | 11(0.1–97.8) | 0.812 |
| After $CD34^+$ cell selection | | | |
| Nucleated cells $\times 10^6$/kg | 5.5(2.8–15.5) | 7.9 (1.6–16.3) | 0.473 |
| % $CD34^+$ cells | 66.3 ± 19.7 | 60.0 ±18.4 | 0.063 |
| $CD34^+$ cells $\times 10^6$/kg | 3.8 (1.3–12.1) | 4.1 (1.5–13.5) | 0.847 |
| Enrichment | 55.7 (6.0–799) | 52.9 (5.9–159.9) | 0.315 |
| Yield | 43.6 (0.2–1205) | 39.1 (8.6–80.3) | 0.339 |

*Data shown are for 30 patients (74 leukaphereses) in the immediate processing group and for 23 patients (62 leukaphereses) in the overnight storage group. The number of nucleated cells and $CD34^+$ cells are the results of the total of all leukaphereses administered to the patients. Results for purity, enrichment, and yield are presented for each product processed. Because of missing data, the n for some cells does not equal the number of patients or the number of leukaphereses.*

products (obtained from 23 patients) stored overnight. After storage and before selection, the number of mononuclear cells, percent $CD34^+$ cells, and total number of $CD34^+$ cells in the product did not differ significantly between groups (Table 2, $P \geq 0.659$). After selection, the number of mononuclear cells, percent $CD34^+$ cells, and the total number of $CD34^+$ cells in the product, as well as the enrichment and yield of the selection process, were similar for those patients whose product was stored overnight compared with patients whose product was not stored overnight (Table 2, $P \geq 0.063$).

## Hematopoietic engraftment by enriched peripheral blood $CD34^+$ cells

Hematopoietic engraftment also did not differ significantly for patients whose leukapheresis products were stored overnight compared with those patients whose collections were processed immediately. Blood neutrophil count reached 500/μL in a median of 10.5 days (range 9 to 14) after immediate processing compared with 11 days (range 9 to 16) after overnight storage ($P$=0.421). Platelet count exceeded 20,000/μL for 7 consecutive days without a transfusion in a median of 13.5 days (range 8 to 53) after immediate processing compared to 13 days (range 7 to 43) after overnight storage ($P$=0.933, Table 3). Delay in platelet engraftment (over 21

**Table 3.** Effect of overnight storage of PBPC collections on engraftment after reinfusion

| | *Median (range)* | | |
|---|---|---|---|
| | *Immediate processing (n=30)* | *Overnight storage (n=23)* | *P value* |
| Median day neutrophils ≥500/μL | 10.5 (9–14) | 11 (9–16) | 0.421 |
| Median day platelets ≥20,000/μL | 13.5 (8–53) | 13 (7–43) | 0.933 |

*Platelet recovery was defined as the first of 7 consecutive days of sustained platelet count ≥20,000/μL without the need for platelet transfusions.*

days) was observed in seven patients, four whose product was processed immediately and three whose product was stored overnight. In addition, there was one patient in the overnight storage group for whom time to engraftment was censored since death occurred at day 30, prior to engraftment.

Four patients, two in each study group, were given high-dose chemotherapy and underwent reinfusion with a CD34-selected cell dose $<2.0\times10^6$ $CD34^+$ cells/kg. Neutrophil engraftment occurred by day 12 and platelet engraftment on day 14 for two of these patients, one in each group of the study. A third patient, whose products were processed immediately, experienced neutrophil and platelet recovery on days 21 and 38, respectively. The remaining patient died before engraftment, as described above.

There were no significant differences between the study groups in the number of platelet transfusions administered from day 0 to day 100 ($P$=0.704) (mean 6.6 after immediate processing vs. mean 5.1 after overnight storage). Furthermore, the arms were comparable in the percent of patients with platelet transfusions after platelet recovery (20% after immediate processing and 17.4% after overnight storage).

No data were collected on infections, bleeding episodes, or duration of hospitalization and rehospitalization. No adverse events were reported as related to the use of the CEPRATE SC System. Data on survival and progression-free survival are too immature to report at this time.

## DISCUSSION

High-dose chemotherapy and stem cell transplantation have significantly improved the outcome for patients with relapsed lymphoma.[24] More than half of the patients, however, will eventually die of their disease. Given these statistics, various strategies to improve outcome, including purging of the autograft, should be pursued. The clinical efficacy of purging is extremely difficult to demonstrate given the large number of patients required in a randomized trial to demonstrate a

difference. Furthermore, in patients with leukemia and lymphoma there is a positive relationship between the patient's tumor burden and the amount of contamination in the autograft.[25] Despite these difficulties, it is important to continue to study the role of purging in the overall outcome of the patients. This is especially true if the means to accomplish purging are relatively simple, there is no clinically significant effect on engraftment, and the costs can be minimized.

We reported previously the successful use of autologous $CD34^+$ enriched peripheral blood mononuclear cells for hematopoietic reconstitution after myeloablative chemotherapy in patients with advanced lymphoma.[18] $CD34^+$ cells selected using the CEPRATE SC System resulted in rapid engraftment of hematopoietic function at a tempo similar to studies in which unselected PBPC were given. On the other hand, it appeared that efforts to optimize the use of labor and the number of immunoaffinity columns might be hampered, since overnight storage of the product before CD34 cell selection appeared to have a negative effect on cell yield (mean 2.4-fold fewer $CD34^+$ cells reinfused) and increased the time to platelet engraftment as well.

These results, however, were inconsistent with previous reports. Storage of PBPC for periods up to 5 days has been reported to have minimal effects on cell count, cell viability, and granulocyte-macrophage colony-forming units (CFU-GM).[26–29] More importantly, overnight storage before processing had been reported to have no effect on platelet recovery after transplant.[30,31] Storage of the PBPC product overnight before processing allows schedule flexibility as well as using fewer immunoaffinity columns.

We designed this prospective, randomized study to systematically investigate the apparent negative effect of overnight storage in a larger population. We demonstrated that overnight storage did not adversely affect neutrophil or platelet engraftment after high-dose chemotherapy. Furthermore, the overnight storage did not affect the cell yield after the selection procedure.

In our previous nonrandomized study, we observed a loss of the association between the number of $CD34^+$ cells infused and platelet engraftment in patients whose collections were stored overnight.[18] In this study, only five patients, evenly distributed between the two groups of the study, experienced a delay in platelet engraftment.

Twelve patients excluded from the analysis clearly were "poor mobilizers," a common problem noted in lymphoma patients.[32,33] This group, however, subsequently underwent high-dose chemotherapy and autotransplant using either unselected PBPC or a combination of selected and unselected cells.

In conclusion, $CD34^+$ cells can be obtained and enriched from the peripheral blood of heavily pretreated lymphoma patients. Cells can be stored overnight before processing to improve the efficiency of the laboratory without having an impact on patient engraftment.

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# The European CUP/UP Trial: A Preliminary Analysis of Autologous Transplantation for Relapsed Follicular Non-Hodgkin's Lymphoma

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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 of 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. Because of insufficient accrual, the trial was closed in 1997. The design of the study and the first interim results are presented.

## INTRODUCTION

About one-third of all patients with NHL have a follicular histology. The majority of these patients have stage III or IV disease at diagnosis and a median age of >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.[2–15] Randomized studies have not been done. 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 have frequent bone marrow infiltration. Therefore, there are some arguments that if a high-dose therapy is considered in the treatment plan of a patient, purging of the graft may be necessary. The first results from

Gribben et al.,[16] although not obtained from a controlled randomized study, do support purging.

To answer the questions on the efficacy of high-dose therapy and purging in patients with follicular non-Hodgkin's lymphoma, 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).

## PATIENTS AND METHODS

Patients were treated with three cycles of chemotherapy. The choice of chemotherapy was free, although CHOP chemotherapy was recommended. In case of a response after three cycles of chemotherapy, defined as at least partial response according to standard criteria and minimal B cell infiltration of the marrow (<20% B lymphocytes), patients were eligible for randomization. From the beginning patients were randomized between three arms (CUP trial), but starting June 1996, patients and doctors also had the choice for a two-arm randomization (transplantation with purged or unpurged stem cells [UP trial]) (Fig. 1).

Stem cells (either blood or bone marrow) were collected as soon as possible after randomization.

Patients randomized to the purging arm had their stem cells purged with a cocktail of anti-CD19, anti-CD20, anti-CD22, anti-CD23, and anti-CD37 plus immunomagnetic beads[17] (supported by Baxter Immunotherapy).

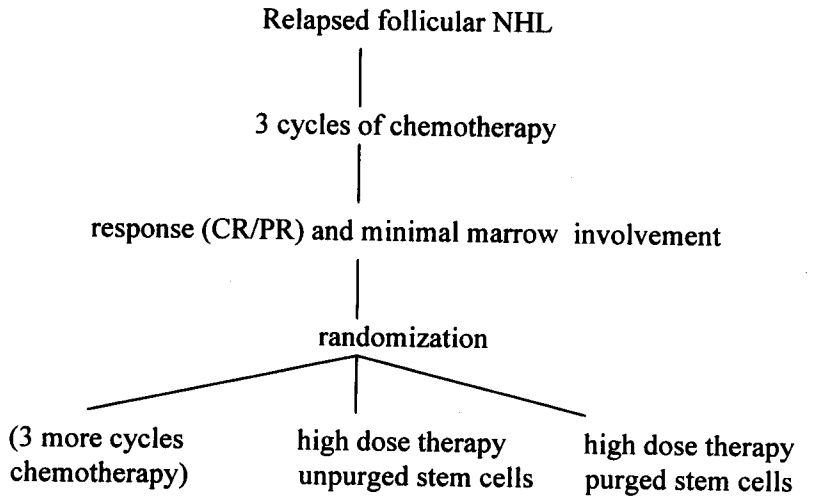


**Figure 1.** *Design European (C)UP trial*

**Table 1.** Patient characteristics at entry

| | |
|---|---|
| Sex | |
| Male | 60% |
| Female 40% | |
| Age (years) | 48 |
| Time from diagnosis to entry | 26 months |
| WHO 0 | 70.8% |
| LDH elevated | 66% |
| Marrow aspirate >20% B-lymphocytes | 57.1% |
| Marrow trephine positive | 60.9% |
| B symptoms | 20% |

Patients randomized to the chemotherapy arm were treated with three more cycles of the same chemotherapy. Patients in the stem cell transplantation arms were treated with high-dose cyclophosphamide (2× 60 mg/kg) followed by TBI and stem cell transplantation.

The trial aimed for 100 patients in every randomization arm. However, because of poor accrual the trial was closed for entry in April 1997.

## RESULTS

A total of 140 patients were entered in the study of whom 89 fulfilled the criteria for randomization. The baseline characteristics are shown in Table 1. Twenty-four patients were randomized to the chemotherapy arm, 33 were transplanted with unpurged stem cells, and 32 with purged autologous stem cells.

With a median follow-up of 30 months after randomization and complete data on 72% of the patients, we did an interim analysis. The results are shown in Table 2.

## DISCUSSION

This is the only randomized trial currently ongoing in patients with relapsed follicular non-Hodgkin's lymphoma assessing the value of high-dose therapy and

**Table 2.** Outcome

| | *Progression/death* | *Survival* |
|---|---|---|
| Chemotherapy | 50% | 87% |
| Purged transplant | 78% | 97% |
| Unpurged transplant | 81% | 85% |
| Not randomized | 67% | 73% |

purging. 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 after the general implementation of peripheral blood stem cells with decreased morbidity and mortality. This has had a great impact on the accrual of patients in this trial, finally resulting in the premature closing of entry.

With current follow-up, which indisputably is too short for this low-grade disease, the results suggest an improved relapse free survival rate in the transplanted patients, not yet reflected in improved survival.

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# Local and Regional Recurrence After Autologous Stem Cell Transplantation for Non-Hodgkin's Lymphoma

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## ABSTRACT

Data from two populations of patients receiving stem cell transplantation (SCT) for non-Hodgkin's lymphoma (NHL) were analyzed for patterns of relapse after transplantation, including time to relapse, the frequency of local vs. distant relapse, and survival after relapse. Group A comprised 106 patients receiving high-dose therapy and autologous SCT at the University of Southampton, U.K. Group B comprised 1339 patients with NHL relapsing after SCT and with documented sites of relapse recorded on the lymphoma registry of the European Group for Blood and Marrow Transplantation (EBMT). In group A, of the 106 patients undergoing autoSCT, 91 had diffuse large B cell NHL (including 13 with transformed follicular lymphoma); 57 (54%) relapsed at a median of 9 months following SCT; 36 (63%) of these relapsed in local/locoregional sites, 15 (27%) in distant sites, and six (10%) at both local and distant sites. The median overall survival (OS) from the date of relapse was 3 months. No difference in OS was seen according to the site of relapse. In group B, of the 1339 patients, 187 had low-grade NHL, 393 intermediate grade, 245 lymphoblastic lymphoma (LBL), 75 Burkitt's or Burkitt-like (BL), and 439 other high-grade subtypes. Two hundred fifty-nine (19%) of patients relapsed at local/locoregional sites, and 1080 at distant sites ± sites of previous involvement. Local relapse was more frequent in patients with low-, intermediate-, or other high-grade disease, compared with LBL or BL histology (21 vs. 22 vs. 20 vs. 12 vs. 15%, respectively, $P$=0.016), in patients with single-site disease before SCT compared with those with multiple sites ($P$<0.0001), and in patients with resistant or untreated relapse compared with those with chemosensitive relapse ($P$<0.0001). Median time to relapse was shorter in patients with local vs. distant relapses ($P$<0.0001). The source of stem cells (autologous bone marrow

vs. autologous peripheral blood progenitor cells vs. allogeneic bone marrow) and purging/positive stem cell selection had no effect on the frequency of local relapse. Median survival after relapse was 15 months, and was inversely correlated with histologic grade ($P<0.000001$). No difference in survival was seen according to local vs. distant relapse. These results confirm the poor outlook for all patients relapsing after SCT for NHL, irrespective of the pattern of relapse. Patterns of relapse after SCT are related to clinical features of the disease before autoSCT rather than to treatment variables. New strategies are urgently required to improve the poor outlook for this group of patients.

## INTRODUCTION

High-dose therapy and autoSCT is a curative treatment modality in some patients with relapsed, aggressive NHL.[1] Its role as a component of first-line therapy in these diseases is the subject of recently completed and ongoing randomized trials.[2,3] Its effectiveness in indolent lymphomas remains uncertain, although evidence from single institution and registry-based studies suggests that it may induce longer remissions than those achieved with conventional dose therapy.[4,5]

Although very few published data exist, the outcome for patients who relapse after autoSCT appears to be very poor. In a report from the University of Nebraska Medical Center, the median survival from progression after autoSCT for patients with NHL was only 3 months, with very few patients surviving beyond 3 years.[6] Attempts at further curative therapy in these patients have included the use of cytokines such as interferon, monoclonal antibodies, and further high-dose therapy using either autologous or allogeneic stem cell transplantation. Although preliminary data suggest that allogeneic transplantation may produce long-term disease-free survival (DFS) in a small proportion of these patients, most die rapidly from recurrent disease.

Only limited data are available on patterns of relapse after high-dose therapy and autoSCT in NHL. Some series, primarily in patients with low-grade lymphoma, have reported a tendency for recurrence to occur at previously involved sites.[7] However, similar data have rarely been reported for aggressive NHL, and the influence of the site or sites of relapse on subsequent outcome has not been investigated.

We have investigated patterns of relapse after high-dose therapy for NHL in a series of patients presenting to one institution over an 8-year period, and in a separate population of patients relapsing after autoSCT who have been reported to the lymphoma registry of the EBMT.

The aims of the study were to identify patterns of relapse and the incidence of local and locoregional relapse in patients with NHL receiving high-dose therapy

and autoSCT, and to determine subsequent survival in these patients. Potential factors contributing to local vs. systemic relapse were investigated, as was the potential value of involved-field radiotherapy given routinely to affected sites at the time of autoSCT.

## PATIENTS AND METHODS

Data from two populations of patients receiving high-dose therapy and autoSCT for NHL were analyzed.

### Group A

Between January 1991 and May 1998, 106 patients with NHL underwent high-dose therapy and autoSCT in the Medical Oncology Unit of the University of Southampton, U.K. Fifty-seven (54%) of these patients have relapsed after autoSCT. The patient characteristics for this group are summarized in Table 1. Most patients ($n$=91) had diffuse large B cell lymphoma, either de novo ($n$=78) or having undergone histologic transformation from previous low-grade disease ($n$=13). Eighty-one of the patients were treated in chemosensitive relapse.

*High dose and transplantation procedures.* Of the total of 106 patients, 97 received BEAM (carmustine, etoposide, cytosine arabinoside, melphalan), two were treated with BEAC (carmustine, etoposide, cytosine arabinoside, cyclophosphamide), and the remaining seven patients received high-dose cyclophosphamide and total body irradiation (TBI). The use of elective involved-field radiotherapy was restricted to patients with sites of bulk disease (>5 cm) at the time of the relapse before high-dose therapy, unless the patient was receiving TBI-based high-dose therapy or the site had been previously irradiated. Seven patients, all with primary mediastinal lymphoma, received involved-field radiotherapy, commencing within 45 days of the stem cell reinfusion.

The source of hematopoietic stem cells was bone marrow in 24 and peripheral blood progenitor cells (PBPCs) in 82 patients. None of the stem cell products underwent positive stem cell selection. Purging with anti–B cell monoclonal antibodies was undertaken in four patients with low-grade NHL as part of a phase II study.

All patients received antimicrobial and blood product support according to established protocols. The routine use of granulocyte colony-stimulating factor (G-CSF) after stem cell reinfusion was introduced for the last 2 years of the study period.

*Assessment of response.* Response to high-dose therapy was assessed at 90 days after stem cell reinfusion. Complete response was defined as the disappearance of all clinical evidence of disease, with normalization of all previously abnormal laboratory and radiological investigations. Partial response was defined as a reduction by at least 50% in the sums of the products of the biperpendicular

**Table 1.** Patient characteristics, group A

| | *All patients* | *Relapsed patients* |
|---|---|---|
| Total | 106 | 57 |
| Median age (range) | 46 (16–66) | 48 (16–66) |
| Male:female | 66:40 | 32:25 |
| Histologic subtype | | |
| DLBC | 78 | 44 |
| Transformed follicular | 13 | 6 |
| Follicular | 2 | 0 |
| Lymphoblastic | 4 | 2 |
| Burkitt's | 4 | 2 |
| Mantle cell | 2 | 2 |
| EATL | 1 | 0 |
| AILD-like | 2 | 1 |
| Status at autoSCT | | |
| CR1 | 5 | 2 |
| Responsive relapse/CR2 | 87 | 45 |
| Resistant relapse | 2 | 1 |
| Untreated relapse | 3 | 0 |
| Primary refractory | 9 | 9 |
| Source of stem cells | | |
| Bone marrow | 24 | 12 |
| Peripheral blood | 82 | 45 |

*DLBC, diffuse large B cell lymphoma; EATL, enteropathy associated T cell lymphoma; AILD, angioimmunoblastic lymphadenopathy–like T cell lymphoma.*

diameters of all measurable disease. Both complete and partial responses were stable for a minimum of 1 month after initial evaluation.

Patients who developed disease progression or relapse after autoSCT were identified for subsequent analysis.

*Diagnosis of relapse/progression.* Relapse or progression was defined as an increase in size at a known site of disease or the appearance of disease at one or more new sites. When possible, histologic confirmation of relapse was obtained, particularly if the disease-free interval was >1 year. However, in the majority of patients, the diagnosis of relapse was based on clinical characteristics only.

*Definition of sites of relapse.* Sites of relapse were defined by comparison with the sites of involvement before autoSCT with the involved sites at relapse. For those patients with active disease at the time of autoSCT, the involved sites at this time were compared with the relapse sites. For those patients transplanted in responding relapse or complete remission, the sites of disease at the relapse immediately before high-dose therapy and autoSCT were compared with the relapse sites after autoSCT.

Local or locoregional relapse was defined as a relapse occurring in a lymph node region or extranodal site that had been previously involved or in an immediately contiguous site. Distant relapse was defined as relapse occurring at sites other than local or locoregional sites. Patients who relapsed synchronously at local and distant sites were identified separately from the other two categories.

*Treatment at relapse.* None of the patients relapsing after autoSCT were treated with curative intent. Therapy was determined by patient and physician preference. Localized relapses at previously unirradiated sites were treated with involved-field radiotherapy (nine patients). Some patients with disseminated disease at relapse declined further therapy ($n$=9). The remaining patients were treated with corticosteroids alone, single-agent—or low dose combination—chemotherapy, or a combination of these.

## Group B

The lymphoma registry of the EBMT was searched, and patients undergoing high-dose therapy and autoSCT for NHL were identified. In addition, a small number of patients undergoing allogeneic bone marrow transplantation were included in this analysis. Of the total of 11,883 patients, 3454 patients who had relapsed after SCT were identified. Of these, data concerning sites of relapse were available for 1339. An initial comparison of these 1339 patients with those on whom relapse sites were not available showed no difference in the distribution of major demographic or prognostic features. The characteristics of these patients are shown in Table 2.

*High-dose therapy and transplantation procedures.* Chemotherapy-only high-dose regimens were used in 749 patients, and chemotherapy plus TBI–containing regimens were used in 590. The precise regimens varied according to the active protocols of the participating institutions. The source of autologous hematopoietic stem cells was bone marrow in 753, peripheral blood in 420, and both in 61. In addition, 105 patients receiving allogeneic stem cells from HLA-identical sibling donors were identified.

Positive stem cell selection was used in 16 patients. Purging of stem cell products was performed in 87 patients, using 4-hydroperoxycyclophosphamide (4HC) in 30 and antibody-based methods in 57.

All patients underwent high-dose therapy in registered transplant centers. Supportive measures including antibiotic and blood product support were given according to the active protocols of the centers, as were hematopoietic growth factors.

*Definition of relapse/progression and relapse sites.* The definition of relapse or progressive disease was the same as for group A. The definition of relapse sites was also the same, except that group B patients with synchronous relapses at local and distant sites were included in the distant relapse category. Insufficient data on treatment after relapse were available to allow analysis of its effectiveness.

**Table 2.** Patient characteristics, group B

| | *Number (%)* |
|---|---|
| Total | 1339 (100) |
| Histologic subtype (WF) | |
| Low grade | 187 (14.0) |
| Intermediate grade | 393 (29.4) |
| High grade | 439 (32.8) |
| Lymphoblastic | 245 (18.3) |
| Burkitt's/Burkitt-like | 75 (5.6) |
| Source of stem cells | |
| Bone marrow | 753 (56.2) |
| Peripheral blood | 420 (31.4) |
| Both | 61 (4.6) |
| Allogeneic | 105 (7.8) |
| High dose regimen | |
| Chemotherapy only | 743 (55.5) |
| Chemotherapy + TBI | 596 (44.5) |

### Statistical analysis

Survival analyses were performed according to the method of Kaplan and Meier.[8] Overall survival was calculated from the date of stem cell reinfusion or the date of relapse/progression to the date of death from any cause. Time to relapse/progression was calculated from the date of stem cell reinfusion until the date of documented disease relapse or progression.

## RESULTS

### Group A

Of the 106 patients undergoing high-dose therapy, 103 achieved a partial or complete response. Two patients had progressive disease, and there was one treatment-related death at day 10 after autologous stem cell reinfusion from pulmonary hemorrhage. Of the 103 responding patients, 57 have relapsed. The median time to relapse was 9 months after autoSCT. The actuarial overall survival from the date of autologous stem cell reinfusion is shown in Fig. 1A and the failure-free survival in Fig. 1B. The median time to relapse for the entire population was 9 months.

Sites of relapse are summarized in Table 3. Most relapses (63%) occurred in local or locoregional sites in this group of patients with predominantly diffuse large B cell lymphoma. The remaining relapses were at distant sites only, or a combination of

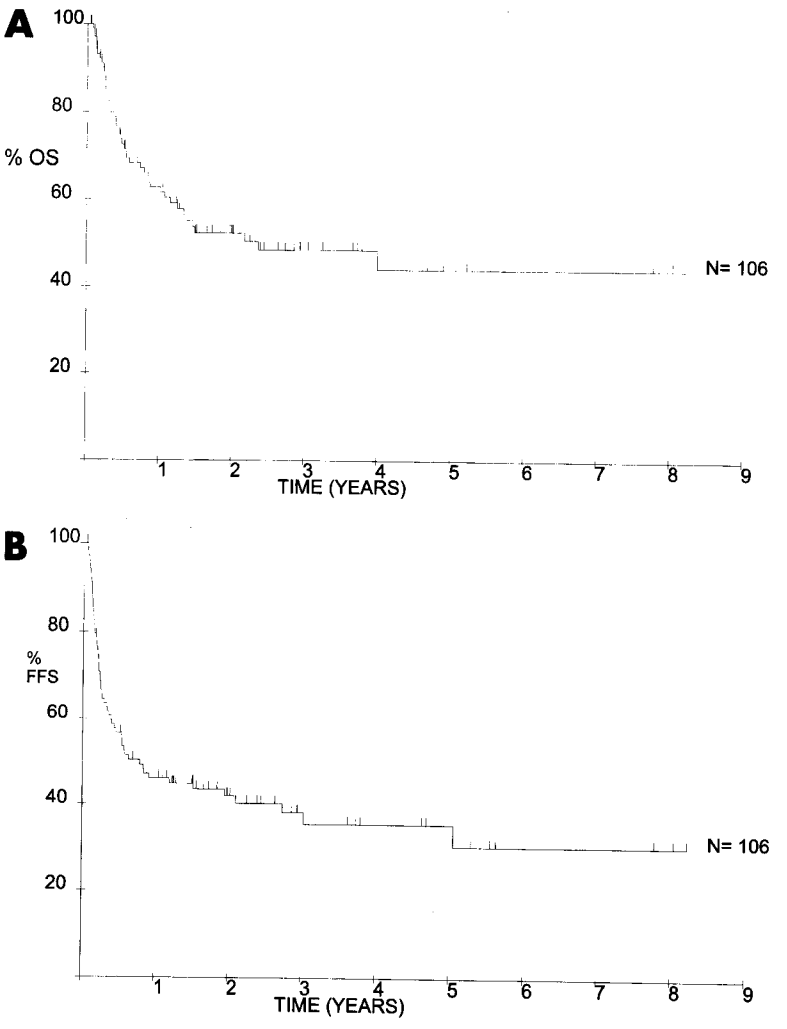


**Figure 1.** *A: Actuarial overall survival calculated from the date of autologous stem cell transplantation for 106 patients with NHL receiving autoSCT in Southampton. B: Actuarial failure-free survival calculated from the date of autologous stem cell transplantation for 106 patients with NHL receiving autoSCT in Southampton.*

distant and previously involved sites. In view of the predominance of patients with diffuse large B cell lymphoma, it was not possible to analyze patterns of relapse according to histological subtype. Although patient numbers are small, no obvious trend for local or locoregional relapse according to involved sites was observed.

Since only seven patients received elective involved-field radiotherapy, its impact on patterns of relapse cannot be fully assessed. However, of the seven

**Table 3.** Sites of relapse, group A

| *Site of relapse* | *n (%)* |
|---|---|
| Total | 57 (100) |
| Local/locoregional | 36 (63) |
| Distant | 15 (27) |
| Local + distant | 6 (10) |

patients receiving mediastinal irradiation, four have relapsed, three with mediastinal involvement and one at distant sites only.

Of the 50 patients who received therapy after relapse, none achieved complete remission. Responses were observed in 27 patients. Three patients, all of whom had local or locoregional relapses, are alive >1 year after relapse. All have active disease.

Figure 2A shows the overall survival for all patients from the date of relapse after transplant. The median overall survival for all 57 patients is 3 months. As shown in Fig. 2B, no difference in survival was observed according to the pattern of relapse.

## Group B

Of the 1339 patients for whom relapse data were available, 259 (19%) relapsed in local or locoregional sites only. The remaining 1080 patients relapsed with disease at distant sites, with or without involvement of previously affected sites. Analysis of the frequency of local vs. distant relapse according to histologic subtype, number of sites of involvement before high-dose therapy, status at autoSCT, and source of hematopoietic rescue is shown in Table 4.

Overall, only 19% of patients relapsed entirely at previously involved sites. The probability of local relapse was significantly lower in patients with lymphoblastic or Burkitt's/Burkitt-like histology compared with the remaining patients ($P$=0.016). The probability of localized relapse was inversely correlated with the number of disease sites involved before autoSCT ($P$<0.0001). Local relapse was more likely to occur in patients with chemoresistant or untested disease than in those with documented chemosensitivity ($P$<0.0001). No difference in the pattern of relapse was observed according to the high-dose regimen used or the source of stem cells. Manipulation of the stem cell product by either $CD34^+$ selection or purging had no apparent effect on the pattern of relapse. The probability of local relapse was also analyzed according to individual disease sites before SCT. No obvious trend was observed for a higher likelihood of local relapse according to any initially involved site. This analysis was also performed for individual histologic subtypes and according to status at autoSCT. Again, no individual disease site was associated with a higher probability of local relapse in any histologic grade or according to status at autoSCT.

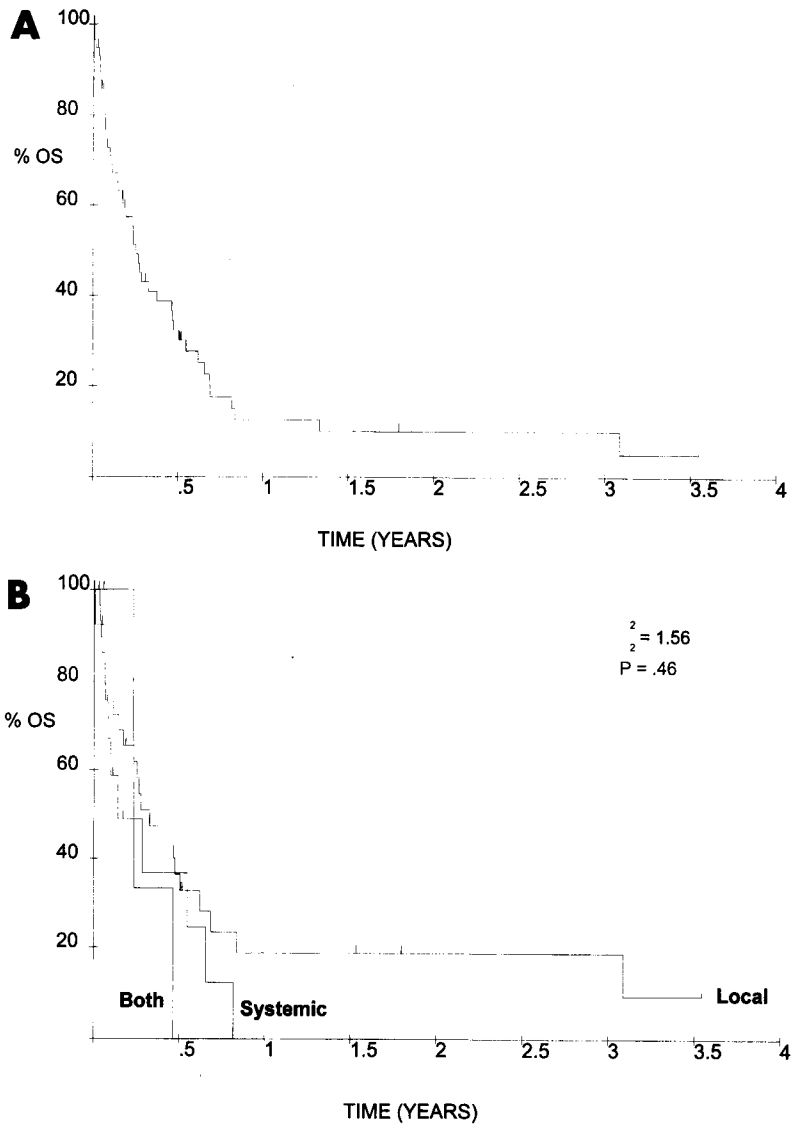


**Figure 2.** *A: Actuarial overall survival from the date of relapse after autoSCT, Southampton patients. B: Actuarial overall survival from the date of relapse according to pattern of relapse, Southampton patients.*

Figure 3A shows the time to relapse after stem cell transplantation for all 1339 patients. The median time to relapse was 6 months. The time to relapse according to site is shown in Fig. 3B. Local relapses occurred significantly earlier after SCT than distant relapses ($P<0.001$). Actuarial overall survival from the time of relapse for the entire population is shown in Fig. 4A, and according to relapse site in Fig.

**Table 4.** Patterns of relapse, group B

| | *Total* | *Local/loco-regional (%)* | *Distant (%)* | *P* |
|---|---|---|---|---|
| All patients | 1339 | 259 (19) | 1080 (81) | |
| Histologic subtype | | | | 0.016 |
| Low grade | 187 | 40 (21) | 147 (79) | |
| Intermediate grade | 393 | 88 (22) | 305 (78) | |
| High grade | 439 | 90 (20) | 349 (80) | |
| LBL | 245 | 30 (12) | 215 (88) | |
| Burkitt's | 75 | 11 (15) | 64 (85) | |
| Number of disease sites before autoSCT | | | | <0.0001 |
| 1 | 527 | 180 (29) | 447 (71) | |
| 2 | 407 | 64 (16) | 343 (84) | |
| 3 | 199 | 12 (6) | 187 (94) | |
| 4 | 77 | 3 (4) | 74 (96) | |
| ≥5 | 29 | 0 | 29 (100) | |
| Status at autoSCT | | | | <0.0001 |
| Chemosensitive | 811 | 156 (19) | 655 (81) | |
| Resistant relapse | 195 | 77 (39) | 118 (61) | |
| Untested relapse | 48 | 22 (46) | 26 (54) | |
| Source of stem cells | | | | NS |
| Allogeneic | 105 | 20 (19) | 85 (81) | |
| Autologous marrow | 753 | 146 (19) | 607 (81) | |
| PBPC | 420 | 85 (20) | 335 (80) | |
| Marrow +PBPC | 61 | 8 (13) | 53 (87) | |

4B. The median OS from the time of relapse for the entire population was 15 months. No difference in OS was observed according to the pattern of relapse.

Figure 5 shows the overall survival from the date of relapse according to histologic subtype. A highly significant correlation was observed between increasing histologic grade and lower survival after relapse ($P<0.000001$). Within each of these subtypes, no difference in overall survival after relapse was observed according to the relapse pattern.

## DISCUSSION

The reported outcome for patients with NHL who relapse after high-dose therapy and SCT is poor. In the series from Nebraska, 74 of 178 patients with NHL undergoing high dose therapy and autoSCT relapsed.[6] The median time from transplantation to relapse was 2 months for those with high-grade disease, 3 months for patients with intermediate-grade disease, and 11 months for patients with low-grade disease. The median overall survival from the date of relapse was only 3

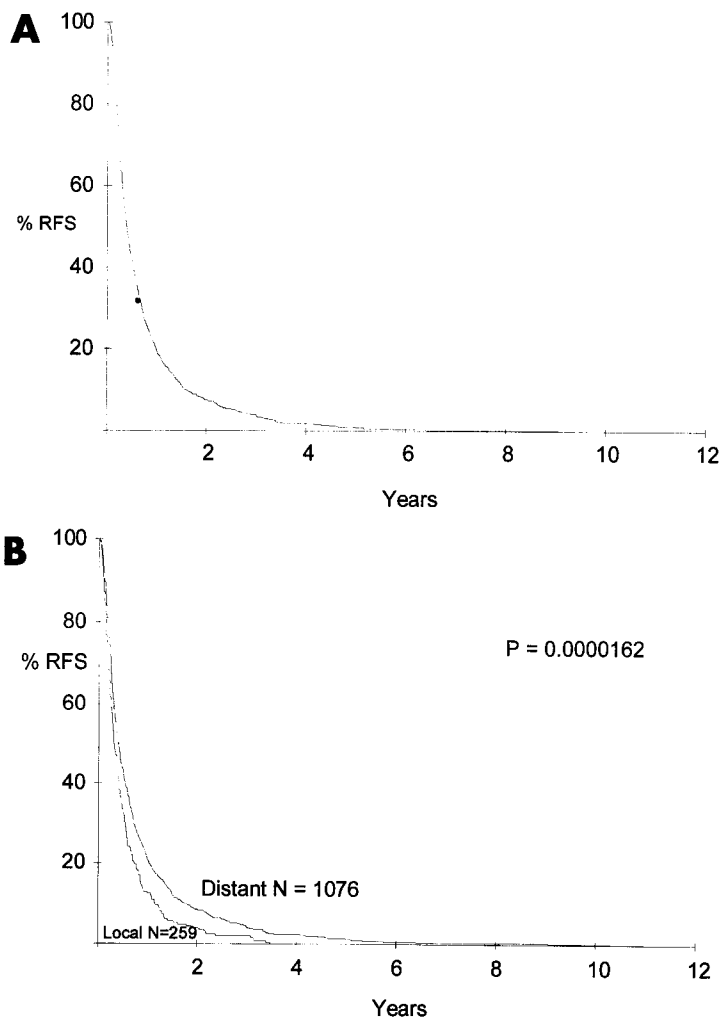


**Figure 3.** *A: Actuarial relapse-free survival from date of stem cell transplantation for 1339 patients undergoing autoSCT in EBMT centers. B: Actuarial relapse-free survival from date of stem cell transplantation according to pattern of relapse for patients undergoing autoSCT in EBMT centers.*

months. At the time of the report, eight patients were alive and progression free, off therapy, six of whom had follicular lymphoma. Five of these patients had received involved-field radiotherapy for localized relapses, two had been treated with combined modality therapy, and one had undergone allogeneic BMT. No data on patterns of relapse are available in this report.

Peterson et al. reported patterns of relapse in 101 patients with malignant lymphoma (20 with Hodgkin's disease, 81 with NHL) undergoing autologous

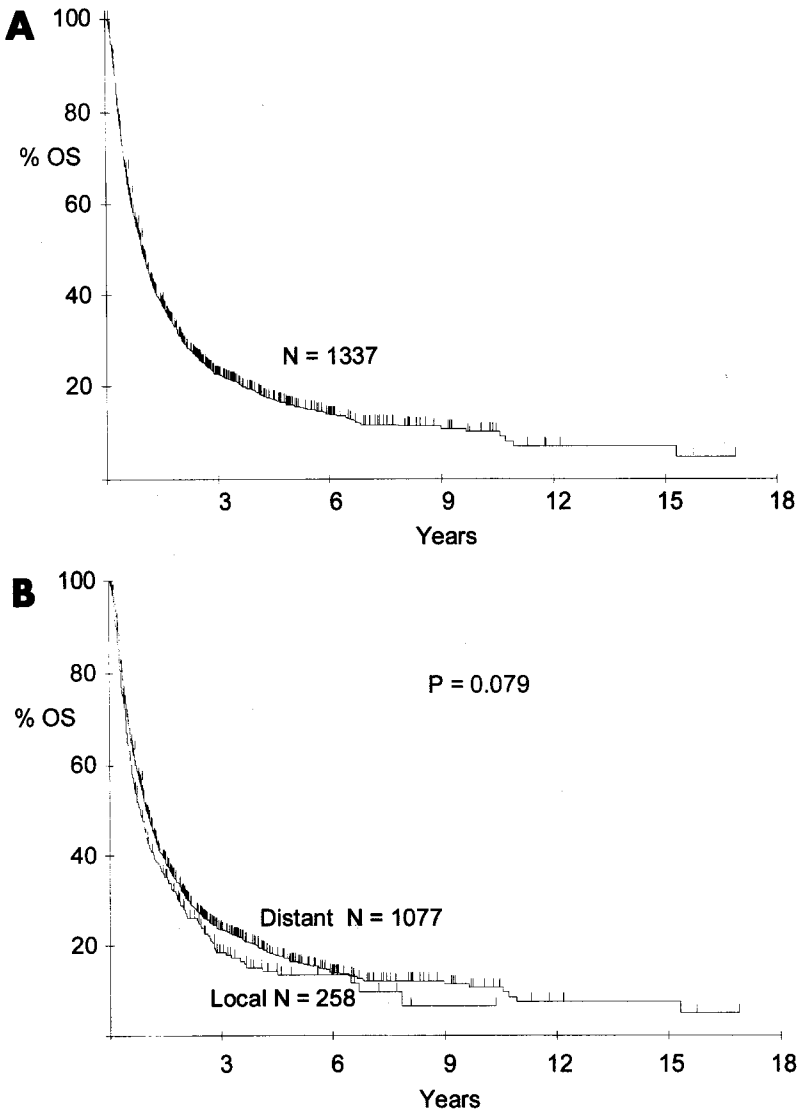


**Figure 4.** *A: Actuarial overall survival from the date of relapse after autoSCT, EBMT patients. B: Actuarial overall survival from the date of relapse according to pattern of relapse, EBMT patients.*

transplantation at Seattle between 1979 and 1988.[9] Sixty-two of these patients relapsed, at a median of 70 days after SCT. Ten of the relapsed patients were alive at a median of 681 days from relapse, all of whom had relapsed at previously involved sites. Five had received involved-field radiotherapy, and five, combination chemotherapy. However, of the 10 patients alive after relapse, five

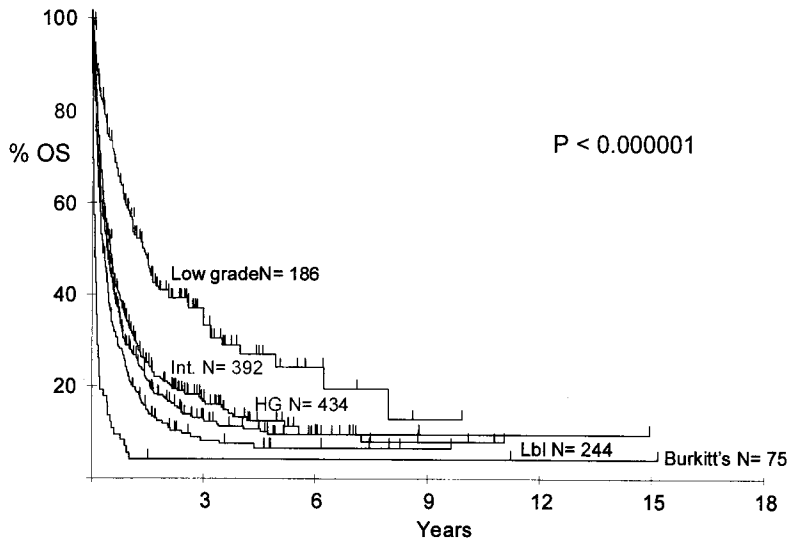


**Figure 5.** *Actuarial overall survival from the date of relapse according to histologic subtype, EBMT patients.*

had Hodgkin's disease, and three of the five patients with NHL were receiving therapy at the time of the report.

Results from both groups of patients in the present study confirm the poor outlook for these patients. In the Southampton series, the median overall survival from the date of relapse was 3 months. Most of the patients in this group had diffuse large B cell lymphoma, and the median survival is identical to that for intermediate-grade patients in the Nebraska series. Although the median survival for all patients in the EBMT series is longer at 1 year, 14% of the patients in this series had low-grade disease, which, as shown in Fig. 5, has a significantly longer survival (median 16 months) after relapse compared with aggressive NHL. Those with aggressive NHL have a median survival of between 2 and 6 months, according to histologic subtype.

## Patterns of relapse

Patterns of relapse have been documented infrequently in previous reports. In the series by Peterson et al.,[9] 59 patients (95%) relapsed in previously involved sites, although the report does not indicate whether these relapses were localized or whether more distant sites were also involved. Of 100 patients with B cell NHL treated with high-dose cyclophosphamide and TBI plus monoclonal anti–B cell purged autologous BMT at the Dana Farber Cancer Institute, 35 patients relapsed.[7] Twenty-three of these patients relapsed only in previously involved sites, seven in

new sites, and five in old and new sites. In a later report, restricted to 69 patients with low-grade or transformed low-grade B cell NHL, the same group observed 23 relapses, 18 of which were limited to sites of previous disease.[10]

Direct comparisons between the two groups of patients in this study and those reported in previous series is difficult in view of differences in patient characteristics, including histologic subtypes, status at autoSCT, etc. The Southampton series is relatively homogeneous in that most patients had diffuse large B cell lymphoma, and the majority were treated in chemosensitive relapse. The 63% incidence of localized relapse in this series is comparable to other published reports. By contrast, the results from the EBMT registry show a much lower overall incidence of localized relapse of 19%. The reason for this apparent discrepancy is unclear. Registry-based data of this type must be interpreted cautiously, in view of the possible selection, referral, and reporting bias which may affect its interpretation. Furthermore, relapse sites were only available for 39% of all relapsing patients. The EBMT series is much more heterogeneous with respect to histologic subtypes of NHL, although the incidence of local relapse, even in low-grade disease, is much lower than that previously reported.

The higher risk of distant relapse for patients with lymphoblastic and Burkitt's or Burkitt-like lymphoma has not been reported previously. However, these diseases are frequently widely disseminated from presentation, with multiple sites of involvement, and localized relapse is therefore unlikely.

The contribution made by reinfused contaminating lymphoma cells to clinical relapse after autologous transplantation remains unclear. Gribben et al. have reported that the presence of low levels of contaminating lymphoma cells, detected by the polymerase chain reaction (PCR), in patients with low-grade B cell NHL predicts for earlier relapse after high-dose therapy and purged autologous BMT.[11] If reinfused contaminating cells contribute significantly to clinical relapse, it is likely that such relapses would be widespread. Unless site-specific homing of lymphoma cells occurs, relapse at previously involved sites is more likely to represent failure to eradicate disease rather than reinfusion of clonogenic cells. In the present study, manipulation of the autologous stem cell product had no observed effect on the frequency of local vs. distant relapse. However, the number of patients who received positively or negatively selected stem cell products was very small. The potential contribution of reinfused lymphoma cells was also examined by analyzing the incidence of local vs. distant relapse according to the hematopoietic stem cell source, including allogeneic cells. No difference was observed according to stem cell source, suggesting that reinfused contaminating cells are not a major source of clinical relapse.

The EBMT data demonstrate that localized relapses occur earlier after high-dose therapy than distant relapses ($P<0.0001$). They also show a higher probability of local relapse for patients with single- vs. multiple-site disease, and for those with resistant

vs. sensitive relapse. Although it is possible that these factors are interrelated, this could not be analyzed reliably because of limited numbers of patients.

The observation that localized relapse is more likely in patients with single-site disease before high-dose therapy suggests a potential role for involved-field radiotherapy in these patients. The effect of local radiotherapy could not be addressed from the EBMT database. From the Southampton series, local radiotherapy was considered as consolidative therapy in those patients with sites of bulk disease before high-dose therapy. However, only seven patients received radiotherapy. Some had already received radiotherapy as a component of their induction therapy and others were receiving TBI-based high-dose regimens. In the seven patients receiving radiotherapy, four relapsed within the irradiated field. These results are similar to those reported from the PARMA randomized trial, in which eight of 22 patients receiving radiotherapy relapsed, compared with 18 of 33 who were not irradiated ($P$=0.19).[1] These results do not, therefore support the routine use of radiotherapy to single sites as consolidation after high-dose therapy, although it may be appropriate for the small proportion of patients who have not received prior radiotherapy.

### Treatment after relapse

The results from both series of patients in this report show no difference in the survival for patients with localized compared with distant relapses. This reflects the limited therapeutic options available at the time of relapse. In the Southampton series, most local relapses occurred in previously irradiated sites, so further radiotherapy was not possible. Some patients with distant relapses at single sites received radiotherapy, with local control of their disease, but all rapidly relapsed at other sites.

Short-term partial responses were achieved in some patients with the use of corticosteroids and low-dose or single-agent chemotherapy.

## SUMMARY

This study confirms the poor outlook for patients with NHL who relapse after stem cell transplantation. The incidence of localized relapse in the large, retrospective EBMT series is lower than that reported for single institutions. Clinical factors such as histological subtype, number of sites involved before autoSCT, and disease status at the time of autoSCT are predictive of localized relapse, whereas treatment variables such as the high-dose regimen and stem cell source have no predictive value. The use of consolidative involved-field radiotherapy to sites of bulk disease at the time of autoSCT could not be accurately assessed, but had no clear effect on subsequent relapse, and was often not feasible, since these sites had been previously irradiated.

Treatment options for patients who relapse after autoSCT are very limited, and the survival after relapse is the same for patients with localized and distant relapses. This is partly due to the fact that sites of localized relapse have often been irradiated earlier in the course of the disease.

New treatment strategies are required for patients who relapse after autoSCT, irrespective of the site of relapse, since prognosis is equally poor for all patients. Local therapy has a very limited role, and novel systemic therapies should be used. Preliminary studies have shown responses to a number of novel agents including monoclonal antibodies and cytokines. The use of allogeneic BMT, including matched unrelated donor transplantation, has been reported to produce long-term disease-free survival in a small proportion of patients relapsing after autoSCT, but has a very high (>40%) regimen-related mortality. This may be a suitable setting in which to consider the use of novel "mini-transplant" strategies.

## ACKNOWLEDGMENTS

We wish to acknowledge all of the EBMT centers who have contributed patients to this study. In addition, we wish to acknowledge the contribution of the medical and nursing staff on the Transplant Unit at the University of Southampton, U.K., for their expert care of these patients.

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# Molecular Remission: A Worthwhile Aim?

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Between 1985 and 1995, 99 patients with follicular lymphoma received high-dose treatment (cyclophosphamide: 60 mg × 2 and total-body irradiation: 200 cGy × 6) with autologous bone marrow transplantation, as consolidation of 2nd or subsequent remission. The bone marrow mononuclear cell fraction was treated in vitro with three cycles of one (74 patients) or four (25 patients) anti–B cell monoclonal antibodies and complement. Thirty-three patients have developed recurrent lymphoma, outcome correlating with the histology at the time of recurrence (follicular, or transformed to a higher grade histology), patients with transformation having a worse survival ($P$=0.01). PCR analysis for t(14;18)-containing cells was performed on bone marrow samples before and after in vitro treatment. PCR status of reinfused cells did not correlate with freedom from recurrence (FFR) ($P$=0.17). However, there was a significant correlation between FFR and PCR status when follow-up bone marrow samples were analyzed, 19/23 patients with consistently negative samples remaining disease-free compared with 13/30 patients with persistently positive or variable results ($P$=0.04). Thus "molecular remission" appears to be a worthwhile aim but longer follow-up is needed, and these results should be seen in the context of other treatments such as fludarabine-containing regimens and antibody therapy, which also results in "molecular remission" being achieved in a proportion of patients.

# High-Dose Therapy Followed by Hematopoietic Stem Cell Transplantation (HSCT) for Mantle Cell Lymphoma (MCL)

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Between June 1991 and January 1998, 46 transplants (autologous, $n$=39; allogeneic, $n$=7; marrow, $n$=5; blood, $n$=41) were performed on 45 patients with MCL. There were 36 males and 9 females. Median age for autologous patients was 56 years (range 34–65). Currently, 28 patients are alive after autologous transplantation and 21 have not progressed (median follow-up 12.8 months; range 3–61). At 2 years, overall survival (OS) and event-free survival (EFS) for these patients are estimated at 65 and 41%, respectively. The actuarial probability of relapse or progression was 56% at 2 years, and there was no evidence of a plateau in EFS. No patients died before day 100, although one patient was diagnosed with a glioblastoma multiforme 18 months after transplantation. Patients with blastic histology ($P$=0.023), any history of bone marrow involvement ($P$=0.008), elevated LDH ($P$=0.15), and three or more prior regimens ($P$=0.001) had significantly worse EFS. Two patients who had allogeneic transplants died 2 and 12 months later from pulmonary complications. Neither had evidence of lymphoma at autopsy. There have been no relapses or progression among the five remaining allogeneic transplant patients (median follow-up 14.2 months; range 3–45). At 2 years, OS and EFS are each projected to be 64%. A case-matching analysis failed to show significant differences in OS or EFS among autologous and allogeneic transplant recipients. Although long-term EFS was observed after autologous HSCT for MCL, a continuous pattern of treatment failure was observed, and there is little evidence that this approach has curative potential. The role of allogeneic transplantation for MCL should be further explored.

# Early or Late Autotransplant in High-Risk Non-Hodgkin's Lymphoma: High-Dose Sequential Chemotherapy and the Role of Involved-Field Radiation Therapy

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## INTRODUCTION

Approximately 45% of adult patients with aggressive non-Hodgkin's lymphoma (NHL) will be cured of their disease with conventional chemotherapy.[1–3] Patients who relapse after multiagent chemotherapy have a particularly poor prognosis.[4] A randomized prospective trial performed by the Parma group[5,6] demonstrated a survival advantage with high-dose chemotherapy and autologous bone marrow transplantation (autoBMT) for patients in sensitive relapse compared with conventional salvage regimens. Multiple institutions have reported pilot studies using high-dose chemotherapy with either peripheral blood stem cell transplantation (PBSCT) or autoBMT as initial therapy for high risk NHL.[7–10]

The French Lymphoma Trials Group (GELA) performed a randomized trial designed to compare standard vs. sequential conventional-dose chemotherapy with high-dose chemotherapy followed by auto-BMT in patients who have achieved complete remission (CR) after receiving conventional chemotherapy. Analysis of the entire cohort did not reveal a significant difference in either disease-free or overall survival. In the cohort of poor-risk patients, disease-free survival (DFS) in the autoBMT arm (59%) was found to be superior to sequential therapy (39%) whereas improvement in overall survival (OS) at 5 years was relatively small (65 vs. 52%, $P$=0.06).[7] This prospective trial indicates the value of high-dose consolidation therapy with autologous stem cell support in younger high-risk patients who have achieved a complete response with the standard induction regimen. An additional nonrandomized pilot trial focusing on early intensification followed by

autoBMT in patients with high-risk large-cell NHL showed 50% DFS rate at a median follow-up of 32 months with a 6% toxic death rate.[11] Use of peripheral stem cell support after cisplatin and etoposide addition to an augmented form of CHOP chemotherapy with escalated doxorubicin and cyclophosphamide doses in high-risk patients resulted in 56% 2-year failure-free survival rate, which is comparable to the other series.[12]

Verdonk and colleagues evaluated the impact of early high-dose consolidative therapy in patients who failed to achieve a complete remission after three cycles of CHOP therapy. Patients who achieved a partial remission after three cycles of CHOP were randomized to high-dose cyclophosphamide combined with single-dose total body irradiation (TBI) and stem-cell rescue or an additional five cycles of the CHOP regimen.[13] The majority of patients enrolled into this trial had low and low to intermediate risk disease. The study failed to demonstrate any benefit from consolidative high-dose chemoradiotherapy in first partial remission. In a smaller, randomized study designed to answer a similar question, partial responders to conventional induction treatment received either dexamethasone, cytosine arabinoside, cisplatin (DHAP) salvage regimen (27 patients) or high-dose BCNU, etoposide, cytosine arabinoside, cyclophosphamide (BEAC) regimen followed by an autologous bone marrow rescue (22 patients). Although the patients randomized to the high-dose chemotherapy arm had a 73% DFS rate compared with 53% observed in patients receiving the DHAP salvage regimen, a statistically significant advantage for transplant was not achieved.[14]

Gianni et al.[15] reported on 98 patients with high risk, diffuse B cell NHL who were prospectively randomized to either standard therapy with MACOP-B (methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone and bleomycin) or high-dose sequential (HDS) chemotherapy followed by myeloablative therapy and stem cell support. With a median follow-up of 55 months. the HDS therapy was found to be superior to standard MACOP-B with an overall survival of 81 vs. 55%.

The rationale for the use of involved-field radiation therapy (IFRT) is supported by the recently completed Eastern Cooperative and Southwest Oncology Group (ECOG and SWOG) trials[16,17] as well as by the pattern of relapse observed by other investigators.[4] However, contemporary practice for the use of radiation therapy in combination with high-dose therapy largely reflects institutional preferences. Neither the appropriate fields nor the appropriate sequencing of radiation in conjunction with bone marrow transplantation has been adequately defined.

This study reports on the impact and toxicity of IFRT in combination with HDS chemotherapy and updates the results reported by Schenkein et al.[2] on the feasibility and efficacy of this treatment approach in newly diagnosed, high-risk patients with NHL.

## PATIENTS AND MATERIALS

### Patient characteristics

Between October 1993 and October 1996, 31 patients were treated according to a prospective phase II protocol at five participating centers. Patients with intermediate- or high-grade NHL, as defined by the Working Formulation, were eligible if at least two of the following high-risk features were present: *1*) stage III or IV disease, *2*) lactate dehydrogenase (LDH) greater than the upper limit of normal, or *3*) an ECOG performance status of 2, 3, or 4. All patients were at least 18 years of age and all were <60 years of age. Patients were staged according to the Ann Arbor Staging System. Clinical staging included routine blood chemistries, computed tomography scans, and bone marrow evaluation. The clinical characteristics of the 27 patients who completed all five phases of therapy and were therefore eligible for consolidative radiotherapy are summarized in Table 1. These patients are the subject of this report.

### Treatment protocol

High-dose sequential chemotherapy was delivered and consisted of intensive, non-cross-resistant agents delivered in five sequential treatment phases, followed by autologous PBSCT as initially described by Gianni[15] and revised by Schenkein.[2] The chemotherapy regimen consisted of adriamycin, vincristine, and prednisone (phase I); cyclophosphamide (phase II); methotrexate and vincristine (phase III); etoposide (phase IV); mitoxantrone and melphalan (phase V); followed by autologous PBSC infusion.[2,11] As part of this phase II protocol, patients with bulky disease or persistent disease were scheduled to receive consolidative IFRT with 2400 cGy after marrow engraftment. Bulk disease was defined as sites of disease measuring ≥5 cm in

Table 1. Patient characteristics

| *Characteristic* | *Number (%)* | *No. receiving XRT* | *No. not receiving XRT* |
|---|---|---|---|
| Total | 27 (100) | 15 (56) | 12 (44) |
| Sex | | | |
| Male | 15 (56) | 7 | 8 |
| Female | 12 (44) | 8 | 4 |
| Median age (years) | 31 | 31 | 36 |
| Disease grade | | | |
| Intermediate | 16 (59) | 8 | 8 |
| High | 11 (41) | 8 | 3 |
| International Index | | | |
| Intermediate–high (2) | 20 (74) | 12 | 8 |
| High (3) | 7 (26) | 3 | 4 |

diameter. Radiation was to be initiated 30–100 days after stem cell infusion. Involved-field radiation therapy was used in 15 of the 27 patients (56%).

Radiotherapy was initiated following a median of 61 days (range 31–136) in the 15 patients receiving radiation as consolidative treatment after high-dose sequential chemotherapy and autologous PBSCT. Involved-field radiation therapy was delivered to two patients achieving CR, 10 patients achieving PR, and three patients with persistent disease. Radiotherapy was delivered to a median of three sites (range one to six) as defined by the Ann Arbor Staging Manual. Patients received a median of 2400 cGy per site (range 1980–5400). Twelve patients did not receive IFRT because of early posttransplant progressive disease ($n$=2), no areas of bulky disease ($n$=5), early complications ($n$=2), and unknown reasons ($n$=3). Toxicity resulting from radiotherapy was determined from medical chart review and graded according to the ECOG system.

## RESULTS

### Overall and disease-free survival

The median follow-up for the entire cohort was 34 months (range 24–63) from the initiation of HDS chemotherapy. The OS and relapse-free survival (RFS) for the entire cohort were 73 and 58%, respectively. The overall survival was not significantly altered by the use of IFRT. Relapse occurred in 11 of 27 patients (40%) at a median of 108 days after PBSC infusion. Two of the patients with late relapses are currently disease-free after undergoing either a second transplant or further multiagent chemotherapy. The RFS was 72% for the IFRT group vs. 40% for the group that did not receive IFRT ($P$=0.08) at a median follow-up of 14 months.

Excluding four patients with small noncleaved non-Burkitt's and lymphoblastic lymphoma, the OS and RFS were 82 and 78%, respectively.

### Patterns of failure

Local failure, after IFRT, occurred in four of 15 patients (27%). Failure in the radiation portal occurred in all three patients considered to have radiographic persistent disease after HDS chemotherapy. One patient considered to have a CR to treatment relapsed in the radiotherapy field. This patient had a high-grade lymphoblastic lymphoma and relapsed both inside and outside of the radiation field. The mean radiation dose in the group of patients with infield failure was 2434 cGy (range 2400–4580), and radiotherapy was initiated within an average of 67 days (range 42–93) from PBSC reinfusion. The mean radiation dose for the patients without evidence of failure was 2929cGy (range 2000–5400), and radiation therapy was initiated within an average of 58 days (range 31–105) from PBSC reinfusion.

## Toxicity

Toxic effects occurred in 10 patients receiving IFRT after HDS chemotherapy and PBSCT. Grade 3 toxicity occurred in three cases, all of which were hematologic. These patients experienced a significant break in the course of their radiation treatments (14–15 days) due to myelosuppression and required transfusions and hematologic growth factors. There were no grade 4 or 5 toxicities. Esophagitis was the most common form of toxicity recorded; however, all cases were minor, grade 1 symptoms.

## DISCUSSION

The International Non-Hodgkin's Lymphoma Prognostic Factors Project[18] identified four risk groups based on prognostic factors. The age-adjusted index used advanced tumor stage, LDH level, and performance status to model the four risk groups. The present study identified and treated newly diagnosed NHL patients with two or three risk factors.

Several studies that incorporate high-dose therapy as part of initial therapy or in first CR/PR have been reported with both positive and negative results. No trial has directly compared early vs. late autotransplant, although the Parma trial clearly demonstrates the utility of this modality in first chemosensitive relapse.

We have confirmed the extremely positive results reported by Gianni with a high OS and RFS at extended follow-up for a cohort of high-risk untreated NHL patients. We have also examined the role of involved-field x-ray therapy in this patient population. Our preliminary results indicate that the addition of IFRT is well tolerated in this group of patients with high-risk, newly diagnosed NHL. While the small patient numbers limit the statistical power of this analysis, there was nonetheless a strong trend toward improved RFS with the use of involved-field radiation therapy. Additional studies, including the ongoing ECOG trial of CHOP vs. HDS and initial PBPC transplantation, are needed to address the controversy of early vs. late transplantation.

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# VACOP-B Plus Autologous Bone Marrow Transplantation at Diagnosis Does Not Improve the Outcome of Aggressive Advanced Stage Non-Hodgkin's Lymphoma: Results of a Prospective Randomized Trial by the Non-Hodgkin's Lymphoma Co-operative Study Group

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## ABSTRACT

The aim of the study was to compare conventional therapy with conventional plus high-dose therapy as a front-line treatment for advanced-stage, intermediate- and high-grade non-Hodgkin's lymphoma in a multicenter randomized trial and

to evaluate the effectiveness of aggressive therapy in improving outcome. One hundred twenty-four patients between the ages of 15 and 60 years were included in the trial. Diagnosis had confirmed diffuse intermediate- to high-grade non-Hodgkin's lymphoma (Working Formulation groups F, G, H, and K) in stages II bulky (>10 cm), III, or IV. All patients were randomized at diagnosis. Sixty-one patients were assessed to receive etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin (VACOP-B) for 12 weeks and dexamethasone, cytarabin, cisplatin (DHAP) as salvage regimen (arm A), while 63 patients were to receive VACOP-B for 12 weeks plus high-dose therapy (HDT) and autologous bone marrow transplantation (arm B). There was no significant difference in the frequency of complete remission (CR) between the two groups: 75% for the VACOP-B plus DHAP regimen group and 73% for the VACOP-B plus autoBMT group. With a median follow-up observation time of 37 months (range 10–72 months), the 6-year survival probability was 64% in arm A and 65% in arm B. There was no difference in the 6-year disease-free survival (DFS) and progression-free survival (PFS) probability of the two groups. DFS was 57% with conventional chemotherapy and 80% with autoBMT ($P$=0.1). PFS was 47 and 60%, respectively ($P$=0.4). Results of patients categorized according to the International Index as being at risk were analyzed. This showed a statistical improvement in terms of DFS ($P$=0.01) and a favorable trend in terms of PFS ($P$=0.08) for intermediate-/high- plus high-risk group patients receiving autoBMT compared with patients categorized in the same groups who were treated with conventional chemotherapy. In this study, conventional chemotherapy followed by HDT and autoBMT as front-line therapy seems to be no more successful than conventional treatment in terms of overall results. However, our study suggests that HDT plus ABMT should be proposed for higher-risk group patients at diagnosis.

## INTRODUCTION

HDT with ABMT represents an efficient approach (30–40% cure rate) for those aggressive non-Hodgkin's lymphoma (NHL) patients who have "chemosensitive relapse" after the failure of conventional chemotherapy.[1–4] Its true role in the treatment of patients in first partial remission (PR) after front-line therapy is still uncertain.[5,6] Haioun et al., in a randomized trial, demonstrated no difference in terms of overall survival and DFS between patients in CR receiving conventional therapy or HDT plus autoBMT.[7] This contrasts with previous reports of nonrandomized trials.[8,9] In a retrospective analysis, the same authors suggested a survival benefit for intermediate-/high- and high-risk patients (as defined by the International Index[10]) treated with HDT and autoBMT.[11] A different approach using sequential chemotherapeutic agents given at full dosage (high-dose

sequential chemotherapy) with peripheral blood progenitor cell (PBPC) support has been associated with improved therapeutic outcome and was demonstrated to be a feasible procedure.[12,13]

The Italian Non-Hodgkin's Lymphoma Study Group (NHLCSG) began a multicenter study in 1991. The aim of the study was to compare, in a prospective and randomized trial, VACOP-B chemotherapy plus DHAP as salvage regimen vs. the same induction protocol plus autoBMT in patients with poor-prognosis intermediate- and high-grade NHL.

## PATIENTS AND METHODS

This prospective, randomized phase III study, with 16 participating centers from the NHLCSG, began in October 1991 and closed in June 1995. Eligibility criteria have been previously described.[14] Patients were 15–60 years old with newly diagnosed intermediate- or high-grade non-Hodgkin's lymphoma according to the Working Formulation, in stage II (bulky), III, and IV. The pretreatment characteristics of patients are reported in Table 1. Three patients were ineligible (one was pretreated and two were in stage II with bulky disease <10 cm), and one patient in PR was lost at the 10-month follow-up.

**Table 1.** Patient characteristics according to treatment

| | *Arm A (n=61)* | | *Arm B (n=63)* | |
|---|---|---|---|---|
| *Characteristic* | *n* | *%* | *n* | *%* |
| Age (years) | | | | |
| Median | 45 | | 40 | |
| Range | 18–59 | | 16–60 | |
| Sex | | | | |
| Male | 34 | 56 | 28 | 44 |
| Female | 27 | 44 | 35 | 56 |
| Performance status | | | | |
| 0 | 30 | 49 | 31 | 49 |
| 1 | 22 | 36 | 20 | 32 |
| ≥2 | 9 | 15 | 12 | 19 |
| Histology | | | | |
| Diffuse mixed | 5 | 8 | 6 | 9 |
| Diffuse large-cell | 32 | 52 | 35 | 56 |
| Large-cell immunoblastic | 12 | 20 | 13 | 21 |
| Other | | | | |
| Anaplastic/Ki-1 | 7 | 12 | 5 | 8 |
| Unclassifiable | 5 | 8 | 4 | 6 |

*Table 1 continued on next page*

**Table 1.** Continued

| Characteristic | Arm A (n=61) | | Arm B (n=63) | |
|---|---|---|---|---|
| | *n* | % | *n* | % |
| Immunophenotype | | | | |
| B | 46 | 75 | 52 | 83 |
| T | 8 | 13 | 6 | 9 |
| Null | 7 | 12 | 5 | 8 |
| Stage | | | | |
| II | 18 | 29 | 22 | 35 |
| III | 12 | 20 | 13 | 21 |
| IV | 31 | 51 | 28 | 44 |
| Symptoms | | | | |
| A | 36 | 59 | 30 | 48 |
| B | 25 | 41 | 33 | 52 |
| Extranodal sites | | | | |
| 0 | 33 | 54 | 37 | 59 |
| 1 | 13 | 21 | 11 | 17 |
| ≥2 | 15 | 25 | 15 | 24 |
| Bulky disease | | | | |
| No | 31 | 51 | 22 | 35 |
| Yes | 30 | 49 | 41 | 65 |
| Lactate dehydrogenase | | | | |
| NV | 27 | 44 | 23 | 36 |
| >1 NV | 23 | 38 | 25 | 40 |
| >2 NV | 11 | 18 | 15 | 24 |
| International Index | | | | |
| Low | 8 | 13 | 6 | 10 |
| Low-intermediate | 17 | 28 | 23 | 36 |
| Intermediate-high | 27 | 44 | 27 | 43 |
| High | 9 | 15 | 7 | 11 |
| Ineligible patients | 1 | 2 | 2 | 3 |

After pretreatment evaluation, 124 patients were randomly assigned to receive either VACOP-B[15] (arm A, 61 patients) for 12 weeks or the same regimen followed by autoBMT as described[14] (arm B, 63 patients).

Arm A patients who obtained a complete remission underwent follow-up. Patients in PR, nonresponders (NR), or relapsed patients underwent salvage DHAP[16] regimen with or without involved-field radiotherapy on residual masses. Arm B patients were to proceed to autoBMT independently of disease status (CR, PR, or NR) at the end of VACOP-B. On completion of treatment, patients with residual masses received involved-field radiotherapy. Relapsed patients were to be treated with DHAP regimen with or without radiotherapy.

Study design and data management of this prospective randomized study were detailed previously.[14] Analysis was based on status of disease on 1 December 1997. Actuarial curves were estimated according to the Kaplan and Meier[17] method and compared by the log-rank test. The relationship between parameters and outcome was examined by univariate and multivariate analysis according to Cox's hazard regression model.[18] Test statistics for comparison of main objectives were regarded as significant if the two-sided *P* value was less than 0.05.

Survival analysis according to a number of prognostic factors was performed. Prognostic factors taken into account in the stepwise Cox analysis were as follows: sex, performance status (0–1 vs. >1), histology (F vs. G vs. H + K), symptoms (A vs. B), bulky disease (≥10 cm vs. others), number of extranodal sites (0 vs. 1 vs. >1), and lactate dehydrogenase (LDH) level (≤1× vs. >1× normal value). Survival, DFS, and PFS were also retrospectively analyzed according to the International Non-Hodgkin's Lymphoma Prognostic Factors Project (International Index). The results were adjusted for age <60 years. Patients were subdivided into four groups (low, low-intermediate, intermediate-high, and high risk) according to the presence of zero, one, two, or three risk factors. These were performance status >1, LDH level >1× normal value, and stage >II. Patients in the two arms also appeared to be balanced if categorized according to the International Index (Table 1).

## RESULTS

### Response to treatment and toxicity

Arm A: after VACOP-B therapy, 34 (56%) and 19 (31%) patients achieved CR and PR, respectively. Six patients progressed, and two died of toxicity. Twelve (19%) additional PR patients obtained CR. Three patients achieved CR after radiotherapy alone, six after DHAP regimen, and three after autoBMT while in persistent PR following DHAP therapy (one of them relapsed). Another three patients received autoBMT while resistant to DHAP regimen but did not respond to the procedure. In conclusion, 46 (75%) of 61 patients achieved CR. Grade 3 and 4 WHO hematological toxicity was observed in <21% of patients, with anemia (10%), infection (10%), and mucositis (5%) during VACOP-B therapy. Treatment-related death occurred in four patients (7%) (three patients from infection and one from cardiac failure). Twelve patients (20%) died of early or late progression. Two other patients (3%) died of a second malignancy while in CR (lung cancer and acute myeloid leukemia, respectively). Currently, 43 of 61 patients are alive.

Arm B: following VACOP-B therapy, 32 (51%) and 19 (30%) patients entered CR and PR, respectively. Ten patients progressed, and two died of toxicity. Eighteen (29%) of 63 patients did not undergo melphalan, carmustine, etoposide,

cytosine arabinoside (BEAM) regimen and autoBMT procedure because of patient's refusal or progression. Forty-five patients underwent autoBMT after which 14 (22%) additional PR patients achieved CR. Five of these patients received involved-field radiotherapy. In conclusion, 46 (73%) of 63 patients achieved CR. Grade 3 and 4 WHO hematological toxicity with leukopenia and granulocytopenia was observed in about 18% of patients with anemia (7%), infection (13%), and mucositis (2%) during VACOP-B therapy. All patients experienced nausea and vomiting (grade 2 and 3). Mucositis was observed in all patients. Most patients suffered infection during the aplastic phase and required antibiotic therapy. Only one patient developed a severe grade 4 liver toxicity. Treatment-related death occurred in four patients (6%), two from infection, one from cardiac failure, and one from liver toxicity. Eighteen (29%) died of early or late progression. Currently, 41 of 63 patients are alive.

According to an intent-to-treat analysis, the difference in complete remission response rate in the two groups of patients was not statistically significant: 75% in arm A and 73% in arm B.

## Survival

The median follow-up survival of the 124 patients is 42 months with 65% of patients estimated to be alive at 6 years. No significant difference in 6-year survival was observed between arms A and B, with 65% (95% confidence interval [CI] 50–79%) and 65% (95% CI 53–77%), respectively ($P$=0.5). Univariate analysis for overall survival showed B symptoms to be the only adverse factor ($P$=0.01). Multivariate analysis showed that B symptoms remained as an adverse factor ($P$=0.03) while all other factors were not significant. There was no significant difference in DFS between the two randomized groups. The 6-year probability of DFS was 60% (95% CI 44–76%) in arm A and 80% (95% CI 68–92%) in arm B ($P$=0.1). Univariate analysis showed that male sex and bulky disease (≥10 cm) were adverse factors predicting a poor outcome ($P$=0.02 and 0.05, respectively), while all other factors were not significant. Multivariate analysis maintained bulky disease as an adverse factor ($P$=0.01). At 6 years, the rate of PFS was 48% (95% CI 33–61%) for the patients receiving conventional treatment and 60% (95% CI 48–72%) for those treated with HDT plus autoBMT ($P$=0.4). Univariate and multivariate analysis did not show any adverse factor affecting outcome (Fig. 1).

## Subgroup analysis

The International Prognostic Index (IPI) model[4] was used for a retrospective analysis of patients randomized to arm A or B. Arm A: low risk, eight patients; low/intermediate risk, 17 patients; intermediate-high risk, 27 patients; high risk,

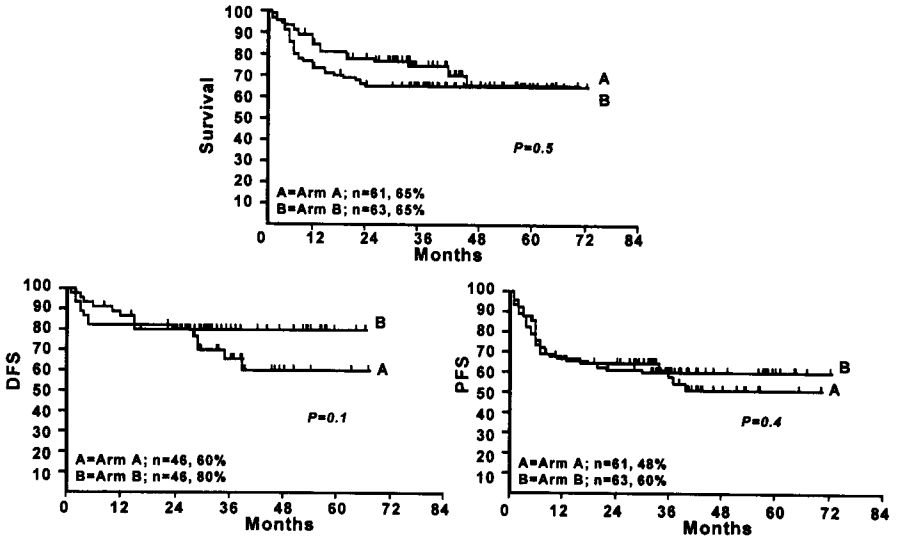


**Figure 1.** *Estimated 6-year overall survival, disease-free survival (DFS), and progression-free survival (PFS) according to treatment arm (arm A, VACOP-B; arm B, VACOP-B plus autoBMT).*

nine patients. Arm B: low risk, six patients; low/intermediate risk, 23 patients; intermediate-high risk, 27 patients; high risk, seven patients (Table 1). Because of the low number of patients in low- and high-risk groups, patients with no negative factors were combined with patients with one negative factor, and patients with three negative factors were combined with patients with two negative factors. In the combined low- plus low-/intermediate-risk groups, the 6-year statistical analysis did not show any difference in terms of overall survival, DFS, and PFS between arms A and B. In the combined intermediate-/high- plus high-risk groups, the 6-year statistical analysis did not show any difference in terms of overall survival between the two arms of patients. However, for DFS, a statistical advantage was evident for arm B patients (48 vs. 87%, respectively; $P$=0.008). A positive trend was also observed for PFS in favor of the HDT plus autoBMT (37 vs. 65%, respectively; $P$=0.08) (Fig. 2).

## DISCUSSION

The VACOP-B regimen as front-line therapy offers a good percentage of complete remissions in aggressive NHL. This is in line with previous reports.[19] The addition of a salvage non–cross-resistant chemotherapeutic protocol (DHAP) in arm A or HDT plus autoBMT in arm B gives similar results for response rate.

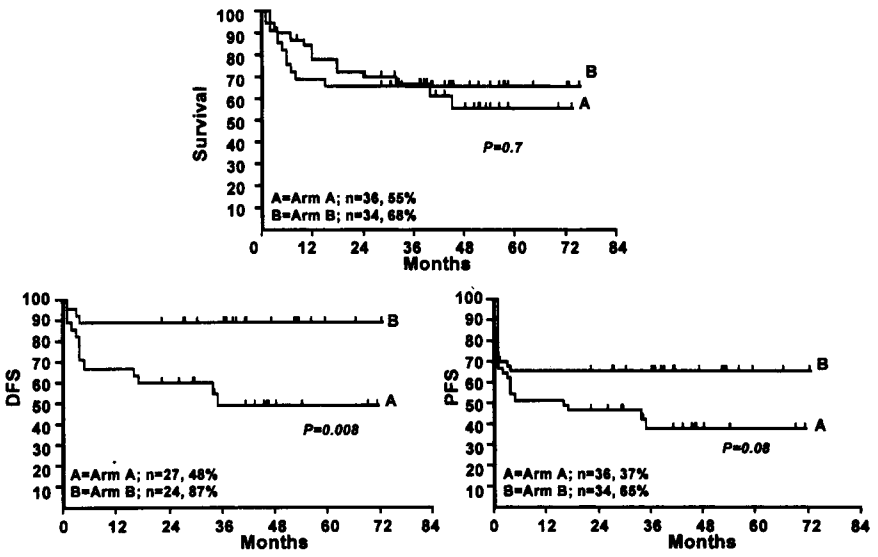


**Figure 2.** *Estimated 6-year overall survival, disease-free survival (DFS), and progression-free survival (PFS) for intermediate-/high- plus high-risk patients according to treatment arm (arm A, VACOP-B; arm B, VACOP-B plus autoBMT).*

Projected 6-year actuarial curves show a similar probability in terms of overall survival, DFS, and PFS in both arms. Trends favor HDT plus autoBMT only for DFS. Results were not modified when three patients in arm A were removed from the analysis. These three patients were in PR after VACOP-B and DHAP regimens and obtained a CR following HDT plus autoBMT. The similarity in projection of the overall survival curves of the two arms reflects the activity and the effectiveness of salvage treatment for patients relapsed after conventional treatment and the problems of feasibility in performing autoBMT procedure. The positive trend for DFS in favor of HDT suggests the usefulness of HDT plus autoBMT as front-line therapy in achieving a thorough eradication of the disease. But this advantage is balanced in terms of overall survival by the more concrete possibility of salvage treatment for patients in arm A.

According to our experience, the use of high-dose therapy did not increase procedure-related death, which was very similar in both arms. The major problem of our study was its feasibility. A high number of patients in arm B (29%) did not receive HDT and autoBMT. There were many reasons for this, the most common being death, progression, and patients' refusal to undergo treatment while in first CR.

Univariate and multivariate analysis of the negative factors presented at diagnosis failed to show any factor able to predict a poor outcome, excluding B symptoms, in terms of overall survival, and bulky disease in terms of DFS.

Patients were stratified in two groups (low plus low/intermediate risk and intermediate/high plus high risk) according to the International Index. The lower-risk patients did not demonstrate any advantage in terms of overall survival, DFS, or PFS when treated with aggressive therapy. In contrast, higher-risk patients undergoing autoBMT showed a significant improvement in DFS ($P$=0.008) and a positive trend in PFS ($P$=0.08). This is partly in line with the results of other reports.[11] It follows that the actual benefit of HDT and autoBMT as part of front-line therapy may be restricted to higher-risk patients. These data suggest that this working hypothesis, based on a retrospective analysis, must be further explored in prospective randomized trials.

Gianni et al.[20] demonstrated a significant advantage of high-dose sequential chemotherapy (HDS) followed by autografting procedure over conventional methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin (MACOP-B) chemotherapy for aggressive B cell lymphomas. With the exception of overall survival probability, all other parameters suggested an advantage in the use of HDS followed by HDT plus autografting. These results cannot be compared with our own since Gianni et al. included multiple courses of high-dose chemotherapy in their trial and patient selection was more restricted. The activity and effectiveness of this approach may be explained either by the relative simplicity of the PBPC procedure compared with the difficulties of autoBMT or by the impact of sequential chemotherapeutic agents used at full dosage. Although this single randomized trial is in favor of the use of HDS and PBPC autografting for improving the outcome in aggressive NHL, further testing is required.

The Non-Hodgkin's Lymphoma Co-operative Study Group has initiated a new randomized trial in high-risk, poor-prognosis non-Hodgkin's lymphoma to explore the possible superiority of HDS with PBPC autografting over conventional chemotherapy treatment.

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# CHAPTER 5
# MYELOMA

# Total Therapy With Tandem Transplants for Newly Diagnosed Multiple Myeloma

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Between August 1990 and August 1995, 231 patients (median age 51, 53% Durie-Salmon stage III, median serum beta-2-microglobulin 3.1 g/L, median C-reactive protein 4 g/L) with symptomatic multiple myeloma were enrolled in a program that employed a series of induction regimens and two cycles of high-dose therapy ("total therapy"). Remission induction used non-cross-resistant regimens (vincristine-doxorubicin-dexamethasone [VAD]); high-dose cyclophosphamide and GM-CSF with peripheral blood stem cell collection; and etoposide-dexamethasone-cytarabine-cisplatin [EDAP]). The first high-dose treatment comprised melphalan 200 mg/m$^2$ and was repeated if complete or partial remission (CR or PR) was maintained after the first transplant; in case of less than PR, total-body irradiation or cyclophosphamide was added. Interferon-(-2b maintenance was used after the second autotransplant. Fourteen patients with HLA-compatible donors underwent an allograft as their second high-dose therapy cycle. Eighty-eight percent completed induction therapy, while first and second transplants were performed in 84 and 71% (the majority within 8 and 15 months, respectively). Eight patients (3%) died of toxicity during induction, and two (1%) and six (4%) during the two transplants. True CR and at least a PR (PR plus CR) were obtained in 5% (34%) after VAD, 15% (65%) at the end of induction, and 26% (75%) after the first and 41% (83%) after the second transplants (intent-to-treat). Median overall and event-free survival (OS and EFS) durations were 68 and 43 months, respectively. Actuarial 5-year OS and EFS rates were 58 and 42%, respectively. The median time to disease progression or relapse was 52 months. Among the 94 patients achieving CR, the median CR duration was 50 months. On multivariate analysis, superior EFS and OS were seen in the absence of unfavorable karyotypes (11q breakpoint abnormalities, −13 or 13-q) and with low beta-2-microglobulin at diagnosis. CR duration was significantly longer with early onset of CR and favorable karyotypes. Time-dependent covariate analysis suggested that timely application of a second transplant extended both EFS and OS significantly,

independent of cytogenetics and beta-2-microglobulin. Total therapy represents a comprehensive treatment approach for newly diagnosed myeloma patients, employing multiregimen induction and tandem transplantation followed by interferon maintenance. As a result, the proportion of patients attaining CR increased progressively with continuing therapy. This observation is particularly important because CR is a *sine qua non* for long-term disease control and, eventually, cure.

# Immune-Based Strategies to Improve Hematopoietic Stem Cell Transplantation for Multiple Myeloma

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## ABSTRACT

We are developing posttransplant immune therapies to treat minimal residual disease (MRD) after high-dose therapy and hematopoietic stem cell transplantation (HSCT) in an attempt to improve outcome. Of 61 patients with multiple myeloma (MM) who underwent CD6-depleted allogeneic bone marrow transplantation (alloBMT), there were 28% complete responses (CR), 57% partial responses (PR), 3% no responses (NR), and only 5% transplant-related deaths. Forty-seven percent of patients developed no graft-vs.-host disease (GVHD) and 36% had grade I GVHD; only 17% patients developed higher than grade II GVHD. Median progression-free survival (PFS) and overall survival (OS) were 1.03 and 1.95 years, respectively. Importantly, five of six patients with relapsed MM post–CD6-depleted allografting have responded (three CR and two PR, in two cases without GVHD) to CD8-depleted donor lymphocyte infusions ($CD4^+$ DLI). Based on T cell Vβ repertoire analysis, distinct T cell clones appear and coincide with the appearance of GVHD vs. graft-vs.-MM (GVM) effect. We are now using $CD4^+$ DLI at 6 months post–CD6-depleted alloBMT to enhance GVM and thereby improve outcome: six patients have received $CD4^+$ DLI, and there are two CR, two PR, and two patients too early to evaluate. To date we have also carried out high-dose therapy and autologous hematopoietic stem cell transplantation (HSCT), including monoclonal antibody (mAb)-purged BM or $CD34^+$ peripheral blood stem cells (PBSC), in 105 patients with MM. There were 30% CR, 62% PR, 3% NR, and one transplant-related death; median PFS and OS from time of autoHSCT are 2.21 and 3.56 years, respectively. Attempts to improve outcome include purging autografts by ex vivo transduction of PBSCs with thymidine kinase gene using adenoviral vectors with a tumor-selective promoter followed by ganciclovir treatment, a strategy that can deplete >6 logs MM cells without adversely affecting normal HSC. In addition, we are attempting to enhance autologous immunity to MM to treat minimal residual

disease (MRD) posttransplant: first, CD40 activation of MM cells enhances their antigen presenting cell (APC) capacity for the generation of autologous MM-specific T cells ex vivo to be used in adoptive immunotherapy; and second, MM cells are being fused with dendritic cells (DCs) to enhance their APC function, since MM-DC fusion vaccines can induce in vitro specific responses and have demonstrated activity for both prophylaxis and treatment in syngeneic murine MM models. Our studies also confirm that it is possible to generate large numbers of DCs from patients with MM which retain both normal phenotype and APC function. The stage is therefore set for adoptive immunotherapy and/or vaccination trials to treat MRD posttransplant and thereby improve outcome.

## INTRODUCTION

Multiple myeloma (MM) will account for 13,800 (1.1%) new cancer cases in the United States in 1998, including 7200 (1.1%) new male and 6600 (1.1%) new female cancer cases, and will be responsible for 11,300 (2.0%) cancer deaths.[1] Although MM cells are responsive to radiotherapy and chemotherapy, durable complete responses (CR) are rare. Chemotherapy with oral melphalan and prednisone (MP) produces response rates as high as 50–60%; however, prospective randomized trials of MP vs. various combinations have failed to clearly show that combination chemotherapy can improve outcome.[2,3] Although maintenance recombinant alpha interferon (IFN) prolongs the response to conventional therapy in patients with near CR to chemotherapy or those with IgA or light-chain MM in some reports, other studies have not confirmed a benefit for IFN therapy.[4] To date, therefore, conventional therapy approaches have not resulted in long-term disease-free survival. The mean age of affected individuals for men is 62 years (75% <age 70) and for women is 61 years (79% <age 70),[5] highlighting the importance of developing new treatment strategies for this presently incurable disease.

A most encouraging lead for new treatment approaches for MM stems from reports of CR after the administration of alkylating agents in higher than conventional doses with or without total body irradiation (TBI), followed by syngeneic or allogeneic bone marrow transplantation (BMT).[6,7] Reduction in tumor mass in some cases has been dramatic, with CR rates commonly in the 40% range and a similar number of partial responses (PRs). Syngeneic BMT has been done infrequently, but patients reported from Seattle remain progression-free at long intervals post-BMT.[8] The European Bone Marrow Transplant Group (EBMT) has reported on allografting in 162 patients with MM: overall actuarial survival was 32% at 4 years and 28% at 7 years for the 72 (44%) patients who achieved CR after BMT. However, overall PFS was 34% at 6 years, and only nine patients remain in continuing CR at >4 years after allografting.[9,10] Favorable pre-BMT prognostic factors for both response to and survival after BMT were female sex, IgA myeloma, low serum β2 microglobulin

(sβ2M), stage I disease at diagnosis, having received one line of previous treatment, and being in CR prior to BMT. Of major concern, however is the 40% transplant-related mortality (50% in males) in the EBMT experience.[11] In a report of allografting for MM in Seattle, actuarial probabilities of overall and event-free survival (OS and EFS) for the 36% patients achieving CR were 0.50 + 0.21 and 0.43 + 0.17, respectively, at 4.5 years. Adverse prognostic factors included: transplantation >1 year from diagnosis, sβ2M >2.5 at transplant, female patients transplanted from male donors, having received >8 cycles of chemotherapy, and Durie Salmon Stage III disease at the time of BMT. Again, toxicity was common, with 35 (44%) patients dying of transplant-related causes within 100 days of BMT.[12] Finally, there were 15 deaths among 36 patients within the first 100 days after allografting in an ongoing multigroup randomized trial of high-dose versus conventional therapy for MM in the United States. As a result of this excessive toxicity, the allografting arm of this study has been closed, and individual academic centers are encouraged to develop strategies to achieve and maintain high remission rates, while avoiding transplant-related morbidity and mortality.

High-dose chemoradiotherapy followed by transplantation of either autologous BM or PBPCs has also achieved high (40%) CR rates, but the median duration of these responses has unfortunately been only 24–36 months at best.[13,14] Patients with sensitive disease and who are less heavily pretreated have the most favorable outcomes. The EBMT Registry has compiled data on 907 patients who underwent autologous stem cell grafting for MM and reported that autografting is most effective when applied early in the course of disease in younger, chemotherapy-responsive patients.[15] The achievement of CR, a non–TBI containing preparative regimen, and alpha IFN maintenance treatment is associated with more favorable outcome. As in other settings, engraftment in patients with MM who receive PBSC autografts is more rapid than in recipients of BM.[16] Most importantly, a national randomized trial in France of 200 patients with MM who received two courses of vincristine, melphalan, cyclophosphamide, prednisone (VMCP) alternating with vincristine, carmustine, doxorubicin, prednisone (VBAP) and then were randomized to receive either conventional chemotherapy (eight additional courses of VMCP/VBAP) or high-dose therapy (melphalan and TBI) followed by autologous BMT has demonstrated significantly higher response rates, EFS, and OS for those patients treated with high-dose compared with those receiving conventional therapy.[17] Response rates in the high-dose and conventional arms were 81 vs. 57%, respectively. The 5-year probability of EFS and OS was 28 and 52%, respectively, in recipients of high-dose therapy and only 10 and 12%, respectively, in patients treated with conventional therapy; treatment-related mortality was comparable between the two groups. However, with further follow-up, the 6-year postdiagnosis EFS in the high-dose arm was only 24%.[18] Therefore, although this study is encouraging, and additional randomized trials in the United States,

Scandinavia, Spain, and England are comparing the outcome of conventional therapy versus high-dose therapy and autografting, it is unlikely that any patients are cured after a single high dose and stem cell autografting regimen. Nonetheless, the importance of randomized trials is highlighted by a recent study which demonstrated that the median survival of patients with MM who are <65 years old and respond to initial chemotherapy, who would be good candidates for autografting protocols, is 5 years.[19]

Experience from around the world therefore now suggests that high response rates can be achieved in affected patients using high-dose therapy followed by HSCT; however, patients are destined to relapse, and few, if any, are cured. Major obstacles to cure are the excessive toxicity noted after allografting in MM, contaminating tumor cells in MM autografts, and most importantly, the persistence of MRD after high-dose therapy followed by either allogeneic or autologous HSCT. In this context, we are first developing improved strategies to decrease MRD in patients with MM using high-dose therapy followed by allogeneic or autologous stem cell grafting. Second, we are evaluating multiple approaches for the generation and enhancement of allogeneic and autologous anti-MM immunity in vitro and in animal models. Based on these studies, we will design clinical trials that couple our treatments to achieve MRD with these novel immune-based therapies for MRD posttransplant, in an attempt to achieve long term disease-free survival and potential cure of MM.

## METHODS

### Allogeneic BMT

Patients with MM sensitive to chemotherapy who are <60 years of age with acceptable cardiac, pulmonary, liver, and renal function are eligible for T (CD6) cell depleted allogeneic BMT. Ablative therapy consists of either cyclophosphamide 60 mg/kg or melphalan 70 mg/m$^2$ given on 2 consecutive days, followed by TBI (1200 rads total dose, with 200 rads given twice a day for 3 days). Patients in whom previous local radiation therapy precludes TBI received busulfan administered over a 4-day period in 16 equal divided doses (1 mg/kg every 6 hours for a 16 mg/kg total dose) followed by cyclophosphamide (60 mg/kg/d for 2 days). T cells are depleted from allogeneic BM using CD6 mAb and complement lysis, as previously described.[20] No other prophylaxis for GVHD is routinely administered.

### CD4$^+$ DLI

Leukocytes are harvested from marrow donors by leukapheresis. Mononuclear cells are isolated by density gradient centrifugation using Ficoll-Hypaque. CD8$^+$

cells are removed from the donor leukapheresis product with anti-CD8 mAb and rabbit complement lysis.[21] The dose of DLI administered is $3\times10^7$ $CD4^+$ cells/kg. No GVHD prophylaxis is given post-DLI.

### Autologous HSCT

Patients with MM sensitive to chemotherapy who are <70 years of age with acceptable cardiac, pulmonary, liver, and renal function are eligible for autologous HSCT. Ablative therapy is as described above for allografting. Fifty patients received autologous BM which was purged of tumor cells using CD10, CD20, and PCA-1 mAbs and complement lysis, as in previous studies.[22–25] Fifty-five patients received PBSCT, $CD34^+$ enriched using the CEPRATE system, as in prior studies.[26–28]

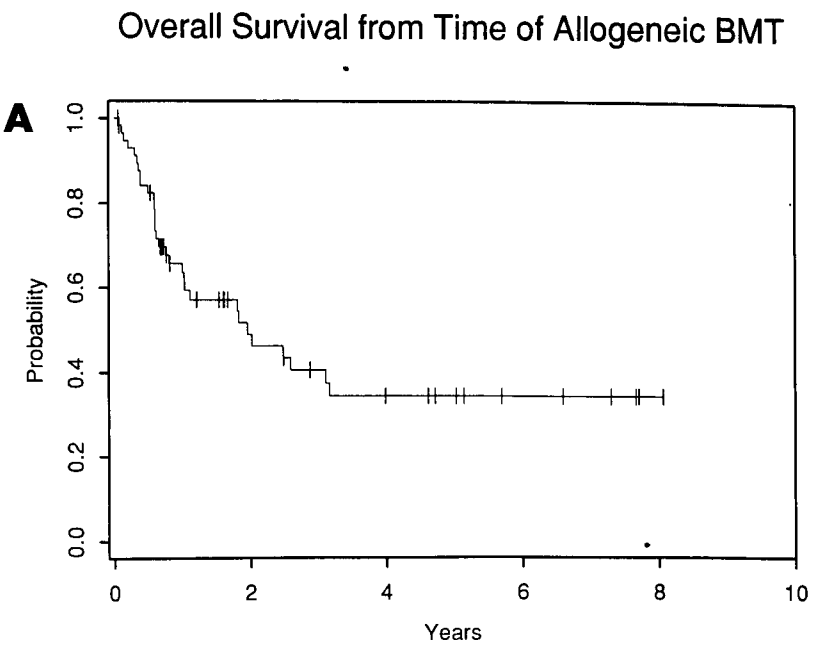
## RESULTS

### Allogeneic BMT

In an attempt to improve the outcome of allografting in MM by avoiding transplant-related toxicity, we have carried out T (CD6) depleted allografting using histocompatible sibling donors in 61 patients with MM whose disease remained sensitive to conventional chemotherapy.[20,25] This included 39 men and 22 women with median age of 44 (32–55) years. At time of diagnosis nine, 17, 20, and 11 patients presented with stages IA, IIA, IIIA, and IIIB disease, respectively, and three patients presented with plasmacytomas. Median time to neutrophil and platelet engraftment was 12 (7–24) and 18 (10–46) days, respectively. There were 17 (28%) CR and 34 (57%) PR, two (3%) NR, three (5%) transplant-related deaths, and four (7%) patients too early to evaluate. Twenty-eight (47%), 21 (36%), seven (12%), two (3%), and one (2%) patients developed maximum grades of 0, 1, 2, 3, and 4 GVHD, respectively. Median PFS and OS are 1.03 and 1.95 years, respectively (Fig. 1). Median follow-up among those alive is 1.6 years, with maximum follow-up time of 9.5 years.

### $CD4^+$ DLI treatment

We have demonstrated that the graft-vs.-leukemia (GVL) effect of DLI can be preserved and that associated GVHD can be abrogated in some patients by using $CD4^+$ DLI.[21] To date, we have used $CD4^+$ DLI to treat 40 patients with relapsed hematologic malignancies post–CD6-depleted allografting. Overall only 12 of 38 (32%) patients developed GVHD, and treatment-related mortality was low (3%), with one death related to infection in the setting of immunosuppression for GVHD.



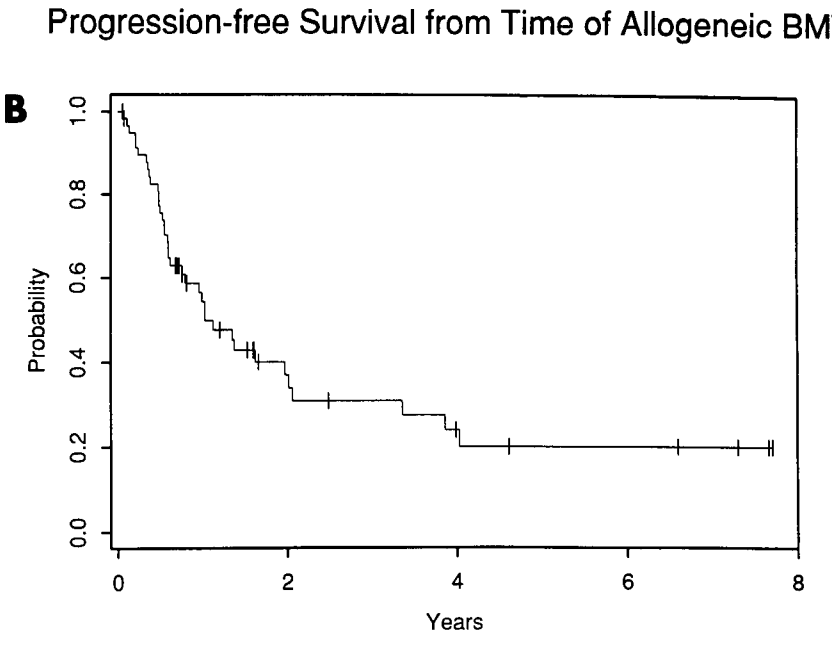




**Figure 1.** *Progression free and overall survival after CD6 depleted allogeneic bone marrow transplantation for multiple myeloma.*

Cohorts of patients received 0.3, 1.0, and $1.5 \times 10^8$ $CD4^+$ cells/kg. Responses were seen in 15 of 19 (79%) patients with early relapsed CML. Of note, five of six (83%) evaluable patients with MM responded (three CR and two PR) at a median of 26 (18–62) weeks after DLI. Responses were associated with GVHD in three cases, but in two patients there were no clinical or laboratory abnormalities consistent with GVHD. Three responders progressed at 26, 38, and 62 weeks post-DLI.

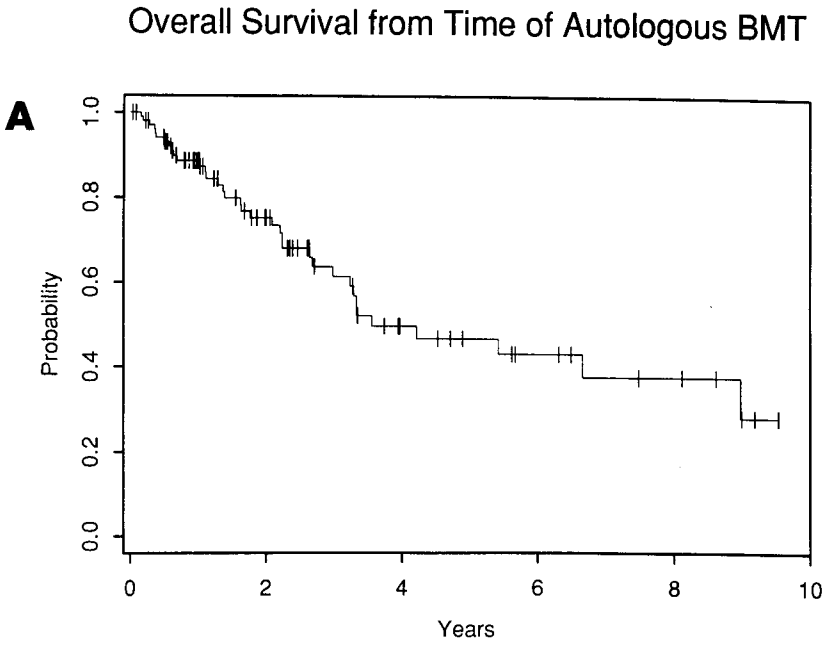
Given the high response rates but inevitable relapses observed in the setting of allografting for MM,[9,10,12,25] we are using $CD4^+$ DLI at 6 months after CD6-depleted BMT to treat MRD posttransplant and thereby improve outcome. Patients who are 6–9 months post-CD6 allografting and who have less than grade 2 GVHD are eligible to receive $CD4^+$ DLI. To date, 17 patients have undergone CD6-depleted allografting, and 12 patients developed grades 0–1 GVHD. Six of these patients are >6 months post-BMT and have received $CD4^+$ DLI. After $CD4^+$ DLI, three patients each have developed grades 0–1 GVHD and grades 2–4 GVHD. There are two CR, two PR, and two patients too early to evaluate post–$CD4^+$ DLI.

## Autologous HSCT

We have carried out autografting (using mAb-purged BM or $CD34^+$ PBPCs) in 105 patients with MM whose disease remained sensitive to conventional chemotherapy.[22–25] This includes 68 men and 37 women with median age of 52 (30–69) years. At the time of diagnosis, 19 (18%), 16 (15%), 52 (50%), and 15 (14%) patients had stages IA, IIA, IIIA, and IIIB disease, respectively; three (3%) patients presented with plasmacytomas. Median time to neutrophil engraftment was 22 (10–53) and 11 (8–17) days in recipients of autologous BM and PBSCs, respectively. Median time to platelet engraftment was a median of 29 (10–371) and 13 (8–97) days in recipients of autologous BM and PBSC grafts, respectively. There were 31 (30%) CR, 64 (62%) PR, three (3%) NR, and one (1%) toxic death; four (4%) patients are too early to evaluate. Median PFS is 2.2 years, with median OS from time of BMT of 4.2 years (Fig. 2). The PFS and OS of patients treated at our institute do not significantly differ (Fig. 3).

## DISCUSSION

High-dose therapy followed by allografting is associated with high response rates but no cures in MM. Allogeneic stem cell grafting is further limited by excessive toxicity. By using CD6 depletion, we are attempting to achieve MRD in MM using allografting and developing immune therapies to treat MRD posttransplant and thereby achieve long-term disease-free survival and potential cure. Our first efforts are to use CD6 T cell–depleted allografts to abrogate GVHD and then use $CD4^+$ DLI to augment GVM posttransplant. The use of DLI to treat



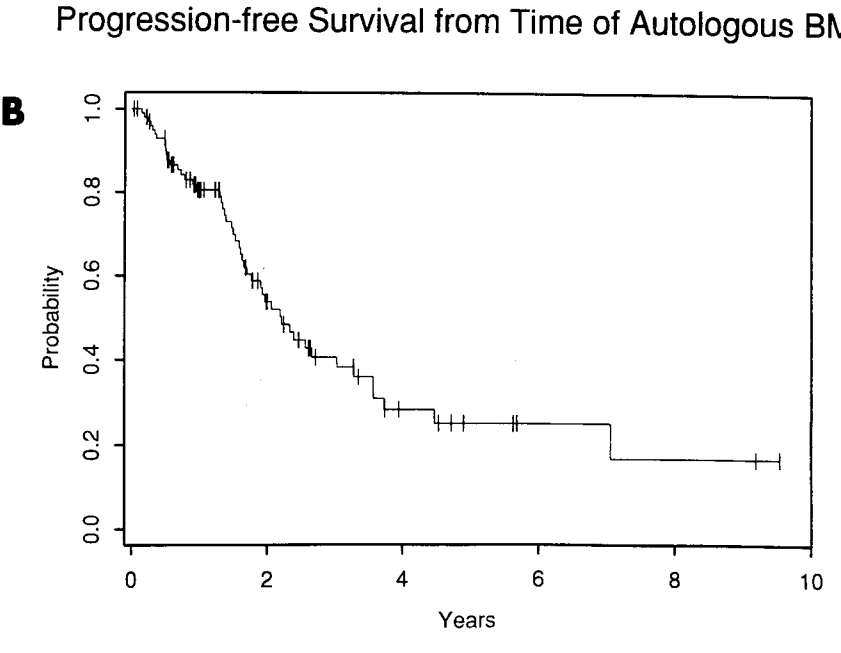




**Figure 2.** *Progression free and overall survival after autologous hematopoietic stem cell transplantation for multiple myeloma.*

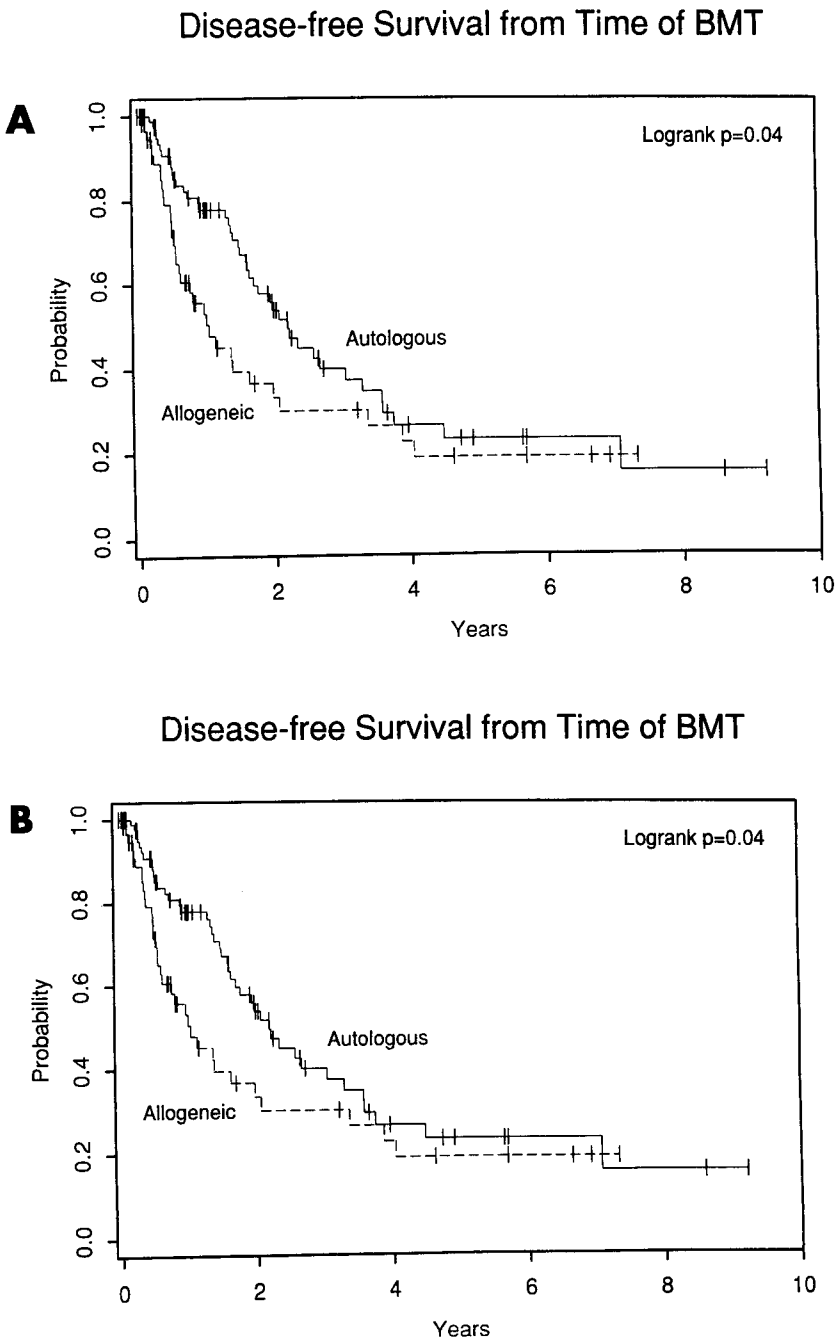



**Figure 3.** *Comparison of progression free and overall survival after allografting and autografting for multiple myeloma.*

patients with relapsed MM after allografting has provided unequivocal evidence of GVM.[29,30] The largest single-center experience showed that eight of 13 patients treated with DLI for relapsed MM after allografting responded, including four CR and four PR.[31] In these cases, GVHD was observed after DLI; however, infusion of thymidine kinase gene–transduced DLI followed by treatment of the recipient with ganciclovir has been proposed as one potential strategy to treat DLI-associated GVHD.[32]

The observations that total T cell and $CD8^+$ numbers correlate with GVHD post–CD6-depleted BMT,[33] coupled with the demonstration by Champlin and colleagues that $CD8^+$ T cell depletion of donor BM or DLI can avoid GVHD,[34] suggested to us that $CD4^+$ enrichment of DLI could perhaps preserve GVM while avoiding GVHD. Our early experience suggests that $CD4^+$ DLI, both in the context of treating relapsed MM after allografting and when given at 6–9 months after allografting to treat MRD, can be associated with GVM in the absence of clinical GVHD in some patients.[21] Moreover, we are evaluating T cell repertoire, assessed by Vβ T cell receptor gene (TCR) rearrangement, to identify clonal T cells associated with clinical GVM and to delineate potential target antigens on tumor cells. To date, we have completed molecular analysis of the TCR repertoire in four patients with relapsed MM who received $CD4^+$ DLI, three of whom responded but also developed GVHD (Orsini et al., manuscript submitted). PCR amplification of 24 Vβ subfamilies determined the relative utilization each Vβ gene subfamily, and analysis of complementarity-determining regions 3 (CDR3) for each TCR Vβ gene subfamily identified clonal and oligoclonal T cells. Interestingly, distinct clonal T cell populations were evident at the time of GVM vs. GVHD in these three patients. In a single patient, we have identified two distinct GVM clones, both of which are $CD8^+$ cytolytic T cells and one of which targets the idiotypic protein. The target antigen of the other GVM clone remains under active investigation. The results of these studies will lay the groundwork for ex vivo expansion of antigen-specific T cells targeting MM cells to be used in trials of adoptive immunotherapy to treat MRD posttransplant.

We are also attempting to improve the outcome of autografting in MM by increasing the efficacy of PBSC purging and, importantly, by developing methods for enhancing autologous anti-MM immunity to treat MRD posttransplant. In terms of autograft purging, mAb-based techniques deplete only 2–3 logs of tumor cells.[22–25] CD34 selection of autologous PBPCs can achieve up to 5 logs' depletion of tumor cells,[26] and in a larger randomized multicenter trial has recently been shown to deplete 3.1 logs of MM cells.[28] The clinical significance of this degree of depletion of tumor cells remains to be determined. Moreover, although depletion of up to 5 logs of MM cells can be achieved using these approaches,[26,35] residual tumor cells are detectable in the autograft, as well as in the patient, after transplant.[27] To improve depletion of MM cells from autografts, we have recently

taken advantage of the selective presence of adenoviral receptors (Teoh et al., manuscript submitted) and Muc-1 (Treon et al., manuscript submitted) antigens on MM cells, but not on HSCs. We have used tumor-selective promoters and adenoviral vectors to transfect thymidine kinase gene into MM cells and, with ganciclovir treatment, effectively purged >6 logs of MM cells within BM without adversely affecting normal HSCs (Teoh et al., manuscript submitted).

If one is to attempt postautografting immune therapies, one must first address whether MM patients can respond to their own tumor cells. Multiple reports suggest that patients may mount an anti-MM immune response,[36,37] but whether this can be of clinical significance is unclear. In a recent study, five MM patients were repeatedly immunized with autologous monoclonal Ig: in three patients, an anti-idiotypic interferon γ and IL-4–secreting T cell response was amplified 1.9- to 5-fold, and B cells secreting anti-idiotype Ab increased during immunization; in two patients, this was associated with a gradual decrease in $CD19^+$ monoclonal B cells. However, the induced T cell response was eliminated during repeated immunization.[38] Osterborg and colleagues have vaccinated patients with MM using autologous M component and soluble granulocyte-macrophage colony-stimulating factor (GM-CSF) and evoked idiotype-specific, predominantly MHC I–restricted, type I T cell responses.[38] Most recently, Hsu and co-workers have investigated the possibility of using autologous DCs pulsed ex vivo with tumor-specific idiotypic protein to stimulate host antitumor immunity: nine patients developed T cell–mediated anti-idiotype and anti-KLH proliferative responses that were specific for the immunizing Ig.[39] In one case, T cells were expanded in vivo which specifically lysed autologous tumor; interestingly, anti-idiotypic Abs were not detected, consistent with the view that the idiotype-pulsed DCs induced a T helper 1 (TH1), rather than a TH2, response against idiotypic determinants on the MM cell. Recent studies have shown that MM cells have can be good APCs.[40] Further studies are needed to optimize the immunization schedule to achieve long-lasting T cell immunity and determine its effect on clinical outcome.

Several attempts are currently underway to enhance autologous immunity to MM to treat MRD after autografting and thereby improve outcome. Champlin and colleagues have recently reported on the use of cyclosporine to induce GVHD postautografting in an attempt to generate associated autologous GVM.[41] We are currently evaluating two strategies to enhance autologous immunity to MM. The first is based on the demonstration that CD40 activation can enhance antigen-presenting function of MM cells, assessed in an autologous MLR.[42,43] This is likely related to the upregulation of class I and II HLA antigens as well as costimulatory molecules on the MM cell surface, although its mechanism remains under investigation. Based on this finding, we are currently harvesting T cells from MM patients who are to undergo autografting before they are treated with chemotherapy. The patients then proceed with initial and then ablative chemotherapy and autologous

PBSC transplantation; concomitantly, an ex vivo expansion of their T cells is undertaken, using CD40-activated γ-irradiated autologous MM cells as stimulators. Preliminary studies suggest that these T cells can be expanded effectively to the level of $10^8$–$10^9$ and safely administered as adoptive immunotherapy. In vitro studies show their reactivity with autologous MM cells, but not with autologous B cells, CD40-activated autologous B cells, or allogeneic MM cells. The clinical utility of this treatment is under active investigation. A second strategy for enhancing autologous immunity to MM posttransplant involves vaccines. One example at our institute involves vaccinations with fusions of MM cells with DCs (MM-DC fusions). We have pioneered vaccinations with MM-DC fusions or with transduced DCs in breast cancer models at our institute[44,45] and demonstrated both triggering of specific in vitro T cell reactivity and clinically significant in vivo responses in animal models. MM-DC fusions used as vaccines have similarly induced in vitro and in vivo specific responses in animal models; to date, prophylactic MM-DC vaccinations have inhibited the subsequent ability to induce MM in syngeneic mouse models; and in animals already bearing MM, MM-DC vaccinations appear to prolong survival. Trials are now underway to test the safety and efficacy of MM-DC vaccinations in humans. Finally, to facilitate these and other novel immune approaches using DCs, we and others have shown that it is possible to generate large numbers of DCs from both the BM and PB of patients with MM which retain normal phenotype and APC function (Chauhan et al. and Raje et al., manuscripts submitted).[46,47] This is true even though our studies suggest that a fraction of these DCs contain human herpesvirus 8 gene sequences(Chauhan et al. and Raje et al., manuscripts submitted). The stage is therefore set for posttransplant immune-based treatments in patients with MM.

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# Autologous Transplantation in Multiple Myeloma: The IFM Experience

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## INTRODUCTION

In the absence of any significant improvement of conventional dose chemotherapy (CC), high-dose therapy (HDT) has been widely explored in multiple myeloma (MM) during the last 10 years. High doses of melphalan (HDM) (≤140 mg/m$^2$ intravenously) yield high remission rates, including apparent complete remissions (CR), but at the expense of severe and durable myelosuppression.[1,2] Autologous transplantation of hematopoietic stem cells (autoSCT), collected in the marrow or in the peripheral blood, reduces the hematologic toxicity of HDM and allows the use of myeloablative regimens with total body irradiation or higher doses of melphalan (200 mg/m$^2$). A number of single-center or cooperative studies have been published reporting the results of HDT followed by autoSCT.[1–3] These uncontrolled studies show that autoSCT is a useful salvage therapy for primary refractory MM and for chemosensitive relapses but is of limited value in patients with resistant disease.[1–3] For patients responding to initial induction chemotherapy, autoSCT is a safe (<5% toxic deaths) and effective consolidation therapy. Most importantly, some of these studies have shown that in newly diagnosed MM, CR rates of 30–50% can be achieved, suggesting that the greater reduction in tumor burden with this approach may lead to prolonged remission and survival.[1,2] However, these pilot studies are difficult to analyze because the recruitment of patients is subject to selection bias regarding age, performance status, renal function, and response to initial chemotherapy. Historical comparisons have suggested that survival of patients <65 years of age responding to initial chemotherapy and receiving only standard therapy was similar to that reported in selected series of patients given HDT early.[4] Therefore, prospective randomized trials were needed to compare conventional dose chemotherapy with HDT. In 1990, the Intergroupe Français du Myélome (IFM) began a trial designed to address this issue.

## THE IFM 90 TRIAL

Between October 1990 and May 1993, 200 patients <65 years of age with Durie-Salmon stage II or III MM were recruited by 33 centers. At diagnosis, they

were randomly assigned to receive either CC (VMCP [vincristine, melphalan, cyclophosphamide, prednisone]/BVAP [carmustine, vincristine, adriamycin, prednisone]) or HDT (140 mg/m$^2$ melphalan plus total body irradiation (8 Gy) followed by an unpurged autologous bone marrow transplant (autoBMT) (collected after four to six cycles of VMCP/BVAP). Maintenance therapy with interferon alpha was administered in both arms. The initial publication showed that, on an intent-to-treat analysis, HDT significantly improved the response rate with 38% CR or very good partial remissions (VGPR) vs. 14% with CC.[5]

These results have been recently updated (November 1997). With a median follow-up of 5 years, the median event-free survival (EFS) and overall survival (OS) are 18 and 42 months, respectively, in the CC arm and 28 and 57 months in the HDT arm (Fig. 1). The 6-year EFS and OS are, respectively, 14 and 28% in the CC arm and 25 and 43% in the HDT arm. HDT significantly improves both the EFS ($P$=0.01) and the OS ($P$=0.03).

## CURRENT IFM STUDIES

In the IFM90 trial, the 6-year EFS was only 25% in the HDT arm, and there was no plateau of the survival curves. Strategies to improve these results were clearly warranted. Since in this trial achievement of CR or VGPR was significantly associated with a prolongation of survival (Fig. 2), the aim of the following IFM trials was to increase the CR rate. Three issues have been addressed with this objective in mind:

- the impact of tandem transplants (IFM 94),
- the source of stem cell (IFM 94), and
- the conditioning regimen (IFM 9502).

### The impact of tandem transplants (IFM 94)

The Little Rock Group has shown that repeating intensive treatments is well tolerated as a result of autologous peripheral blood progenitor cell transplantation (PBPCT) and hematopoietic growth factors. In newly diagnosed patients, with the so-called "total therapy," the CR rate increased at each step of the procedure.[6] However, the impact of such an aggressive strategy on OS warranted further evaluation. In 1994, the IFM has initiated a randomized study comparing one and two autoSCTs in patients up to the age of 60. At diagnosis, patients were randomly assigned to receive either one course of HDT (melphalan 140 mg/m$^2$ plus 8 Gy total body irradiation) followed by autoSCT (arm A) or two courses of HDT, the first with HDM 140 mg/m$^2$ followed by PBPCT, the second with HDM 140 mg/m$^2$ plus TBI followed by autoSCT (arm B). The outline of the trial is in Fig. 3.

From October 1994 to March 1997, 405 patients with symptomatic stage I and

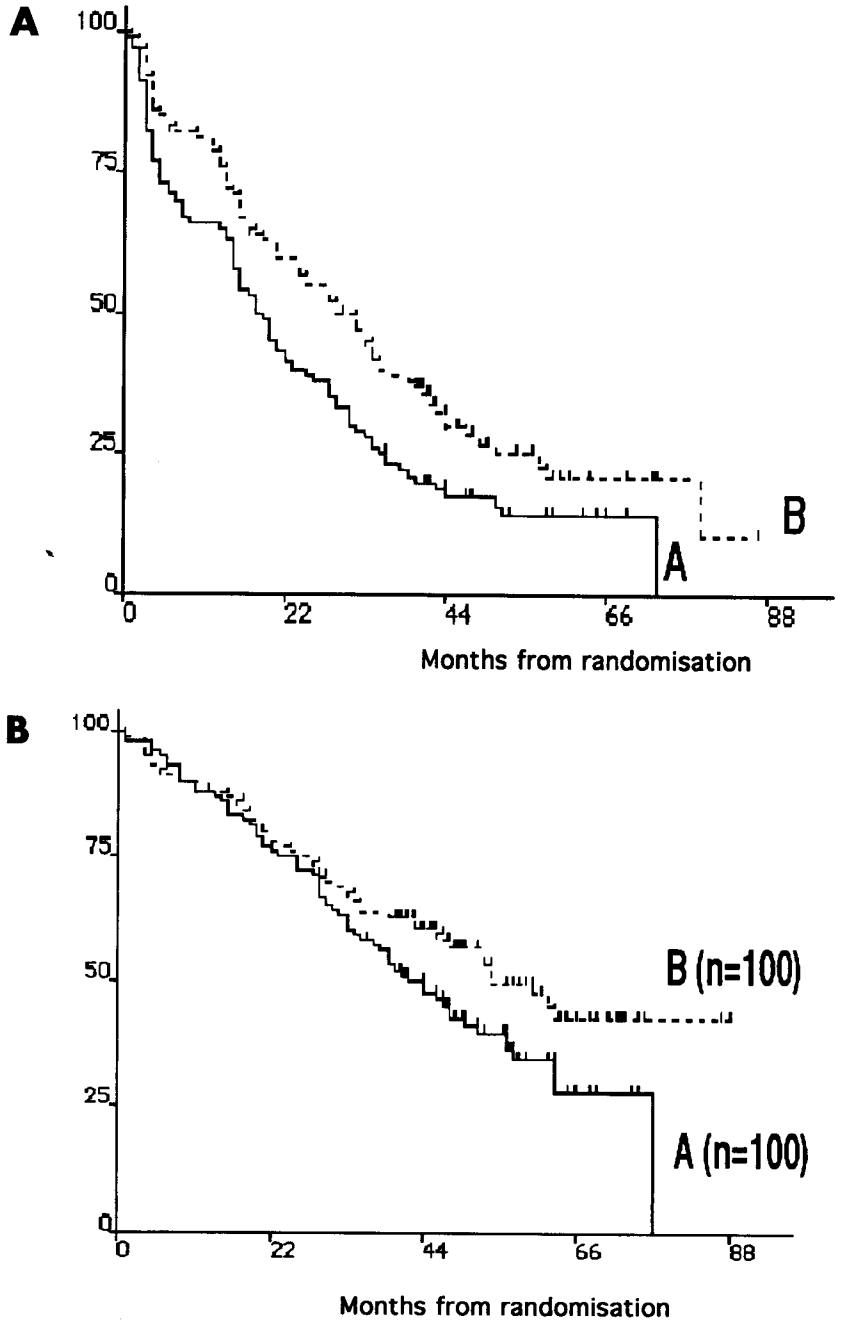



**Figure 1.** *A: IFM 90, event-free survival according to treatment arm. —, arm A (conventional chemotherapy); - - -, arm B (autoBMT). B: IFM 90, overall survival according to treatment arm. —, arm A (conventional chemotherapy); - - -, arm B (autoBMT).*

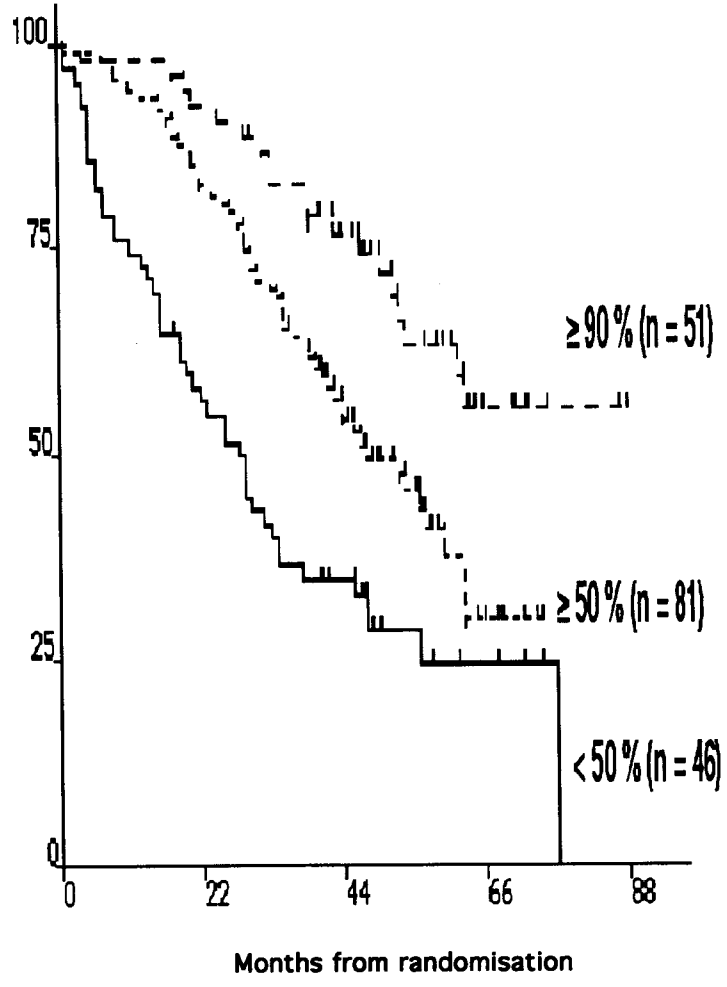



**Figure 2.** *IFM 90, overall survival according to response to treatment.*

II–III MM have been enrolled by 36 centers. A first interim analysis was performed in November 1997 on the first 200 patients with a follow-up of at least 1 year from diagnosis (median follow-up 26 months) (105 arm A, 95 arm B). The two groups are well balanced regarding the initial characteristics. (age, sex, Durie-Salmon stage, type of M-component, β2 microglobulin level, hemoglobin and calcium levels). In arm A, the ASCT has been performed in 84% of cases. In arm B, the first HDT has been performed in 85% of cases and the second HDT in 73%. The toxic death rate was low in both arms (2%).

When looking at the treatment actually received, the results were in favor of arm B (two HDT): the rate of CR + VGPR was 57% in arm B vs. 46% in arm A.

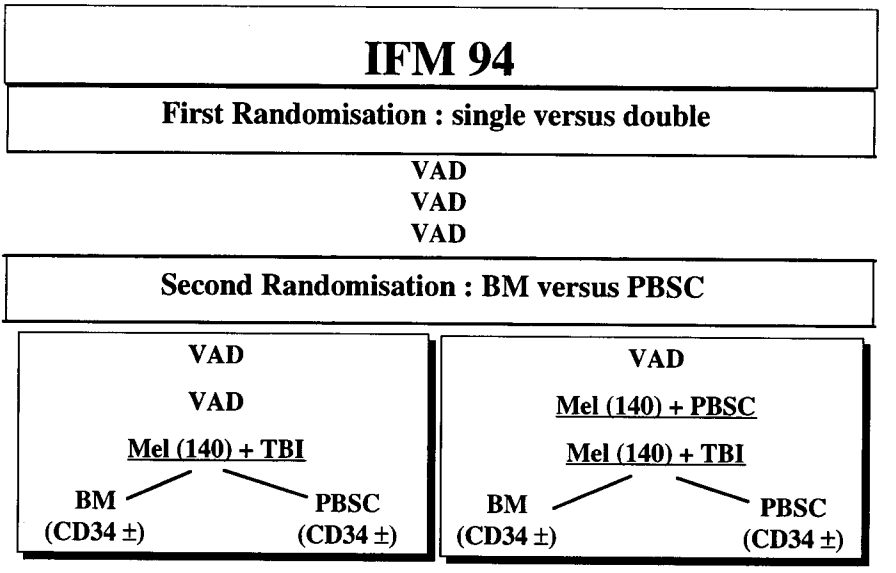



**Figure 3.** *Outline of the IFM 94 protocol.*

Although the 2-year OS was not significantly different between both arms, the 2-year EFS was 70% after two HDT vs. 57% after one HDT ($P$=0.02)

However, when the analysis was performed on an intent-to-treat basis, there was no significant difference between arm A and arm B regarding the CR rate, the 2-year EFS, and the 2-year OS (Table 1).

By multivariable analysis, the only parameter related to OS was the initial β2 microglobulin level. In the group of patients with a good prognosis (β2 microglobulin level ≤3 mg/L) the probabilities of survival at 2 years appeared to be higher in arm B (Fig. 4A) than in arm A. Conversely, for patients with a poor prognosis (β2 microglobulin level >3 mg/L), the 2-year OS appeared to be slightly better in arm A (Fig. 4B).

However, it should be emphasized that these results are preliminary and that analysis of the whole group of patients, as well as a longer follow-up, is needed before drawing any definite conclusion.

## The source of stem cells

Peripheral blood progenitors collected after moderately intensive chemotherapy (high-dose cyclophosphamide) plus granulocyte or granulocyte-macrophage colony-stimulating factor (G-CSF or GM-CSF) or after priming with G-CSF alone are currently preferred to bone marrow, mainly because of more rapid hematopoietic reconstitution. The use of hematopoietic growth factors (G-CSF or

**Table 1.** IFM94 study, comparison of one and two autoSCTs (intention to treat analysis)

| | *Arm A, one autoSCT* | *Arm B, two autoSCTs* | *P value* |
|---|---|---|---|
| *n* | 105 | 95 | |
| CR | 33% | 34% | NS |
| VGPR | 7% | 11% | NS |
| 2-year EFS | 52% | 58% | NS |
| 2-year survival | 73% | 69% | NS |

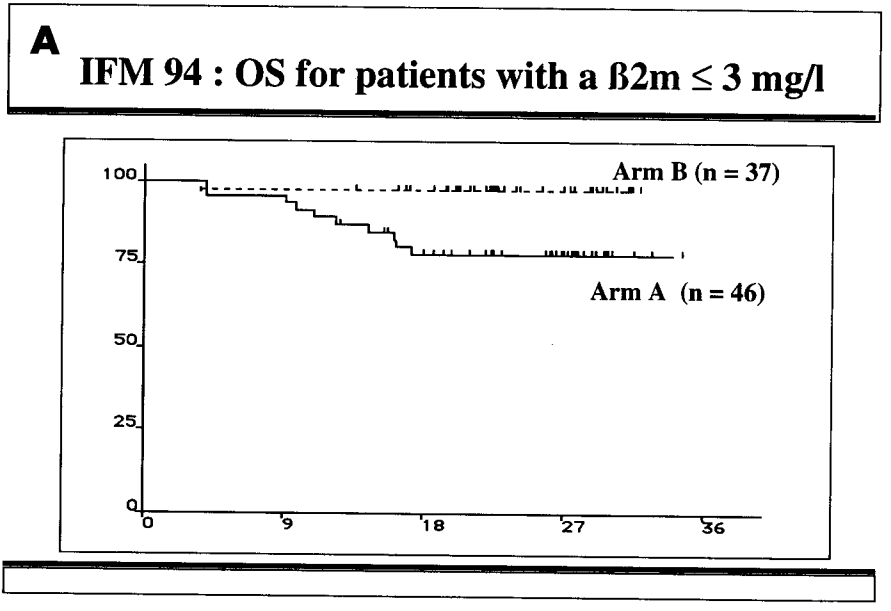
GM-CSF) after transplant and of peripheral blood instead of bone marrow progenitors has dramatically reduced the duration of myelosuppression induced by HDT. As a consequence, the duration of hospitalization and, it is hoped, the costs have been significantly reduced. However, contamination of the autologous graft by the malignant clone remains a critical concern even with blood progenitors. Sensitive methods, including amplification of the patient's rearranged gene, allow myeloma cells to be detected in blood and apheresis products. Attempts to purge marrow with cyclophosphamide derivatives or monoclonal antibodies are feasible but induce prolonged myelosuppression. Selection of $CD34^+$ progenitors appears to be a promising alternative. Tumor depletion up to 4.5 logs can be obtained with this technique, but sophisticated assays for detection of minimal residual disease show the persistence of myeloma cells in the $CD34^+$ cell fraction. The clinical impact of this cumbersome and expensive procedure has not yet been fully evaluated.[2]

The question of the source of stem cells has been also addressed by the IFM94 trial. At the time of stem cell collection, patients were randomized between autoBMT and PBPCT. PBPC were collected after priming with G-CSF alone (10 μg/kg/d for 7 days starting on day 17 after VAD course). However, investigators were allowed to perform a $CD34^+$ cell selection either on bone marrow or PBPC. Of the first 200 patients, 172 underwent the second randomization (78 BM, 94 PBPC).

However, 20 patients have not been transplanted (10 ABMT, 10 PBSCT), 22 patients assigned to receive ABMT were actually transplanted with PBPC, and one patient randomized to receive PBPC was actually transplanted with BM.

According to the protocol, CD34 selection was performed in 35 cases (nine ABMT, 26 PBPCT). Finally, 129 of the 172 randomized patients (75%) were transplanted as per protocol (37 BM, 35 CD34, and 57 PBPC). There was no significant difference between the three groups regarding the main initial characteristics, the first randomization, and the response to initial CC. With a short follow-up (median 26 months) the clinical outcome did not appear to differ significantly (Table 2).

The only difference between the three groups concerned hematopoietic reconstitution. The median durations of neutropenia ($<0.5\times10^9$/L) and thrombocy-



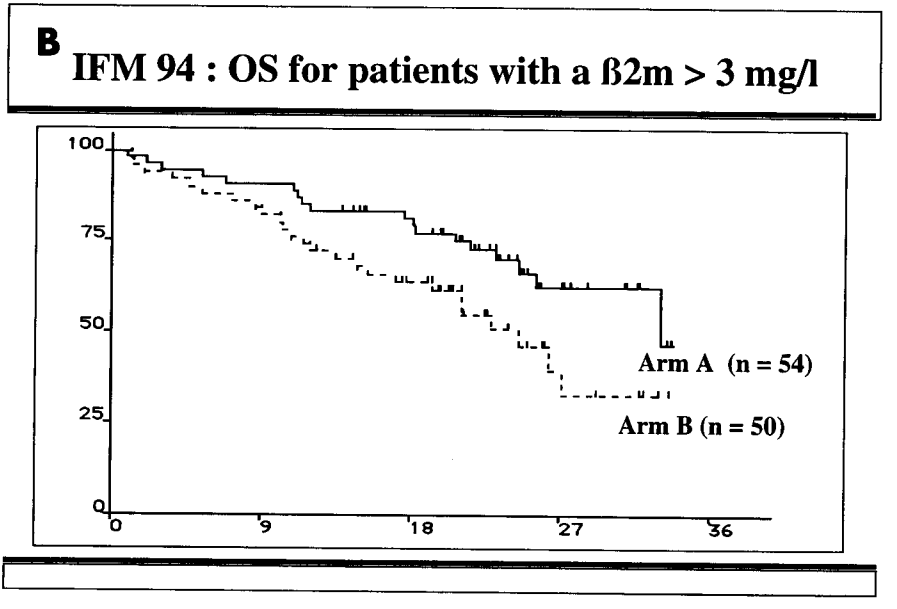




**Figure 4.** *A: IFM 94, overall survival of patients according to the initial b2 microglobulin ≤3 mg/L. — arm A (single transplant); - - -, arm B (double transplant). B: IFM 94, overall survival of patients according to the initial b2 microglobulin >3 mg/L. —, arm A (single transplant); - - -, arm B (double transplant).*

**Table 2.** IFM 9502 study, preliminary results

| | *TBI* | *HDM 200* | *P value* |
|---|---|---|---|
| *n* | 65 | 61 | |
| Status at transplant (% responding patients) | 64 | 58 | NS |
| CR (%) | 29.5* | 34.5† | NS |
| CR + VGPR (%) | 41 | 54.5 | NS |
| Response (%) | 84 | 89 | NS |
| Toxic death | 2 (4%) | 1 (2%) | NS |

**Including 3 patients in CR before transplantation. † Including 2 patients in CR before transplantation.*

topenia ($<50\times10^9$/L) were 10 and 14 days with PBPC and 11 and 13 days with $CD34^+$ cells, respectively, with no significant difference between the two groups. However, after autoBMT, the median duration of neutropenia was significantly longer (11.5 days, $P$=0.001) as well as the median duration of thrombocytopenia (21 days, $P$=0.001).

The preliminary conclusion is that there is no apparent difference in the outcome, although there was more rapid hematologic reconstitution after PBPC and $CD34^+$ cell autologous transplantation. Being easier and less expensive, PBPCT may be preferable to $CD34^+$ cell transplantation.

## The conditioning regimen

The standard conditioning regimen in the context of autoSCT is the combination of HDM 140 mg/m$^2$ and total body irradiation.

However, with this regimen, the CR rate remains <50% in newly diagnosed patients. Moreover, the feasibility of total body irradiation raises concerns in patients >60 years of age. In the IFM90 trial, 42% of the patients could not receive the assigned autoBMT in this age subgroup (compared with only 18% in patients aged ≤60 years, $P$=0.01). The Royal Marsden Group has reported an impressive 70% CR rate with HDM 200 mg/m$^2$ in newly diagnosed patients and low extramedullary toxicity.[7] The Little Rock Group also questioned the role of total body irradiation in a population of refractory patients, since HDM 200 mg/m$^2$ appeared to be superior to HDM 140 mg/m$^2$ plus total body irradiation and to be less toxic, with 1 vs. 36% early death.[8] In 1995, the IFM has initiated a randomized study comparing HDM 200 mg/m$^2$ and HDM 140 mg/m$^2$ plus total body irradiation followed by PBPCT in patients aged 60 to 65 years.

Three hundred eighty-six patients have been currently enrolled in this study. A preliminary analysis of the first 126 patients has been performed (HDM 200 mg/m$^2$, 61 patients; HDM 140 mg/m$^2$ plus total body irradiation, 65 patients). The

two groups are well balanced in regard to the initial characteristics, the response to initial CC, and the modalities of PBPC collection. The only differences between the two groups were the median number of $CD34^+$ cells in the graft, which was slightly higher in the HDM 200 mg/m$^2$ group ($8.5\times10^6$/kg vs. $6\times10^6$/kg, $P$=0.08), and the number of patients receiving growth factors after transplantation (78% in the total body irradiation group vs. 96% in the HDM200 group, $P$=0.008). Currently, there is no significant difference in clinical outcome between the two groups (Table 2). However, HDM 200 mg/m$^2$ appears to be less toxic. The median duration of neutropenia is significantly shorter (8 vs. 10 days, $P$<0.001). The difference remains significant if the analysis is restricted to the patients receiving growth factors after transplant. The median duration of thrombocytopenia is also shorter after HDM 200 mg/m$^2$ (5 vs. 6.5 days, $P$=0.03). As a consequence, the median number of platelet transfusions is reduced (one vs. three, $P$<0.001). Finally, the incidence of WHO grade ≥3 mucositis is dramatically reduced after HDM 200 mg/m$^2$ (3 vs. 52%).

If these results are confirmed by further analysis, HDM 200 mg/m$^2$ should be preferred to the standard regimen HDM 140 mg/m$^2$ plus total body irradiation, since this regimen has similar efficacy but is easier to perform and is, apparently, less toxic.

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# Syngeneic Transplantation in Multiple Myeloma

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## ABSTRACT

Syngeneic transplantation is an option for very few patients with multiple myeloma. Among more than 2500 transplants in the EBMT registry, only 25 were syngeneic. A case-matched comparison of these 25 transplants with 125 autologous and 125 allogeneic transplants was recently performed. Seventeen (68%) of the patients receiving twin transplants entered a complete remission following transplantation compared to 48% of those who received autologous transplants and 58% of those who received allotransplants. The median survival and progression-free survival for the transplants were 73 and 72 months, respectively. Overall survival was significantly better than for autologous transplants (median 44 months) and allogeneic transplants (median 16 months). Progression-free survival was significantly better with syngeneic transplants than with either autologous transplants (median 25 months) or allotransplants (median 9 months). Transplant-related mortality was low; nly two patients died of transplant-related causes at the time of follow-up. The relapse rate was significantly lower than for autologous transplants and similar to the relapse rate in allogeneic transplants. Only three of the patients who entered complete remission had relapsed at the time of follow-up. Syngeneic transplantation in multiple myeloma appears to be the treatment of choice if a twin donor is available. A lower relapse risk compared with autologous transplantation may be due to infusion of malignant cells in the autologous setting or to the presence of a graft-vs.-myeloma effect in syngeneic transplants.

## INTRODUCTION

Myeloma is a disorder characterized by a median survival of less than 3 years using conventional chemotherapy.[1] Autologous transplantation has been shown to

increase both survival and progression-free survival.[2] Allogeneic transplantation has a high transplant-related mortality with presently used transplantation techniques but is associated with a lower relapse rate than autologous transplantation.[3]

Until recently, syngeneic transplantation has been attempted only in occasional patients.[4,5] However, in 1996 results in 11 patients were reported by the Seattle group.[6] Some of these patients were in an advanced stage of the disease. Still, two patients are surviving 9 and 15 years without disease progression.

In the registry of the European Group for Blood and Marrow Transplantation (EBMT), 25 patients have been reported to have received a syngeneic transplant. Recently, we performed a case-matched analysis among syngeneic, allogeneic, and autologous transplantation, comparing one syngeneic transplant to five auto- and allotransplants, respectively (Gahrton G, Svensson H, Björkstrand B, Apperley J, Carlson K, Cavo M, Ferrant A, Fouillard L, Gratecos N, Gratwohl A, Guilhot F, Lambertenghi Deliliers G, Ljungman P, Masszi T, Milligan DW, Powles RL, Reiffers J, Samson D, Stoppa AM, Vernant JP, Volin L, Wallvik J, for the European Group for Blood and Marrow Transplantation, manuscript submitted). Such a comparison can generate information not only about the outcome following syngeneic transplantation, but also about mechanisms of relapse in allogeneic and autologous transplantation.

## PATIENTS AND METHODS

Twenty-five patients, 12 males and 13 females, underwent syngeneic transplantation. The median age was 49 years. Four patients were in stage IA at diagnosis, six in stage II, 11 in stage IIIA, two in stage IIIB, and in two the stage was not reported. Sixteen patients had IgG, four IgA, one IgD, three light chain, and one IgG + IgA myeloma. The median time from diagnosis to transplantation was 10 months (0–98).

At the time of transplantation, four patients were in complete remission, 14 were in partial remission, five were nonresponsive, and two had progressive disease. The patients had received a variety of pretransplant regimens. The conditioning regimen was either TBI + cyclophosphamide, TBI + melphalan alone or combined with other cytotoxic drugs, melphalan alone, or busulfan + cyclophosphamide. The median follow-up after transplantation at the time of analysis was 55 months (2–111).

Previous studies have identified prognostic factors for both autotransplantation[7] and allotransplantation.[8,9] The syngeneic transplants were therefore matched for those prognostic factors that had been shown to be most important in this previous prognostic factor analysis; i.e., for autologous transplantation, the number of treatment regimens before transplantation and the response status at transplantation, and for allogeneic transplantation, the sex of the recipient and the number of treatment regimens before transplantation.

There was no significant difference in stage at diagnosis, age, or response status before transplantation among the groups. However, in the autologous group, fewer

patients were male and the follow-up time was shorter, while in the allogeneic transplant group the follow-up was longer.

## RESULTS AND DISCUSSION

Among the twin transplants, three patients had entered complete remission before transplant, and of the remaining 22, 14 (64%) entered complete remission after transplantation. The patients in complete remission before transplant remained in this state after transplant, thus a total of 17 patients (68%) were in complete remission after transplantation. There were two transplant-related deaths (viral infection and veno-occlusive disease), and at the time of follow-up, six additional patients had succumbed to progression of multiple myeloma. Five patients had graft-vs.-host disease, four with grade I and one grade II. The median overall survival was 73 months, and the median progression-free survival, 72 months. Three of 17 patients who entered a complete remission following transplantation are still in complete remission 5–111 months posttransplant. Three patients currently survive in complete remission beyond 8 years.

The response rate (64%) with syngeneic transplantation was not significantly different from the response rate with autologous transplantation (38%) or allogeneic transplantation (50%). However, the overall survival tended to be better with twin transplantation than with autotransplantation (median survival 44 months) or with allogeneic transplantation (median survival 16 months). The progression-free survival was significantly better than for both autologous transplantation (median 25 months) or allogeneic transplantation (median 9 months). The relapse rate was significantly lower than with autologous transplantation and similar to the relapse rate in allogeneic transplantation.

Thus, transplantation using monozygous twins appears favorable compared with both autologous transplantation and allogeneic transplantation. Also, most importantly, the relapse rate is significantly lower than with autologous transplantation, which may be due to reinfusion of myeloma cells from the autograft, but perhaps also to some extent to the presence of a graft-vs.-myeloma effect, the mechanism of which is unknown. However, graft-vs.-host disease was seen in five of our patients and has been reported previously in syngeneic transplantation for other disorders.[10–12] Thus, a graft-vs.-myeloma effect cannot be excluded in the syngeneic setting.

In conclusion, syngeneic transplants have the advantage of having a lower transplant-related mortality than allogeneic transplants and a lower relapse rate than autologous transplants. Therefore, overall survival and progression-free survival appear superior to both other transplant-related modalities. Syngeneic transplantation should therefore be performed whenever a monozygous twin is available.

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# Pretreatment With Alkylating Agents Predicts for Tumor Cell Content and CD34+ Stem Cell Yield in Leukapheresis Products of Patients With Multiple Myeloma

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## ABSTRACT

The aim of our study was to examine whether pretreatment regimens affect the tumor load and the stem cell yield in leukapheresis products (LP) of patients with multiple myeloma (MM) harvested after high-dose cyclophosphamide and granulocyte colony-stimulating factor (G-CSF). We assessed the percentages of tumor cells and $CD34^+$ cells in LP collected on day 1 (LP 1) from 30 patients with MM. The proportion of clonotypic cells was quantitated by an allele-specific oligonucleotide (ASO) polymerase chain reaction (PCR) assay based on limiting dilutions. $CD34^+$ cells were determined by flow cytometry. The percentages of malignant cells in the LP were in the range of 0.0 to 0.71% (mean 0.04%). $CD34^+$ cells ranged between 0.1 and 9.72% (mean 2.05%). We could demonstrate for the first time that conventional therapy with alkylating agents was able to significantly lower the tumor numbers of the first leukapheresis collected (Spearman test: total number of cycles with alkylating agents, $P$=0.035; pretreatment with alkylating agents in the last three cycles before mobilization, $P$=0.016). Pretreatment with alkylating agents (kind and number of pretreatment cycles) also significantly reduced the stem cell yield ($P$=0.00004, $P$=0.0004, respectively). Thus, the positive impact of pretreatment with alkylating agents in lowering the tumor load in apheresis products must be balanced against the adverse effect on the stem cell yield.

## INTRODUCTION

MM is a B cell malignancy characterized by the accumulation of clonal plasma cells in the bone marrow (BM) secreting a monoclonal immunoglobulin.

Intensified high-dose chemotherapy regimens followed by autologous hematopoietic stem cell transplantation resulted in a significant prolongation of overall survival and an increased number of patients achieving complete remissions.[1,2] Peripheral blood stem cells mobilized with G-CSF and high-dose chemotherapy are increasingly used autotransplants. The influence of pretreatment regimens on lowering the collection efficacy of peripheral $CD34^+$ stem cells has been demonstrated for several disease entities.[3–5] Tumor cells in leukapheresis products as a possible relapse source remain a further concern in the autologous peripheral blood progenitor cell transplant (PBPCT).[6–8] $CD34^+$ purging strategies have been established to reduce the numbers of reinfused tumor cells.[9,10] The impact of the kind or the duration of pretreatment on the tumor cell load in apheresis products has not been described for MM. However, effect of previous treatment regimens on the number of circulating plasma cells in LP was shown by immunofluorescence analysis.[11]

We were particularly interested in evaluating the predictive value of pretreatment regimens on tumor cell numbers in leukaphereses collected on day 1 calculated with a quantitative PCR assay based on a limiting dilution method.

## METHODS AND MATERIALS

### Patients

The characteristics of the 30 patients enrolled into the study are shown in Table 1. The diagnosis of MM was made by using standard criteria.[12] All patients have been included in a phase II study evaluating the efficacy of high-dose chemotherapy and PBPCT. Informed consent was obtained from each patient.

At the time of mobilization, four patients were in complete remission (CR), 21 patients had achieved partial remission (PR), and five showed minimal response (MR) according to the EBMT criteria.[1] All patients had received one or more cycles of conventional treatment. Pretreatment consisted of a median of five cycles. Of the 30 patients evaluated, four were pretreated with alkylating agents only, predominantly with melphalan, 11 had received VA[I]D (vincristine, doxorubicin, [idarubicin,] and dexamethasone), and 15 received both regimens.

### PBPC mobilization

Patients were treated with high-dose cyclophosphamide (HD-CY) (7 g/m$^2$, $n$=20, or 4 g/m$^2$, $n$=5) (Asta Medica, Dresden, Germany) or, in cases of preexisting heart disease or amyloidosis, with ifosfamide/mitoxantrone (Ifo/Mito) ($n$=5, Ifo 4 g/m$^2$ days 1 and 2 [Asta Medica] and Mito 10 mg/m$^2$ days 2 and 3 [Lederle, Münster, Germany]) plus G-CSF (Amgen-Roche, München, Germany). G-CSF

**Table 1.** Patient characteristics

| | |
|---|---|
| Number of patients | 30 |
| Age (years) | |
| Median | 50 |
| Range | 30–65 |
| Men/Women | 23/7 |
| Ig type | |
| A/D/G | 6/1/20 |
| A+G | 1 |
| BJ | 2 |
| Stage at transplantation | |
| I | 1 |
| II | 10 |
| III | 19 |
| Status at mobilization | |
| CR/PR/MR | 4/21/5 |
| Pretreatment | |
| Number of cycles | |
| Median | 5 |
| Range | 3–21 |
| Cycles with alkylating agent | |
| Median | 3 |
| Range | 0–12 |

administration was started at the dose of 300 or 600 µg/d 24 hours after chemotherapy and continued until PBPC harvesting was completed. Harvesting began when more than 20 $CD34^+$ cells/µL were found by immunofluorescence analysis. If no $CD34^+$ cells were detected, aphereses were started when the leukocyte counts reached more than $25\times10^9$ cells/liter and platelets were above $80\times10^9$ cells/liter. Harvesting was performed using a CS 3000 (Baxter Deutschland GmbH, München, Germany) or a Cobe Spectra (Cobe, Lakewood, CO). To support melphalan-containing high-dose chemotherapy, an autograft containing more than $2.0\times10^6$ $CD34^+$ cells/kg body weight was considered necessary for a successful engraftment.[3] PBPC were cryopreserved as previously described.[13]

## Patient samples

BM aspirates obtained before mobilization therapy were used for the identification of the complementarity-determining region 3 (CDR3) of the immunoglobulin heavy chain gene. LP of the 30 patients collected on day 1 were assessed for tumor cell load by a quantitative ASO-PCR assay and for the number

of $CD34^+$ cells by fluorescence-activated cell sorter (FACS) analysis. Mononuclear cells (MNC) from BM and LP samples were obtained by Ficoll-Hypaque density centrifugation (Biochrom, Berlin, Germany).

## Immunofluorescence analysis

$1\times10^6$ MNC of the LP or 20–50 µL of whole blood were incubated for 30 minutes at 4°C with fluorescein isothiocyanate-conjugated anti-CD34 antibody (HPCA-2) obtained from Becton Dickinson (Heidelberg, Germany). Isotype-identical antibodies served as controls. The immunofluorescence analysis was performed using a five-parameter FACScan (Becton Dickinson) as previously described.[13]

## Quantitative assessment of tumor cells and statistical analysis

RNA and DNA extraction, CDR3 consensus PCR, cloning, and sequencing of the patient-specific CDR3 regions with the subsequent generation of allele-specific oligonucleotides complementary to the specific CDR3 regions were performed as described previously.[14]

Quantitative ASO-PCR was carried out essentially as reported.[15] In brief, per aliquot of LP, 2 µg of DNA (equivalent to $3.3\times10^5$ cells) was serially diluted in 10-fold steps with placental DNA (Clontech, Palo Alto, CA) and amplified using the patient-specific ASO together with the antisense $J_H$-consensus primer.[16] At each dilution level, five or 10 identical PCR reactions were performed simultaneously in a volume of 50 µL with 2 µg genomic DNA, 2.5 U AmpliTaq Gold DNA-polymerase (Perkin Elmer, Weiterstadt, Germany), 2 mM $MgCl_2$, 5 µL GeneAmp 10× PCR buffer II, 0.2 mM of dNTPs, and 0.6 µM of each primer.

The percentages of malignant cells in LP were determined by using a maximum likelihood function based on the Poisson distribution of positive and negative reactions at each sample dilution. The calculation was done by $\chi^2$ minimization. The absolute number of malignant cells in LP was deduced from the MNC obtained from the differential white cell count less the number of neutrophils.

Tests whether two quantitative variables are uncorrelated were performed using Spearman's rank correlation coefficient. Linear regression analysis was used to examine the influence of the pretreatment cycles on tumor cell load and stem cell yield of LP using Box-Cox power transformed data for tumor cell load and stem cell yield (y^lambda) with parameter lambda = 0.25. Graphical displays of the model fit also include 95% confidence intervals. An effect was considered statistically significant if the $P$ value of the corresponding test value was $\leq 0.05$. Statistical calculations were done using Statistica for Windows (StatSoft, Tulsa, OK), and S-Plus, Version 3.4 for Sun SPARC (Math Soft, Seattle, WA).

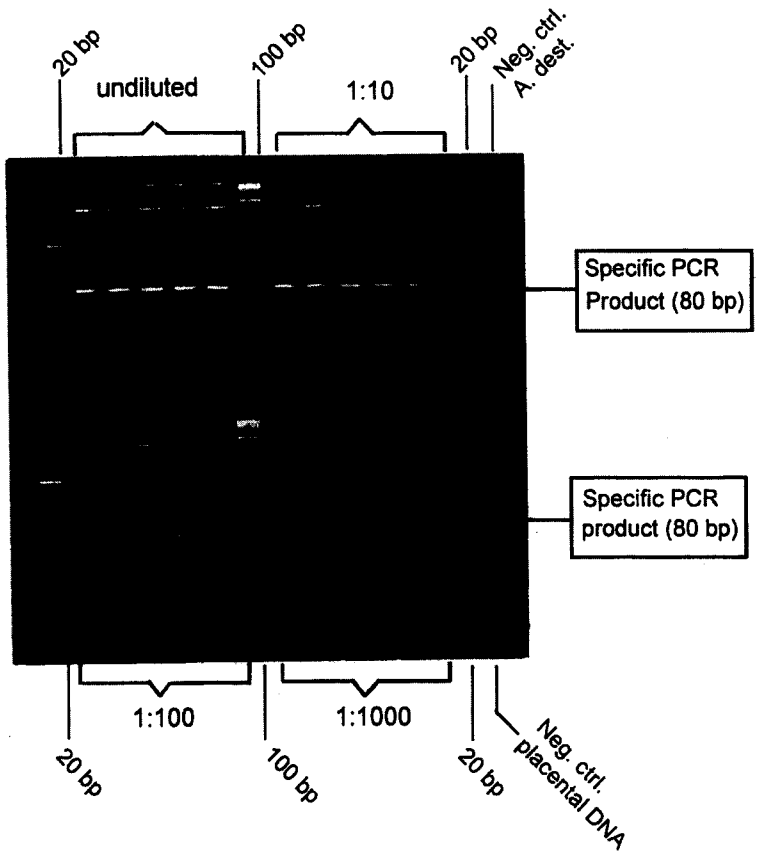


**Figure 1.** *Quantitative ASO-PCR analysis of the tumor load of LP 1 from one patient. DNA from 330,000 cells was serially diluted in 10-fold steps with placental DNA (undiluted, 1:10, 1:100, 1:1000). At each dilution level, five PCR reactions were performed simultaneously. The length of the specific PCR product is 80 bp.*

## RESULTS

### Quantitative assessment of tumor and stem cells in LP 1

In three of 30 LP examined, no tumor cells could be detected. The percentages of tumor cells varied between 0 and 0.71% (mean 0.04%), resulting in a tumor cell number in LP 1 ranging from 0 to $2.73 \times 10^8$. Results of a PCR assay with a DNA dilution series from LP 1 of one patient are shown in Fig. 1. The proportion of $CD34^+$ stem cells in LP 1 ranged from 0.1 to 9.72% (mean 2.05%) resulting in a stem cell yield from $9.2 \times 10^6$ to $3.6 \times 10^9$ cells. Tumor and stem cell numbers of the 30 LP 1 are summarized in Table 2.

**Table 2.** Descriptive statistic of tumor cell content and stem cell yield

| | *n* | *Mean* | *Minimum* | *Maximum* | *SD* | *SE* |
|---|---|---|---|---|---|---|
| MNC of LP | 29 | 41.3 | 4.5 | 117.6 | 30.38 | 5.64 |
| Tumor cells (%) | 30 | 0.04 | 0.0 | 0.71 | 0.13 | 0.024 |
| Number of tumor cells ($\times 10^6$) | 29 | 17.91 | 0.0 | 273 | 51.47 | 9.56 |
| $CD34^+$ stem cells (%) | 30 | 2.05 | 0.1 | 9.72 | 2.15 | 0.39 |
| Number of $CD34^+$ cells ($\times 10^6$) | 29 | 816.71 | 9.20 | 3591 | 987.72 | 183.42 |

*SD, standard deviation; SE, standard error.*

### Statistical analysis

Correlating the kind and the number of pretreatment cycles with the tumor cell load in LP 1, a significant reduction in the number of tumor cells could be observed as a consequence of the treatment with cycles of alkylating agents at all ($P$=0.035). The corresponding regression curve is shown in Fig. 2. Focusing on the inclusion of alkylating agents in the last three treatment cycles before mobilization confirmed this significant effect on lowering the tumor cell numbers in LP 1 ($P$=0.016). Determination of the impact of both parameters on the stem cell yield revealed a concomitant significant reduction in the stem cell yield ($P$=0.00004; $P$=0.0004) (Fig. 3).

## DISCUSSION

Tumor cell contamination in autologous grafts as a possible source of relapse is still a major concern in autologous transplantation. For MM, a borderline significance for the number of plasma cells autografted in predicting relapse-free survival has been demonstrated.[17] Purging procedures have been proposed for tumor cell reduction.[9,10] Whether the pretreatment protocols used have an influence on the tumor load of the harvests has been studied recently. Cremer et al.[18] demonstrated that chemotherapy-containing mobilization regimens were able to significantly lower the tumor cell number in leukapheresis products compared with the use of growth factor alone. Omede et al.[11] presented the possibility of reducing tumor cell contamination in LP by repeated cytotoxic chemotherapy courses before PBSC collection. Whether different conventional pretreatment regimens also have an effect on the number of tumor cells in apheresis products is not yet determined. We described here for the first time that pretreatment with alkylating agents in MM resulted in lower tumor cell numbers in aphereses harvested after chemotherapy and G-CSF mobilization compared with the pretreatment with VA[I]D. This effect was after administration of alkylating agents in the last three cycles of conventional chemotherapy before mobilization regimens. Our study also confirmed the adverse

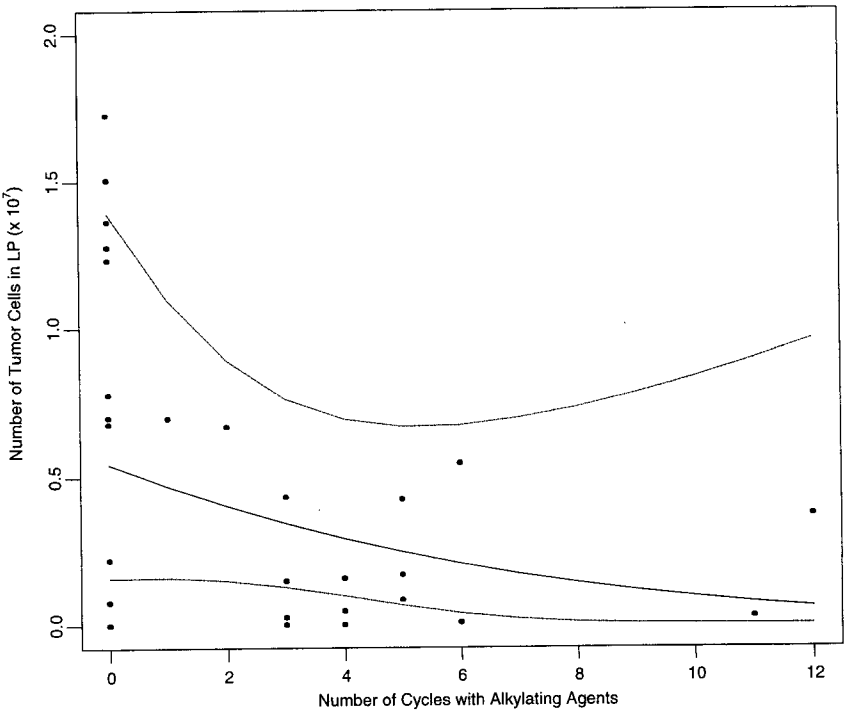


**Figure 2.** *Correlation of the number of tumor cells in LP 1 with the number of pretreatment cycles with alkylating agents. Linear regression analysis was used to examine the influence of the pretreatment cycles on tumor cell load in LP 1 using Box-Cox power transformed data for tumor cell load. The graphical display also includes the 95% confidence interval.*

effects of alkylating agents on the stem cell yield described by other authors. The use of these drugs should be balanced against the adverse effects on the stem cell yield.

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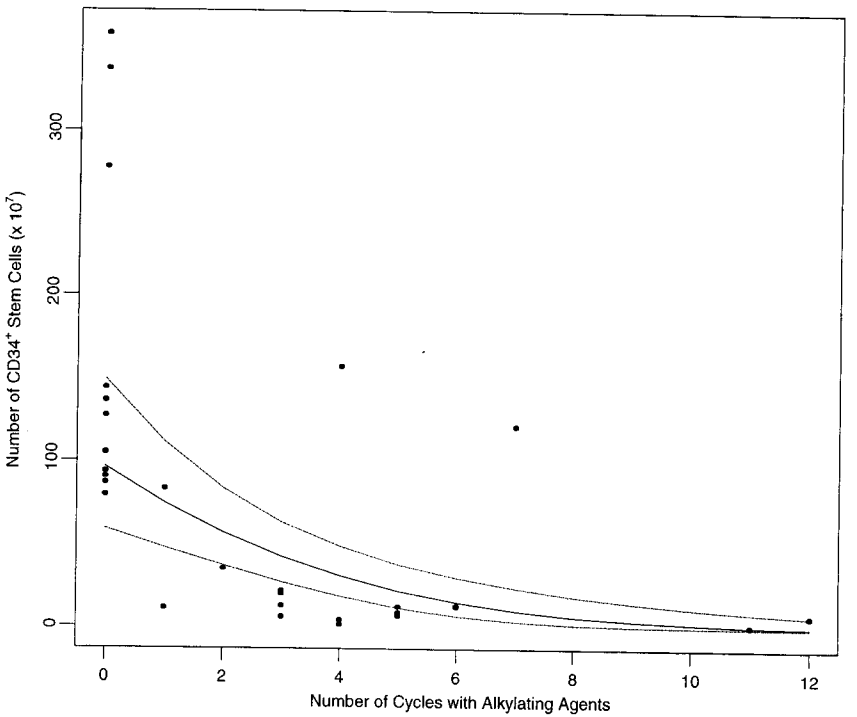


**Figure 3.** *Correlation of the number of CD34⁺ stem cells in LP 1 with the number of pretreatment cycles with alkylating agents. Linear regression analysis was used to examine the influence of the pretreatment cycles on stem cell yield in LP 1 using Box-Cox power transformed data for tumor cell load. The graphical display also includes the 95% confidence interval.*

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# CHAPTER 6
# BREAST CANCER

# The Presence of Micrometastases in Bone Marrow and Blood in High-Risk Stage II Breast Cancer Patients Before and After High-Dose Therapy

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## INTRODUCTION

The majority of breast carcinoma patients are not in advanced stages when first diagnosed and are considered potentially curable. However, about 50% of these patients will subsequently develop recurrent disease. Even in patients without tumor involvement in axillary lymph nodes (LNs) at surgery, there is a death rate of 25% over a decade. Previous reports have demonstrated that adjuvant chemotherapy improves the outcome of both lymph node positive ($LN^+$) and negative ($LN^-$) breast cancer patients.[1] Prognostic factors such as tumor size and histological grade have been reported to be of clinical value in selecting unfavorable prognostic $LN^-$ and $LN^+$ patients.[2] Despite these factors, however, a high proportion of patients in the low-risk group will experience recurrences of the disease. This underscores the need for more basic knowledge about individual breast cancer patients.

New treatment modalities for breast cancer patients are under development. High-dose chemotherapy with autologous hematopoietic progenitor cell support is being used with increasing frequency to treat breast cancer patients with poor prognosis.[3–5] The most common cause for the ultimate failure of an autologous transplant is not lack of engraftment, toxicity of therapy, or infection, but rather relapse of disease. There is evidence that reinfusion of autografts containing tumor

cells can contribute to relapse and influence patient outcome after high-dose treatment.[6] Tumor cell contamination can be observed in histologically normal bone marrow autografts in patients undergoing high-dose treatment using sensitive immunocytochemical techniques. Peripheral blood progenitor cell (PBPC) autografts are increasingly used in the belief that these products will have a low probability of containing tumor cells. Recent findings confirm that although tumor cell involvement is less extensive in PBSC autografts than in bone marrow, it is still frequently found in PBPC collections from breast cancer patients.[7–9]

Nonetheless, relapse after high-dose chemotherapy will most likely be the result of persisting tumor cells in the patients. Consequently, detection methods for minimal residual disease in the bone marrow before and after therapy and in autografts are of growing importance in the evaluation of patients receiving high-dose chemotherapy with stem cell support.

Immunocytochemistry employing monoclonal antibodies (mAbs) is the most frequently used method to detect tumor cells in bone marrow and blood. The method should be specific, and the mAbs applied should not crossreact with normal cells. Since the mAbs frequently used are tumor-associated and not specific against breast cancer cells, immunostaining protocols have some limitations. The presence of minimal residual disease in bone marrow detected by immunocytochemistry has frequently been studied in breast cancer patients at diagnosis (Table 1). Experience has shown that despite the need for standardization and improvement of immunocytochemical protocols, meta-analyses confirm the presence of micrometastases in bone marrow at diagnosis as an independent predictor of both reduced disease-free survival and overall survival.[10–16]

In Scandinavia, we have recently finished an adjuvant study in high-risk stage II breast cancer. Patients were randomized to have dose escalation of chemotherapy with hematopoietic growth factor or high-dose therapy with PBPC support (Protocol FBG 9401 chaired by J. Bergh, Uppsala, Sweden). Among patients entering this study in Norway, micrometastatic detection was performed on bone marrow and blood before treatment, in PBPC, and on bone marrow and blood 6–12 months after treatment. Even though the observation time is short, the presence of circulating tumor cells appears to be associated with early relapse after high-dose therapy and stem cell support.

## MATERIALS AND METHODS

### Patients and high-dose therapy

Breast cancer patients with a life expectancy of less than 30% after 5 years were randomized to have either nine cycles of dose escalation of FEC (5-fluorouracil, epiadriamycin and cyclophosphamide) supported by subcutaneous (s.c.)

**Table 1.** Immunocytochemical detection of breast cancer cells in bone marrow

| *Marker* | *Patients (n)* | *Detection ratio (LN+:LN–)* | *Prognostic value* |
|---|---|---|---|
| EMA13 | 350 | 33:19% | + |
| 2E1112 | 727 | 59:31% | + |
| CK-214 | 95 | 11.9:7.5% | ND |
| C26,T16,AE115 | 51 | 41:27% | + |
| AE1,AE35 | 124 | 29%:— | ND |

*EMA, epithelial membrane antigen; 2E11, breast mucin antigen; CK-2,CK-8,18,19,AE1,AE2, mAbs against different cytokeratin antigens; C26, T16, mAbs reacting with distinct epithelial specific antigens; ND, not determined.*

granulocyte colony-stimulating factor (G-CSF) (filgrastim 5 μg/kg; Amgen/Roche) or three cycles of FEC plus high-dose therapy followed by stem cell support. Mobilization and collection of PBPC was done by combining the third cycle of FEC plus G-CSF. Three days after the end of chemotherapy treatment, the patients were given daily s.c. G-CSF (filgrastim 5 μg/kg). When a peak of CD34-positive cells appeared in the blood, usually at day 11 to 12 after initiating the chemotherapy, leukapheresis was performed with a CS 3000 Fenwal Cell Separator with a flow rate of 70 mL/min. Ten liters of blood were processed. $CD34^+$ cell numbers were calculated, and the cells were frozen until use. $CD34^+$ cell enrichment was performed in 30 patients using the ISOLEX 300 procedure as reported previously.[17] The mean purity of $CD34^+$ cells after positive selection was 98% (91.5–99.7%) with a mean yield of 48 (35–81). High-dose chemotherapy for breast cancer patients consisted of daily administration for 4 days with cyclophosphamide 1.5 g/m$^2$, carboplatin 200 mg/m$^2$, thiotepa 125 mg/m$^2$, and uromitexan 15 mg/kg every 4 h. Three days after finishing the chemotherapy, the cells were reinfused. From day 2 after reinfusion of PBPC or $CD34^+$ cells, G-CSF (filgrastim 5 μg/kg s.c.) was administered daily until reconstitution. After high-dose therapy, all patients were given irradiation to the chest wall plus tamoxifen 20 mg/d for 5 years.

## Immunocytochemistry

Sensitive immunocytochemical methods have been developed using tumor-associated mAbs and alkaline phosphatase anti-alkaline phosphatase (APAAP)-staining techniques to detect occult micrometastatic tumor cells in blood and bone marrow. In most reports, cytospins containing a total number of $0.5–1\times10^5$ mononuclear bone marrow cells were tested. Recently, a new cytocentrifuge method has been developed making it possible to test $0.5\times10^6$ cells on each slide.[11] A bilateral bone marrow aspiration from posterior iliac crest and blood were taken before and 6–12 months after therapy from 124 patients entering the randomized

breast cancer study. Mononuclear cells were prepared by Ficoll. Immunocytochemical evaluation of a total of $2\times10^6$ mononuclear cells from the blood or bone marrow of each patient was done.[17] The slides were air-dried overnight, fixed for 10 minutes, and incubated for 30 minutes in a moist chamber with a 1:20 dilution of the anticytokeratin primary antibodies AE1 and AE3 ( Signet Laboratories, Dedham, MA), followed by washing twice with Tris-HCl. As a second step, a polyclonal rabbit anti-mouse antibody (Dako, Glostrup, Denmark) was added, and after 30-minute incubation, the cells were washed twice with Tris-HCl. Finally, preformed complexes of alkaline phosphatase monoclonal mouse and anti-alkaline phosphatase (Dako, Glostrup, Denmark) were added for 30 minutes. After two washings with Tris-HCl, the color reaction of antibody binding cells was obtained after 10-minute incubation with 0.26% New Fuchsin solution (Aldrich Chemical Company, Milwaukee, WI). In addition, all slides were counterstained with hematoxylin to study cellular morphology.

The stained slides were mounted in glycerin-gelatin and examined under a light microscope by an experienced pathologist. In an ongoing study at our hospital, employing anticytokeratin mAbs AE1/AE3 or A45-B/B3 and immunocytochemistry, 26.6% of the bone marrow samples from 257 breast cancer patients tested were anticytokeratin-positive. Among these samples, 5.4% of isotype controls stained positive, suggesting that nonspecific bindings of anticytokeratin mAbs to nonepithelial cells have taken place. Based on this experience, we always employ morphological evaluation and negative controls using slides incubated with isotope-matched mouse myeloma immunoglobulins followed by the APAAP technique as described above.

## RESULTS

Among the 124 patients, 29% presented with tumor cells in bone marrow and 8% in the blood at diagnosis of breast cancer (Table 1). The presence of micrometastases in bone marrow and blood before three cycles of chemotherapy and in the PBPC product was studied in 60 patients (Fig. 1). Despite tumor-reductive therapy with three cycles of chemotherapy, 14 of 60 patients (23%) had tumor cells in their PBPC products. Since eight of these did not have tumor cells detected in blood before treatment, this suggests that chemotherapy and G-CSF can mobilize tumor cells into the blood (Fig. 1).

Six to 12 months after treatment, the patients were tested for the presence of micrometastases in bone marrow. Of the 67 patients treated with high-dose therapy and stem cell support, 36 had immunocytochemical examination of their marrow 6–12 months after finishing the therapy. As can be seen in Fig. 2, eleven patients were positive in the bone marrow at diagnosis. Two had contamination of the PBPC products and six had persistent cytokeratin-positive cells after high-dose

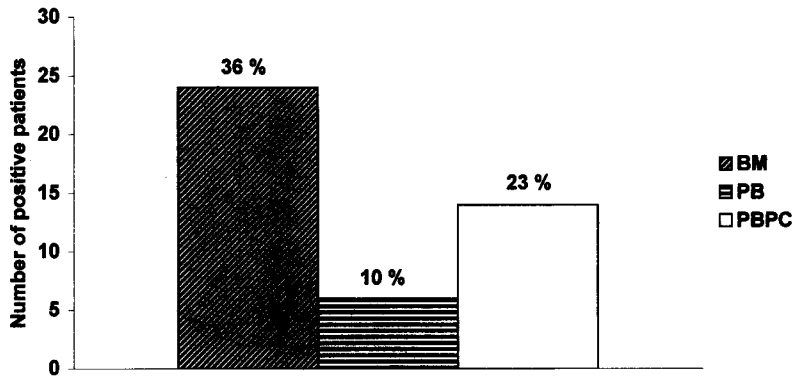


**Figure 1.** *Sixty stage II high-risk breast cancer patients treated with high-dose therapy with PBPC support were examined by immunocytochemistry employing anticytokeratin mAbs. $2310^6$ bone marrow mononuclear cells and peripheral blood before treatment and in PBPC were examined from each patient. Only $CK^+$ cells with epithelial cell–like morphology were scored as positive. BM, number positive in bone marrow at diagnosis; PB, number positive in blood at diagnosis; PBPC, number positive in peripheral blood progenitor cell products.*

therapy. Four patients became positive in the PBPC products without any known micrometastases in bone marrow. Of those four, one had a positive marrow after high-dose therapy. Four patients previously negative in both bone marrow and PBPC became positive in the marrow after high-dose therapy. Among the patients

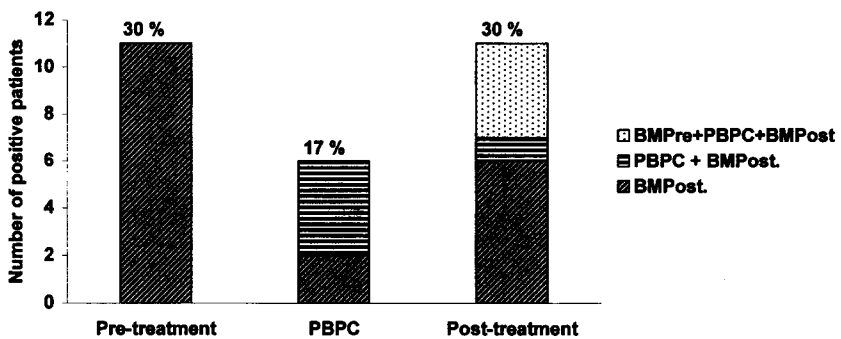


**Figure 2.** *Thirty-six stage II high-risk breast cancer patients treated with high-dose therapy with PBPC support were examined by immunocytochemistry employing anticytokeratin mAbs. $2310^6$ bone marrow mononuclear cells before treatment, in PBPC product and in bone marrow 12 months after therapy were tested from each patient. Only $CK^+$ cells with epithelial cell–like morphology were scored as positive. Pre-treat+PBPC+post treat column, number positive at all stages; PBPC+post-treat column, number that became positive in the PBPC product and after therapy; Post-treat column, number that became positive after therapy.*

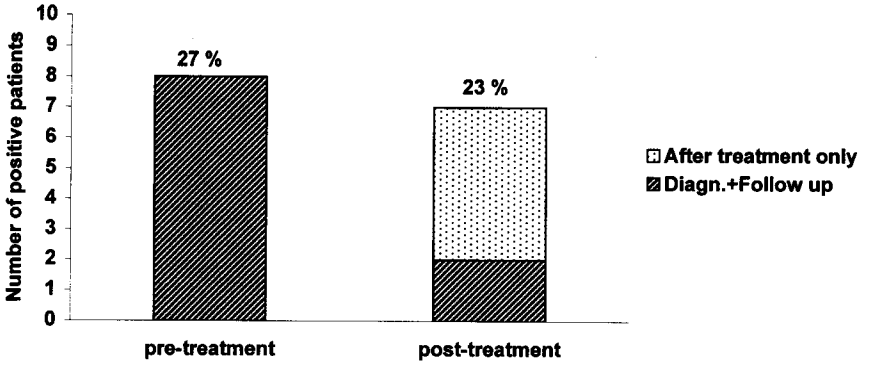


**Figure 3.** *Thirty stage II high-risk breast cancer patients treated with repeated-dose escalating cycles of FEC plus G-CSF were examined by immunocytochemistry applying anticytokeratin mAbs. $2310^6$ bone marrow mononuclear cells before treatment and from 6–12 months after therapy were tested from each patient. Only $CK^+$ cells with epithelial cell–like morphology were scored as positive.*

given nine cycles of dose-escalating FEC and G-CSF, 30 patients are evaluable (Fig. 3). Of those, eight were positive in the bone marrow before treatment, two had persistent tumor cells, and five became positive after therapy. Due to the short observation time, the correlation between the presence of micrometastases and outcome in the patients studied is not yet known.

Despite high-dose therapy and stem cell support, 14 patients relapsed within the first 12 months. Since all were monitored by immunocytochemistry of marrow cells before therapy and of the PBPC products, it was of interest to study the relationship between early relapse and presence of micrometastases (Table 2). As can be seen from the 14 patients studied, 13 had circulating tumor cells either in bone marrow at diagnosis ($n$=9) or in the PBPC ($n$=4). This indicates that early relapse is associated with circulating tumor cells resistant to high-dose therapy.

**Table 2.** $CK^+$ cells in 13 stage II high-risk breast cancer patients treated with high-dose therapy with PBPC support with early recurrence

| | *Patient no.* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12* | *13* | *14* |
| BM at reg. | + | + | + | + | + | + | – | + | – | - | – | + | – | + |
| PBPC | – | – | – | – | – | – | + | – | – | + | + | + | + | – |
| Overall positivity | + | + | + | + | + | + | + | + | – | + | + | + | + | + |

*From each patient, $2\times10^6$ bone marrow mononuclear cells and peripheral blood before treatment and in PBPC were examined by immunocytochemistry applying anticytokeratin mAbs. Only $CK^+$ cells with morphology like epithelial cells were scored as positive.*

## DISCUSSION

In this study, we looked for the presence of occult tumor cells in bone marrow and blood in breast cancer patients treated with adjuvant high-dose therapy. Of 124 patients studied, 29% presented with tumor cells in the bone marrow and 8% in the blood at the time of diagnosis of the breast cancer. The percentage of positive bone marrow among our well-defined high-risk breast cancer patients is generally lower than reported by others (Table 1). The differences observed may be due to the immunocytochemistry protocols used, raising the need to standardize these procedures.

In this study, we confirm a previous report on mobilization of tumor cells occurring simultaneously with the presence of $CD34^+$ cells into the blood.[18] The question of the clinical significance of infusing tumor cells in our patients cannot be adequately answered at present. Among the 67 patients studied, 30 patients were rescued with autografts enriched with $CD34^+$ cells. Such a procedure gives an average of 2.5-log tumor cell depletion, and longer follow-up will show if such highly purified autografts have any influence on disease-free survival. Among patients with early relapse, no differences could be observed between patients given unmanipulated PBPC and enriched $CD34^+$ cells. This indicates that the relapses occur due to an inefficient high-dose therapy regimen incapable of eradicating all tumor cells in the patient. Standard prognostic factors such as tumor size, receptor status, nuclear grading, and number of lymph nodes above eight do not appear to influence the outcome of patients with early relapse after high-dose therapy. Therefore, to further elucidate this problem, we are currently characterizing the primary tumor and the micrometastases with regard to other prognostic factors such as, *p53* mutations, C-erbB2,neu,Her2 oncogene expression, and others.[19–21]

Identification and characterization of micrometastases employing immunocytochemistry are limited by the number of tumor cells found on each slide. Unfortunately, in our study, and supported by the findings of others, only an average of one to five tumor cells per $2\times10^6$ normal bone marrow cells are detected by immunocytochemistry. To improve the sensitivity and specificity of the detection methods, there is a need to analyze a higher number of bone marrow cells from each patient. Standard immunocytochemical methods are cumbersome for the analysis of large numbers of cells. Recently, in our hospital, we tested the efficacy of the anti-$CD45^-$ immunobead depletion method in 165 bone marrow samples and 22 PBPC products.[22,23] After CD45 depletion of $2\times10^7$ cells, the remaining cells were tested for the presence of cytokeratin-positive cells. We found that an average fourfold higher number of positive cells was detected by CD45 depletion compared with the standard immunocytochemistry procedure. These data show that anti-CD45-immunobead depletion before immunocytochemistry permits

the examination of a larger amount of cells, giving a higher tumor cell detection sensitivity that also will permit the detailed study of the biology of tumor cells surviving high-dose therapy regimens.

In summary, although the clinical interpretation of our study is limited due to a short observation time after therapy, our results suggest the potential value of monitoring the presence of micrometastases before and after therapy.

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# Detection of Cytokeratin$^+$ Cells in Marrow as a Predictor for Tumor Cell Contamination and Measurement of Tumor Cell Loss in Apheresis Products

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## ABSTRACT

Metastasis of solid tumors, such as breast cancer, to bone marrow can be detected using an immunofluorescent assay for cytokeratin with a sensitivity of 1 tumor cell in $10^6$ cells. Initial comparisons to pathology in 70 consecutive aspirates revealed that our methodology not only confirmed positive pathology but was 3 times more likely to identify micrometastases; 35 of 70 vs. 12 of 70. Between January 1996 and April 1998, 79 stage III and IV breast cancer patients were leukapheresed after cytoreductive therapy. Marrow samples from each patient were assayed for the presence of cytokeratin$^+$ cells before apheresis.

| | *Aspirate results* | *Mean tumor frequency* | *– apheresis* | *+ apheresis* |
|---|---|---|---|---|
| Negative | 70/79 (89) | 0 | 64/70 (91%) | 6/70 (9%) |
| Positive | 9/79 (11%) | $1/3\times10^5$ | 1/9 (11%) | 8/9 (89%) |

Of the patients with a negative aspirate, 91% had negative collections. Similarly, 89% of the patients with a positive aspirate had at least one positive apheresis procedure, suggesting that marrow status is a strong predictor of tumor involvement in apheresis products.

Flow cytometric analysis of $10^6$ stained cells identified 12 positive apheresis from eight patients, containing a mean of $2.3\times10^5$ cytokeratin$^+$ cells. Apheresed cells were not immediately frozen but were centrifuged, resuspended in an isotonic electrolyte solution with 10% autologous plasma, and held overnight at 4°C. Repeat staining and analysis of 24-hour-old specimens revealed that tumor cells had fallen below the detection level of $1/10^6$ cells in 10 of 12 apheresis products. The other two specimens still contained positive cells but showed decreases in total tumor cell numbers: $1.6\times10^5$ to $8.8\times10^3$ and $1.1\times10^5$ to $2.1\times10^3$. These overnight changes emphasize the necessity of assaying fresh apheresis samples.

In an effort to increase the sensitivity to $1/10^8$ cells and quantitate log reductions in tumor burden, fluorescent microscopy replaced flow cytometry. In nine consecutive positive apheresis products collected from six patients, the mean number of cytokeratin$^+$ cells was $1.3\times10^5$ in the fresh sample and $<8.9\times10^2$ 24 hours later. The cells that disappeared may have died as the result of cytotoxic therapy and/or the inability to survive ex vivo in suspension. This 2–3 log reduction in cytokeratin$^+$ cells requires minimal manipulation, adds no additional cost to processing, may enhance the efficacy of other purging methods, and is achieved without significant loss of CD34$^+$ cells.

## INTRODUCTION

Clonogenic tumor cells in the stem cell graft have been implicated as a cause of recurrent disease following high-dose chemotherapy with autologous transplant in several types of cancer.[1–4] Using mobilized peripheral stem cells rather than bone marrow as the source of stem cells may[5,6] or may not[7,8] reduce the level of malignant cells. Further reduction can be accomplished by purging in vivo with cytoreductive therapy[9,10] or ex vivo using techniques such as tissue culture or CD34$^+$ cell selection.[11–13] The ability to detect micrometastases, monitor the efficacy of purging, assess the role of reinfused tumor cells in posttransplant relapse, and identify a safe threshold of transplanted tumor cells requires an extremely sensitive and reproducible assay.

We previously described an immunofluorescent flow cytometric assay for detecting cytokeratin$^+$ epithelial cells in marrow and stem cell products with a sensitivity of 1 in $10^6$ cells. Initial studies using parallel immunohistochemical staining and morphology confirmed the malignant character of these cells.[14]

In the current study, fluorescent microscopy replaced flow cytometry, thereby permitting morphologic examination while increasing the sensitivity to 1 tumor cell in $10^7$–$10^8$, depending on the number of nucleated cells available for review. This approach was used to determine the relationship between the presence or absence of micrometastases in marrow and tumor involvement in leukapheresed cells. This method also revealed an inverse relationship between the number of tumor cells detected in an apheresis product and time, suggesting a simple and economical way to remove 2–3 logs of tumor cells.

## MATERIALS AND METHODS

### Bone marrow aspirates

Under local anesthesia, 1–2 mL marrow was aspirated from a single bone puncture. Half of the sample was placed in a tube containing EDTA and sent for

cytokeratin staining. The balance of the specimen was used to make slides for morphologic examination by a pathologist.

## Leukapheresis samples

Between January 1996 and April 1998, 79 consecutive stage III and IV breast cancer patients underwent stem cell leukapheresis during recovery from standard-dose chemotherapy with one of the following regimens: FAC: 5-fluorouracil, 500 mg/m$^2$, adriamycin, 50 mg/m$^2$, cyclophosphamide, 500 mg/m$^2$; CAVe: cyclophosphamide, 500 mg/m$^2$, adriamycin, 50 mg/m$^2$, VP-16 (etoposide), 240 mg/m$^2$; CAT: cyclophosphamide, 500 mg/m$^2$, adriamycin, 50 mg/m$^2$, taxol, 175 mg/m$^2$.

Growth factor support consisted of 5 μg/kg G-CSF (Amgen) beginning 2 days after completion of therapy and continuing through leukapheresis. Daily apheresis began when the total circulating CD34$^+$ cell count exceeded $50\times10^6$ (days 12–15) and continued until $5\times10^6$ CD34$^+$ cells/kg had been collected. High-speed (85–110 mL/min) large-volume (18–20 liters) procedures were performed on a Cobe Spectra (Cobe BCT) or Baxter CS3000 (Baxter Fenwal). A single sample was taken from the final product bag for total nucleated cell count, CD34$^+$ analysis, sterility testing, and cytokeratin staining.

## CD34 labeling

Samples were counted using a Sysmex F800 (Baxter) or a Cell Dyne 3500 (Abbott) hematology instrument. Based on the white blood cell count, 0.5 to $1.0\times10^6$ nucleated cells from whole blood or leukapheresis products were incubated with 20 μL anti-HPCA-2-PE (Becton Dickinson) at room temperature in the dark for 20 minutes. Additional incubation for 10 minutes with 2 mL FACSlyse (Becton Dickinson) followed by vortexing and centrifugation for 2 minutes at 1000*g* effectively removed the red blood cells. Cells were washed once with phosphate-buffered saline (PBS) and resuspended in 1 mL PBS + 0.2% paraformaldehyde. A minimum of $7.5\times10^4$ cells was acquired on a flow cytometer. Analysis was performed with a FACSort flow cytometer (Becton Dickinson) using Paint-a-Gate software by gating on a plot of CD34 fluorescence (X) vs. side scatter (Y). Positive cells appeared in a discreet cluster as reported by Sandhaus et al.,[15] exhibiting CD34 fluorescence in the fourth decade and low side scatter. We analyzed samples using this strategy vs. sequential gating as recommended by Sutherland et al.[16] and found no difference between the two methods, *P* values of 0.5620 and 0.6727 for peripheral stem cells and whole blood, respectively.

## Calculations

#CD34$^+$ cells in blood =
(%34$^+$cells/100) × [nucleated cells($\times 10^6$)/mL × blood volume (mL)]
#CD34$^+$ cells in apheresis =
(%34$^+$cells/100) × [nucleated cells($\times 10^6$)/mL × product volume (mL)]

## Cytokeratin labeling

Samples were counted using a Sysmex F800, and 30–50$\times 10^6$ nucleated marrow cells or 50–100$\times 10^6$ nucleated leukapheresed cells were incubated with 4 mL FACSlyse for 30 minutes at room temperature. Cells were centrifuged at 1000*g*, decanted, and washed once in 2 mL PBS. Cells were resuspended in 200 µL PBS + 0.5% bovine serum albumin, split in half, and incubated with either 20 µL IgG2 isotype antibody or 20 µL CAM 5.2-FITC anti-cytokeratin antibody (Becton Dickinson) at room temperature in the dark for 45–60 minutes. Cells were washed once with 2 mL PBS and resuspended in 50 µL PBS + 0.2% paraformaldehyde. The actual volume was measured with a micropipette and typically ranged between 50 and 80 µL. Using fluorescent slides with 15 mm etched circles, 5 µL cells was placed per field and reviewed under fluorescence and bright-field microscopy until positive cells were identified or the sample was depleted. Positive control cells, such as the breast cancer cell line MCF-7, were set up concurrently with patient's samples and analyzed with flow cytometry. To minimize cell loss, large numbers of cells from unprocessed specimens were used; working volumes were kept small by using 12×75-mm tubes in a tabletop serofuge, and large numbers of cells ($10^6$–$10^8$) were reviewed.

## Calculations

# of cells labeled = [starting cell count ($\times 10^6$) × volume used (mL)] ÷ 2
Estimated # cells reviewed =
[# labeled cells ($\times 10^6$) ÷ cell pellet volume (µL)] × volume examined
% Positive = (# positive cells ÷ estimated # cells reviewed) × 100
Tumor Frequency =
(# cells reviewed ÷ # positive cells) (expressed as 1 positive cell/# cells examined)
Total Tumor Cell Content = (# of nucleated cells ÷ tumor frequency)

## RESULTS

Comparison of immunofluorescent staining of cytokeratin$^+$ cells to routine pathology is summarized in Table 1 for 70 consecutive bone marrow aspirates.

**Table 1.** Flow cytometry on cytokeratin-stained aspirates versus routine pathology

| *Flow cytometry* | *Pathology* |
|---|---|
| 50% positive (35/70) | 17% positive (12/70) |
| 12 positive samples | 12 positive pathology |
| 23 positive samples | 23 negative pathology |
| Maximum sensitivity: | Maximum sensitivity: |
| 1 tumor cell/$3.0\times10^5$ cells | 1 tumor cell/$7.0\times10^3$ cells |

Flow cytometry allowed analysis of $10^5$–$10^6$ cells, resulting in an assay that detected the presence of epithelial cells three times more often than pathology. All specimens with morphologically confirmed metastases were also positive using our approach, lending credence to the accuracy of cytokeratin$^+$ events identified with flow cytometry. Aspirates determined positive by both methods had the highest level of tumor contamination, with an estimated frequency of at least 1 tumor cell in $7\times10^3$ nucleated cells. Flow cytometric analysis of immunofluorescently stained cells was 40 times more sensitive than pathology and could detect as few as 1 tumor cell in $3\times10^5$ cells.

Tumor contamination of mobilized peripheral stem cell collections (PSCC) has been associated with overt disease in the marrow.[7,17,18] We found that the presence of tumor cells with a frequency as low as 1 in $10^6$ cells still predicted tumor contamination in PSCC from stage III and IV breast cancer patients (Table 2). Despite the inclusion of cytoreductive therapy in the mobilizing regimen, 89% of the patients with cytokeratin$^+$ marrow had detectable tumor cells in one to two leukapheresis products. Designating a negative marrow as prerequisite to apheresis reduced the risk of tumor presence in the stem cell product. Of the 70 patients with cytokeratin-negative marrow, 91% also had negative leukaphereses.

Initial development and adaptation of the staining procedure from cell lines to primary tissue was dependent on the availability of positive samples. Bone marrow aspirates collected in EDTA anticoagulant could be stored in aliquots and studied for 2–3 days because tumor cells remained detectable in the unprocessed portions. Conversely, whole blood samples, which contained cytokeratin$^+$ cells on the day of collection, had no detectable cells 24 hours later. This observation raised concerns

**Table 2.** Cytokeratin status of marrow vs. peripheral stem cell collections: 79 stage III and IV breast cancer patients

| *Aspirate results* | *Tumor frequency* | *− apheresis* | *+ apheresis* |
|---|---|---|---|
| 70 negative patients | 0 | 64 (91%) | 6 (9%) |
| 9 positive patients | $1/1.2\times10^5$ ($1/9\times10^3$–$1/10^6$) | 1 (11%) | 8 (89%) |

**Table 3.** Frequency of cytokeratin$^+$ cells in apheresis products: $10^6$ cells analyzed with flow cytometry

| | *Day 0* | *24 hours* |
|---|---|---|
| 10 procedures | $25/10^6$ (2–346) | $<1/10^6$ |
| Two procedures | $5/10^6$ | $1/10^6$ |
| | $26/10^6$ | $1/10^6$ |

about the timing of cytokeratin testing of leukapheresed cells, since they resemble blood more closely than marrow.

Twelve consecutive leukapheresis products determined to be cytokeratin$^+$ on the day of collection demonstrated decreases when restained and analyzed with flow cytometry after plasma depletion and overnight storage at 4°C (Table 3). Cytokeratin$^+$ cells were detectable at a level of 1 in $10^6$ cells in two of the stem cell products but fell below our sensitivity in the other 10 collections. The effect of overnight storage on the stem cell content was examined and is summarized in Table 4.

Overnight storage of plasma-depleted leukapheresed cells, collected with ACD-A (acid citrate dextrose) anticoagulant, had no statistically significant impact on measurements of nucleated cell number, % viability, or % CD34$^+$ cells for 17 paired samples. The calculated total number of CD34$^+$ stem cells harvested was unaffected.

Maintenance of CD34$^+$ cells and loss of cytokeratin$^+$ cells with overnight storage suggested a possible approach to tumor cell purging. To estimate the log reduction in tumor cells after 24-hour storage, we had to increase the number of analyzed nucleated cells from $10^6$ to $10^8$. Unfortunately, this exceeded the utility of flow cytometry, leading to false-positive events which could not be confirmed microscopically. Flow cytometry was replaced with bright-field and fluorescence microscopy. The results for nine positive leukaphereses from six patients are presented in Table 5. Six procedures had detectable levels of cytokeratin$^+$ cells the same day as collection but fell below a frequency of $1/10^8$ after 24 hours. Three other procedures still contained positive cells after 24 hours but demonstrated a 2–3 log reduction in the number of cytokeratin$^+$ cells.

**Table 4.** Effect of overnight storage on CD34$^+$ cell content

| | *Day 0* | *24 hours* | *P value* |
|---|---|---|---|
| Total nucleated cells ($\times 10^{10}$) | 4.38 | 3.96 | 0.5988 |
| % viability | 96.9 | 95.8 | 0.4637 |
| % CD34$^+$ cells | 0.49 | 0.52 | 0.8582 |
| # CD34$^+$ cells | 215 | 206 | 0.9125 |

*P values were calculated using the Student's* t *test for paired samples.*

**Table 5.** Frequency of cytokeratin$^+$ cells in apheresis products: $10^8$ cells analyzed with fluorescent microscopy at 24 hours

| | *Day 0* | *24 hours* |
|---|---|---|
| Six procedures | $13/10^6$ | $<1/10^8$ |
| | (1–16) | |
| Three procedures | $3/10^6$ | $13/10^8$ |
| | $23/10^6$ | $4/10^8$ |
| | $32/10^6$ | $2/10^8$ |

## DISCUSSION

Detection of cytokeratin$^+$ cells is a sensitive approach for identification of micrometastases in the marrow and blood of breast cancer patients. Using an immunofluorescent assay with a sensitivity of 1 tumor cell in $10^6$ cells, we found 90% of the patients with occult marrow metastases had tumor contamination in PSCC, even when chemotherapy was used for mobilization. Postponing apheresis until a patient had a cytokeratin-negative marrow reduced the possibility of tumor presence in leukapheresed cells to 9%. The six patients with negative marrow and positive aphereses probably had marrow involvement that was below our detection capability. Regardless of the testing methodology, locating micrometastases using a small representative sample is fraught with problems, inherently leading to false-negative results.

The stability of cytokeratin$^+$ cells in whole marrow but not in whole blood was an unexpected observation. Considering the basic biology of solid tumors, which grow anchored and merely transit the blood, their instability in ex vivo blood suspension is not surprising. However, this raised concerns about when to perform cytokeratin analysis on leukapheresed cells. Assays were conducted the day of collection and repeated 24 hours later on positive stem cell products. All positive apheresis products exhibited a decline in tumor frequency at 24 hours, often below our detection level of 1 in $10^6$ cells. Increased sensitivity to 1 in $10^8$ cells allowed us to estimate a 2–3 log reduction in tumor contamination after 24-hour storage. The use of chemotherapy for mobilization and/or disruption of the cellular environment with plasma depletion may facilitate the decrease in tumor cells.

Until resolution of the debates over the biologic relevance of infused tumor cells and whether there is a safe transplantable threshold, it would seem prudent to infuse the fewest tumor cells possible. The inability of cytokeratin$^+$ cells to survive overnight while CD34$^+$ cells are unscathed, affords a simple method of negative selection for leukapheresis products without increased labor or cost.

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# The Clinical Significance of Breast Cancer Cells in Marrow and Stem Cell Products Taken From Stage IV Patients

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## ABSTRACT

There is mounting evidence that marrow stem cell transplantation can provide significant antitumor benefit to patients with solid malignancies who would otherwise recur using conventional chemotherapy only. This modality is plagued by the presence of breast cancer cells in the stem cell product and marrow. In an effort to determine if micrometastatic disease in stem cell collections is associated with decreased disease-free survival (DFS), we performed immunocytochemical (ICC) analysis on paired peripheral blood progenitor cell (PBPC) collections and marrow samples taken from 246 stage IV breast cancer patients. The ICC method used monoclonal antibodies and avidin-biotin alkaline phosphatase staining. In addition, for a subgroup of patients, we determined the growth potential in vitro of these cells by placing them in a soft agar tumor clonogenic assay. Clinical follow-up was available for all 246 patients, and Kaplan-Meier survival analysis was performed on all patients. Tumor cells were detected in 43 marrow specimens and 40 PBPC specimens. In 15 patients, tumor cells were found in both marrow and PBPC specimens. Tumor colonies were documented in 38 of 130 PBPC specimens and 14 of 66 marrow specimens. The presence of tumor cells in the marrow strongly correlated with a poor posttransplant outcome. Fifteen patients had tumor cells detected in both the marrow and PBSC. All of these patients relapsed within

18 months after transplant. In addition, we found an extremely strong correlation between the presence of clonogenic tumor cells in the marrow or PBPC and a poor posttransplant outcome (relapse in 51 of 52 instances). Taken together, the ICC and clonogenic assay provide complementary and prognostic information. Detection of occult tumor cells with these assays identifies patients who are poor transplant candidates.

## INTRODUCTION

Autologous PBPC transplant following high-dose therapy is being used with increasing frequency for patients with breast cancer. While some patients appear to benefit, many relapse for a variety of reasons. These include a high in vivo tumor burden, the development of drug resistance, lack of a tumor immune response by the patient's hematopoietic cells, and reinfusion of malignant cells that contaminate the stem cell products. The detection and quantitation of the residual breast cancer cells both in vivo and in the graft may be helpful in determining the prognosis of individual patients. We have developed ultrasensitive ICC methods to detect tumor cells in the marrow and PBPC. In addition, we have developed a tumor clonogenic assay that can determine the growth potential of occult residual breast cancer cells. With these new technologies, it is now possible to determine the incidence and clinical significance of tumor cells in the marrow and PBPC in the autotransplant patient. We used these assays to evaluate the marrow and PBPC from 246 patients with stage IV breast cancer.

## MATERIALS AND METHODS

### Participating centers

This protocol was approved by the Institutional Review Board for Human Investigation at each center, and patients gave written informed consent. Participating treating institutions included Ireland Cancer Center at Case Western University, Cleveland, OH; Johns Hopkins Medical Center, Baltimore, MD; the Arthur James Cancer Hospital at Ohio State University Medical Center, Columbus, OH; Response Oncology, Memphis, TN; the James Brown Cancer Center, Humana Hospital-University of Louisville, Louisville, KY; Northern New Jersey Cancer Center, Hackensack, NJ; and Texas Oncology, Dallas, TX. Patients were enrolled into the study from 1991 to 1997.

### Patient population

Patients with histologically documented metastatic breast adenocarcinoma were eligible. Patients were evaluated using physical exam, external imaging

tests, and bilateral posterior iliac crest marrow aspirates and biopsies. Clinical follow-up was calculated using the day of PBPC infusion, and the last follow-up was obtained in November 1997. The information obtained included diagnosis date, mobilization regimen, clinical status at the time of harvesting, date of relapse, site of relapse, date of death, and last clinical follow-up.

## Marrow and PBPC specimens

Approximately 3–5 mL of bone marrow and 1–3 mL of PBPC product were obtained from each patient. Samples from referring hospitals arrived within 24 hours, and all specimens were processed within 48 hours of collection. On arrival in the laboratory, the marrow/PBPC product 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 future immunostaining.

## Immunostaining

Patient specimens were removed from storage the next day 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). Before March 1995, immunostaining was performed using the Zymed immunoperoxidase kit. After fixation, slides were incubated with anti–breast carcinoma antibodies for 45 minutes at room temperature. This was followed by incubation in normal goat serum, followed by Zymed reagents as per protocol. After March 1995, following fixation, slides were placed on the Biotek automated immunostainer (TechMate; Ventana, Tucson, AZ) 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 coverslipped with cytoseal.

Positive control slides consisted of cultured breast cancer cells (e.g., CAMA-l) seeded into normal leukapheresis product or bone marrow and immunostained as above. Negative control slides were the patient's specimen immunostained with normal mouse serum at the same concentration as used for the anti–breast carcinoma antibodies.

## Tumor cell clonogenic assay

The mononuclear cell fraction was placed in medium ready for plating. In some cases where multiple PBPC specimens were obtained, the specimens were pooled and processed as a single specimen. A total of $5.0\times10^5$ to $1.0\times10^6$ mononuclear cells per mL were plated in triplicate in 35 $mm^2$ grid-bottomed petri dishes (Nunc, Naperville, IL) in a soft agar-based medium consisting of final concentration of 15% Iscove's modified Dulbecco's medium (IMDM) (Sigma), 10% fetal bovine serum (Sigma), 0.3% agar solution (Sigma), 2 µg/mL human recombinant epidermal growth factor (Collaborative Research, Bedford, MA), and 10 µg/mL human recombinant insulin-like growth factor I (Collaborative Research). Negative control plates consisted of medium without supplemental growth factors and PBPC specimens from patients with nonepithelial tumors (e.g., normal donors, neuroblastoma, etc.). All plates were incubated in a humidified chamber at 37°C with 7.5% $CO_2$ for 10–14 days. An inverted phase contrast microscope was used to count tumor colonies (>25 cells).

## Tumor colony identification

To definitively distinguish tumor colonies from normal hematopoietic colonies, agar plates were floated onto 2- × 3-inch microscope slides and air-dried at 4°C. Initially, in situ tumor colony verification was performed using immunofluorescence staining with FITC-labeled anti-cytokeratin mAb SB-3 (Caltag, San Francisco, CA) (Fig. 1). In certain cases, granulocyte-macrophage colony-forming unit (CFU-GM) colony verification was performed using immunofluorescence staining with rhodamine-labeled anti-CD11 mAb (Dako, Carpenteria, CA). Later, an immunocytochemical method was developed to verify and count the number of tumor colonies. Slides were placed into cold storage at −70°C. The patient specimens were then removed from storage and tested for immunoreactivity. Immunostaining was accomplished using the alkaline phosphatase–based technique. Slides were baked in an oven, fixed in a paraformaldehyde solution at 4°C for 30 minutes, and washed thoroughly in PBS with 1% triton (Gibco). After fixation, slides were incubated with agarase followed by anti–breast carcinoma antibodies for 45 minutes at room temperature. Biotek immunoreagents for alkaline phosphatase staining were performed as per protocol. The specimen was then incubated with Ehrlich's hematoxylin counterstain and coverslipped with mounting medium.

The number of tumor colonies per plate was then recorded as identified by the above methods. PBPC specimens were excluded from analysis if the cell viability was <50%, if they failed to grow any colonies (including normal hematopoietic colonies), or if they had bacterial contamination.

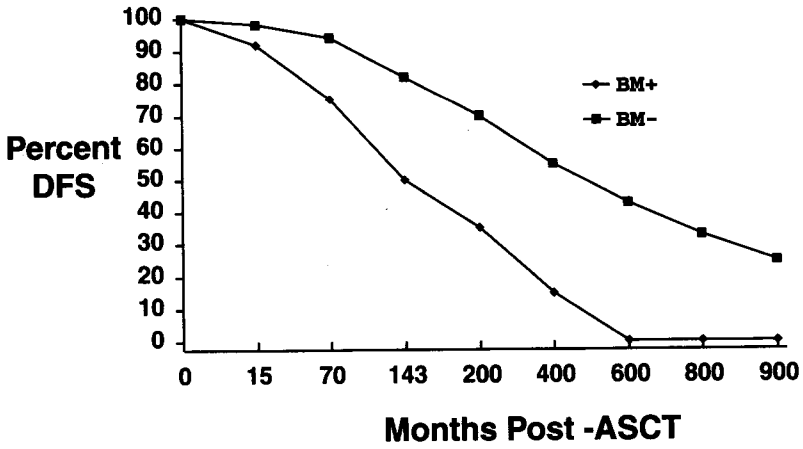


**Figure 1.** *Survival curve comparing the disease-free survival of patients with an ICC-positive marrow to those with a negative sample.*

## RESULTS

Paired marrow and stem cell samples were available from 246 stage IV patients. All samples were evaluated using ICC, and 130 PBPC and 66 bone marrow samples were analyzed using the clonogenic assay.

The marrow was positive in 43 instances (17.5%), and the PBPC was positive in 40 cases (16.2%). In 15 patients, the marrow and PBPC both had breast cancer cells detected. The marrow and PBPC were negative for tumor cells in 178 women (see Table 1).

Clinical follow-up was available for 246 patients. The overall DFS was 15% for all patients at a median follow-up of 30 months. The median DFS was significantly reduced for patients with ICC-positive marrow (146 days) when compared with patients who were ICC-negative (458 days; $P$=0.0001; see Fig. 1).

All 15 patients whose marrow and PBPC samples were positive for tumor relapsed (median DFS 286 days vs. 423 days; $P$=0.015). The patient group with the

**Table 1.** The incidence of breast cancer cells in marrow and PBPC collections

| *Specimen type* | *ICC positive* | *ICC negative* |
|---|---|---|
| Marrow | 43 | 203 |
| PBPC | 40 | 246 |
| Marrow and PBPC* | 15 | 178 |

**53 patients had only one specimen for breast cancer cells (either the marrow or the PBPC).*

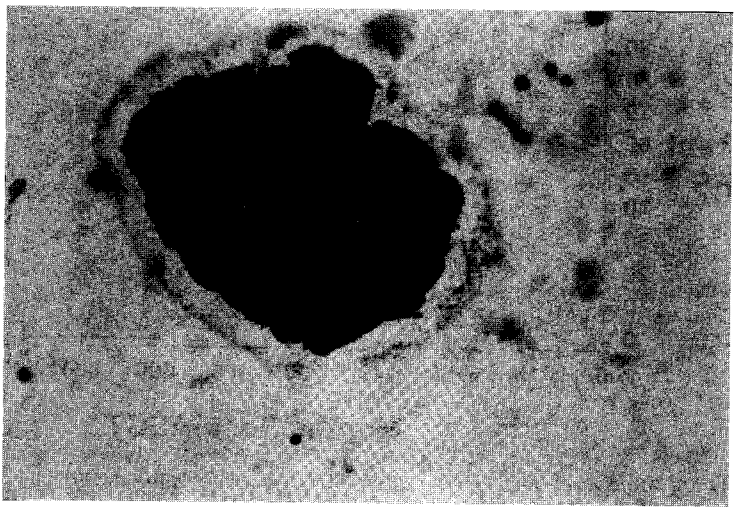

**Figure 2.** *400× picture of a breast cancer colony grown from a PBPC sample.*

best clinical outcome was the group where both the marrow and stem cell were negative for tumor cells (median DFS of 471 days vs. 339 days; $P= 0.007$).

Tumor colonies were found in 38 of 130 patients with stem cell samples and 14 of 66 patients with marrow samples (Fig. 2). Relapse of disease was documented in 37 of 38 patients with positive stem cell samples and 14 of 14 patients with a positive marrow specimen. This compared with a relapse rate of 55 of 92 and 34 of 52, respectively. DFS was significantly reduced for patients with tumor colony growth ($P<0.0001$ for stem cell samples and $P<0.02$ for marrow samples; Table 2). For patients with tumor colonies in the marrow, the median DFS was 266 days compared with 335 days for patients with no tumor colonies. For patients with tumor colonies in the stem cell product, the median DFS was 211 days compared with 369 days for patients with no tumor colonies (Fig. 3).

**Table 2.** The incidence and clinical significance of tumor colonies in PBPC products and bone marrow.

| | *Patients with tumor colonies (relapses/DFS)* | *Patients with no tumor colonies (relapses/DFS)* | *P value* |
|---|---|---|---|
| PBPC | 37/38 | 55/92 | <0.0001 |
| Marrow | 14/14 | 34/52 | <0.02 |

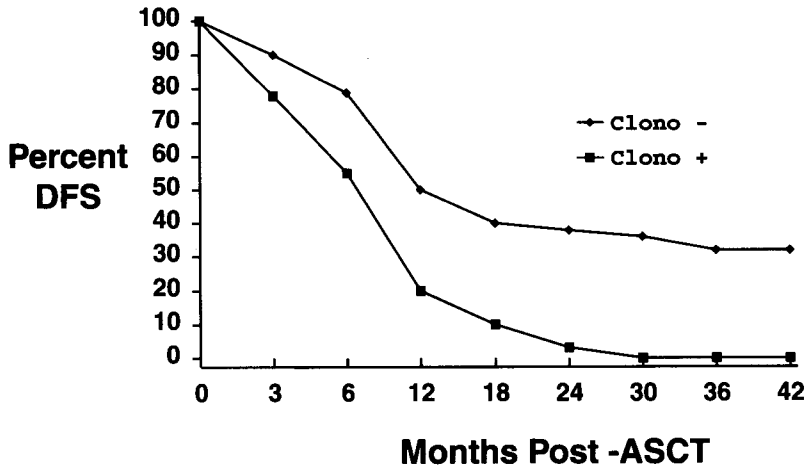


**Figure 3.** *Survival curve comparing the disease-free survival of patients with tumor colonies in the PBPC vs. those with no colony growth.*

## DISCUSSION

Studies have shown that approximately 3–22% of patients with stage IV breast cancer will have contamination of the PBPC product,[1–5] compared with the presence of breast cancer cells in 36–82% of marrow specimens.[1,6,7] Little, however, is known about the clinical significance of these occult micrometastatic cells.

In this study, we demonstrate that the presence of detectable tumor in blood and marrow stem cell collections at the time of transplant is highly predictive of a poor clinical outcome. Patients with both PBPC and marrow positive for breast cancer cells had a 100% relapse rate. On the other hand, patients whose marrow and stem cell product were negative for tumor cells had the best clinical outcome. This is one of the first studies to show that the presence of breast cancer cells in the reinfusion product is predictive of posttransplant outcome.

The clonogenic and ICC assays provide complementary prognostic information. In approximately 10% of cases, tumor colonies will grow despite a negative ICC due to extremely low levels of tumor contamination, poor cell viability, or other factors. In approximately 30–40% of cases, an ICC-positive patient will not grow tumor colonies. All of the positive patients will do poorly. This indicates that the presence of tumor cells in the collection may be a biologic marker of highly resistant disease. Consequently, these patients may be very poor transplant candidates and should be considered for alternative therapy.

Another important consideration is the determination of the growth potential for tumor cells in the marrow and stem cell product. One such method is the use of cell

culture assays to determine the viability and growth capacity of these micrometastatic cells. Some investigators have used such methods to grow tumor colonies from patient marrow in agar or in liquid culture systems.[2,3,8–10] In this study, we used a previously reported soft agar assay to determine the clonogenic growth of breast cancer cells in marrow and PBPC. We found that there was an extremely strong correlation with the presence of clonogenic tumor cells in the marrow and PBPC with a poor posttransplant outcome. In fact, 51 of 52 patients relapsed if clonogenic cells were found in the marrow or blood. This might suggest that patients with clonogenic micrometastatic breast cancer cells should not undergo autologous SCT, should have their graft purged of tumor cells, and/or should receive posttransplant therapy.

## ACKNOWLEDGMENTS

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# Tandem PBSC-Supported High-Dose Therapy for High-Risk Operable Breast Cancer

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We report on the efficacy and toxicity of a tandem high-dose therapy with peripheral blood stem cell (PBSC) support in 85 patients with high-risk stage II/III breast cancer. There were 71 patients with >9 tumor-positive axillary lymph nodes. The patients received an induction therapy of two cycles of ifosfamide (total dose 7.5 g/m$^2$) and epirubicin (120 mg/m$^2$). The PBSC-supported high-dose chemotherapy consisted of two cycles of ifosfamide (total dose 12,000 mg/m$^2$), carboplatin (900 mg/m$^2$), and epirubicin (180 mg/m$^2$). Patients were autografted with a median number of $3.7\times10^6$ CD34$^+$ cells/kg (range, 1.9–$26.5\times10^6$), resulting in hematologic reconstitution within ~2 weeks after high-dose therapy. The toxicity was moderate in general, and there were no treatment-related deaths. The probability of event-free and overall survival at 4 years was 60 and 83%, respectively. According to a multivariate analysis, patients with stage II disease had a significantly better probability of event-free survival (74%) compared with patients with stage III disease (36%). The probability of event-free survival was also significantly better for patients with estrogen receptor–positive tumors (70%) compared with patients with receptor-negative ones (40%). Bone marrow samples collected from 52 patients after high-dose therapy were examined to evaluate the prognostic relevance of isolated tumor cells. The proportion of patients presenting with tumor cell–positive samples did not change in comparison with that observed before high-dose therapy (65 vs. 71%), but a decrease in the incidence and concentration of tumor cells was observed over time after high-dose therapy. This finding was true for patients with relapse and for those in remission, which argues against a prognostic significance of isolated tumor cells in bone marrow. In conclusion, further intensification of the therapy, including the addition of non-cross-resistant drugs or immunologic approaches, may be envisaged for patients with stage III disease and hormone receptor–negative tumors.

# Autotransplantation for Men With Breast Cancer

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## ABSTRACT

We studied thirteen men receiving autotransplants for breast cancer and reported to the Autologous Blood and Marrow Registry (ABMTR) by 10 centers. Six had stage 2 breast cancer, four had stage 3, and three had metastatic breast cancer. Of 12 tumors tested, all were estrogen receptor (ER)-positive. Median age at transplant was 49 years. The most common conditioning regimen was cyclophosphamide, thiotepa, and carboplatin ($n$=5); the remaining eight men received other alkylator-based regimens. Three men received bone marrow, eight received blood stem cells, and two received both for hematopoietic support. All 13 patients had hematopoietic recovery. There were no unexpected regimen-related toxicities. Of 10 men receiving autotransplants as adjuvant therapy, three relapsed at 3, 5, and 50 months and died 16, 19, and 67 months posttransplant. Seven of 10 are disease-free with median follow-up of 23 months (range 6–50). Of three men treated for metastatic breast cancer, one had progressive disease and two recurrent disease at 6, 7, and 16 months posttransplant. Results of autotransplants for male breast cancer appear similar to those reported for women receiving autotransplants for breast cancer.

## INTRODUCTION

Male breast cancer accounts for <1% of all breast cancers, and the annual incidence is <1 in 100,000 males.[1,2] Stage at diagnosis is generally more advanced than in women and may be due to delay in diagnosis or anatomic factors.[3] Outcomes appear to be the same as in women when patients are matched for histopathology, receptor status, and disease stage.[3–6] There are some differences between male and female breast cancer.[1–7] In addition, more men have ER- and progesterone receptor (PR)-positive breast cancer. In one large study, 50–75% of postmenopausal women's breast cancers were ER- and PR-positive,[8] whereas 80–90% of men have ER- and PR-positive disease.[1–7] The standard therapy of

breast cancer in men is similar to treatment in women, including surgery, radiation, hormone therapy, and chemotherapy.[1–7] Autotransplant has been used for the treatment of women with high-risk adjuvant and metastatic breast cancer.[9–12] This is the first reported series of men undergoing autotransplant for male breast cancer (McCarthy et al., manuscript submitted).

## METHODS

Thirteen men were identified among 3254 autotransplants for breast cancer reported to the ABMTR between January 1989 and January 1996 by 107 centers. Detailed patient-, disease-, treatment-, and outcome-related data were obtained on standard ABMTR report forms. The ABMTR is a voluntary working group of more than 120 transplant centers primarily in North and South America that contribute detailed data on their autologous blood and bone marrow transplants to the Statistical Center at the Medical College of Wisconsin. Participants are required to report all consecutive autotransplants, and compliance is monitored by on-site audits. The ABMTR database includes data on ~50% of the autotransplants done in North and South America since 1989. Patients are followed longitudinally. Computerized error checks, physician review of submitted data, and on-site audits of participating centers ensure data quality.

## RESULTS

Thirteen subjects were treated at 11 centers. The median age was 49 years (range 32–60). Of 12 cancers tested, 12 were ER-positive and 10 were PR-positive. Twelve men underwent mastectomies, and one received presurgery chemotherapy. Ten men received autotransplants as adjuvant treatment, six for stage 2 disease (all with >10 positive ipsilateral axillary lymph nodes) and four for stage 3 disease (one with inflammatory breast cancer). All had received prior standard-dose adjuvant chemotherapy. Three men had autotransplants for metastatic disease. One had a complete response, one a partial response, and one stable disease before transplant.

Median interval from diagnosis to autotransplant was 6 months (range 4–9) for men with stage 2 or 3 disease and 12, 31, and 32 months for three with metastatic disease. One patient with metastatic disease received a planned second autotransplant. Mobilized peripheral blood ($n$=9), BM ($n$=3), or both ($n$=2) were used for hematopoietic stem cell (HSC) support. Five peripheral blood collections were mobilized with hematopoietic growth factors alone and six with growth factors and chemotherapy. High-dose therapy regimens consisted primarily of alkylating drugs. The most common regimen was cyclophosphamide, thiotepa, and carboplatin ($n$=5). Twelve of 13 men received hematopoietic growth factors after graft infusion. Nine of 10 men with stage 2 or 3 disease received primary chest wall

radiation, two before and seven after autotransplant. Six of 10 received hormonal therapy (tamoxifen) after autotransplant. One of the men with metastatic disease received local radiation therapy to the chest wall after autotransplant.

Median day to absolute neutrophil count >1000 cells $\times 10^9$/L was 12 days (range 8–22). Median day to platelet count >25,000 $\times 10^9$/L was 14 days (range 6–20). In three autotransplants, the exact day of achieving absolute neutrophil count >1000 cells $\times 10^9$/L was not reported, and in four, the day to platelet count >25,000 $\times 10^9$/L was not available. However, all patients had evidence of hematopoietic recovery and became transfusion-independent. No patient has developed myelodysplasia or other bone marrow disorder. No grade 4 (World Health Organization) nonmyeloid toxicities were reported.

Seven of the 10 men receiving autotransplants as adjuvant therapy are disease-free with median follow-up of 23 months (range 6–50). Three men relapsed at 3, 5, and 50 months posttransplant and subsequently died 16, 19, and 67 months posttransplant. All three men treated for metastatic breast cancer had evidence of progressive or recurrent disease after autotransplant. The patient who achieved a complete response to standard-dose chemotherapy received two autotransplants and relapsed 5 months after his second autotransplant. The patient transplanted after a partial response to standard-dose chemotherapy failed to achieve a complete response and progressed 7 months posttransplant. The patient transplanted with stable disease achieved a complete response after autotransplant but relapsed 16 months later. Two subsequently died 12 and 23 months posttransplant. One is alive with progressive breast cancer 27 months posttransplant.

## DISCUSSION

The ABMTR has reported on the use of autotransplant for the treatment of an unusual disease, male breast cancer (McCarthy et al., manuscript submitted). Autotransplant in this population was well tolerated with no regimen-related deaths and no unexpected nonmyeloid toxicities. The toxicity appears equivalent to previously published reports in women receiving autotransplants for breast cancer.[9–12]

Although the number of subjects is small, the efficacy of autotransplant in 10 men with high-risk breast cancer appears similar to results reported in women.[9,10] Seven of 10 men receiving autotransplants as adjuvant therapy are alive and disease-free. These results are favorable when compared with treatment with standard chemotherapy, radiation, and hormonal therapy for patients with locally advanced disease.[1–7] There were only three patients with metastatic disease, therefore it is difficult to evaluate the role of autotransplant for this indication. Evaluation of more cases and longer follow-up will be necessary to determine the incidence of late recurrence and possibility of cure in this population. The relative

efficacy of standard and high-dose chemotherapy in men with breast cancer is probably not evaluable given the rarity of this disease. However, these data suggest that indications for high-dose therapy developed in the ongoing randomized studies in women will be applicable to men. In addition, it would be reasonable to recommend inclusion of male breast cancer patients in randomized studies that examine the role of autotransplant for breast cancer.

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# High Dose Sequential Chemotherapy With PBSC Support in Inflammatory Breast Cancer: Toxicity and Pathologic Response. A French National Study, Pegase 02 (FNCLCC-SFGM)

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## INTRODUCTION

Inflammatory breast cancer (IBC) is an uncommon disease, occurring in about 2 to 4% of all breast cancers.[1] It is generally defined as a clinical entity, corresponding to the T4d stage of the 1988 International Union against Cancer (UICC) classification.[2]

Despite its low frequency, IBC remains a challenge for oncologists. When treated with surgery or radiotherapy alone, or both, 5-year survival does not exceed 15%.[3] The use of neoadjuvant chemotherapy has improved treatment of IBC, but prognosis is still very poor, with 5-year survival rates between 30 and 50%,[4–7] and there is no current consensus on the "best" induction chemotherapy regimen. Response to initial chemotherapy has been described as predictive of outcome, with progression-free and overall survival significantly higher for responding patients than for nonresponding patients.[8,9] Achievement of complete pathologic response seems a particularly important prognostic factor.[10–14] We can therefore speculate that improving efficacy of first-line chemotherapy could be one method of improving IBC prognosis.

Based on those considerations, the High Dose Chemotherapy for Breast Cancer Study Group (PEGASE) of the French Federation of Anticancer Centers initiated such a study (PEGASE 02) aimed at evaluating toxicity and feasibility of high-dose sequential chemotherapy with recombinant granulocyte colony-stimulating factor (G-CSF; filgrastim) and stem cell support in inflammatory breast cancer. In addition, response to this chemotherapy will be evaluated with an emphasis on pathologic response and impact on disease-free survival and survival. This report examines the toxicity and response rate in 100 patients.

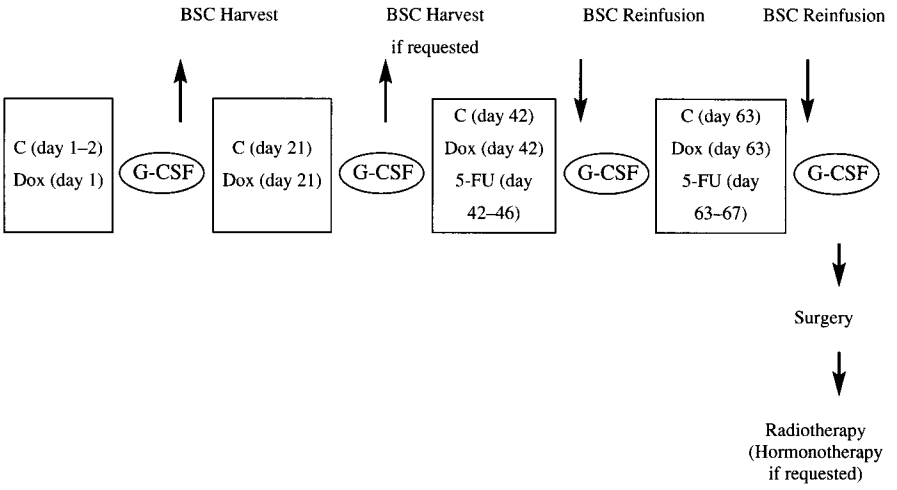


**Figure 1.** *Pegase 02 regimen. C, cyclophosphamide, 3 g/m²/day; Dox, doxorubicin, 75 mg/m²; 5-FU, 5-fluorouracil, 500 mg/m²/day continuous infusion; BSC, blood stem cells.*

## PATIENTS AND METHODS

### Eligibility

One hundred consecutive women with primary inflammatory breast cancer were included in this study. IBC was defined as follows: histologically documented adenocarcinoma of the breast with inflammatory signs (erythema, "peau d'orange" appearance, and increase in local temperature) which involved more than one-third of the breast (T4d of the 1988 UICC classification). Absence of dermal lymphatic carcinomatosis was not a criterion for exclusion.

Patients with locally advanced breast cancer (other T4 of the 1988 UICC classification), secondary IBC, or metastatic breast cancer (including supraclavicular lymph node involvement) were excluded from the study. Baseline evaluation included physical examination, bilateral mammography and breast echography, chest x-ray, radionuclide bone scan, liver echography, bone marrow aspiration, and, if possible, two bone marrow biopsies, standard biologic tests, and CA 15-3 assay.

In accordance with French law, the study was approved by the ethical committee (CCPPRB) of the University of Toulouse, and written informed consent was obtained from patients before study entry.

### Chemotherapy

Four cycles of chemotherapy were administered every 21 days. Cycle 1 consisted of cyclophosphamide 6 g/m² and doxorubicin 75 mg/m², cycle 2 of cyclophosphamide

3 g/m$^2$ and doxorubicin 75 mg/m$^2$, and cycles 3 and 4 of cyclophosphamide 3 g/m$^2$ doxorubicin 75 mg/m$^2$ and 5-fluorouracil (5FU) 2500 mg/m$^2$. Cyclophosphamide was administered as a 1-hour intravenous infusion, the dose was divided in two and administered for two consecutive days in cycle 1, and doxorubicin was given as a 15-min intravenous infusion and 5FU as a 5-day continuous intravenous infusion.

Semisaline hyperhydration (3 L/m$^2$/24 h) was started 4 hours before cyclophosphamide and stopped 20 hours after the end of cyclophosphamide infusion. Uroprotection was assured by uromitexan only at cycle 1.

Chemotherapy was administered if absolute neutrophil count (ANC) was $\geq 1.5 \times 10^9$/L and platelet count $\geq 100 \times 10^9$/L. No dose reduction was planned. If the neutrophil and platelet count did not meet these criteria on day 21, chemotherapy was delayed until adequate count recovery. Exclusion of patients from the study because of absence of hematologic recovery was left to the decision of each investigator.

For doxorubicin and cyclophosphamide, dose intensity was calculated as total chemotherapy administered, divided by body surface area and delay (in weeks) between day 1 of cycle 1 and 3 weeks after day 1 of cycle 4 (or 3 weeks after the theoretical day 1 of cycle 4 for patients who stopped treatment). Relative dose intensity (RDI) was calculated as the dose intensity divided by theoretical dose intensity.

## rG-CSF, stem cell collection, and reinfusion

rG-CSF (filgrastim) was administered at a daily dosage of 5 μg/kg (maximum 300 μg/kg per day) at each cycle of treatment. Administration started at day 4 of cycles 1 and 2 and day 7 (day of stem cell reinfusion) of cycles 3 and 4. rG-CSF was stopped the day before last apheresis or when the ANC reached $0.5 \times 10^9$/L on 3 consecutive days for cycles without apheresis.

Aphereses were performed after the first cycle of chemotherapy and/or after the second, depending on the possibilities of each center. Generally, the procedure was started when the absolute number of CD34$^+$ cells in the peripheral blood rose to 20/μL. Apheresis were stopped when collected CD34$^+$ cells exceeded $4 \times 10^6$/kg. Cells were divided into two bags at least, to allow reinfusion of a minimum of $2 \times 10^6$/L CD34$^+$ cells/kg after cycle 3 and cycle 4, after storage in liquid nitrogen.

No attempt was made to purge hematopoietic stem cells of possible tumor contamination.

Hematopoietic stem cells were reinfused on day 7 of cycles 3 and 4, at least 20 hours after the end of chemotherapy.

## Further anticancer therapy

Mastectomy was performed after induction chemotherapy for nonprogressive patients. Locoregional treatment was completed by radiotherapy, according to

procedures in each center. Finally, patients who were menopausal at diagnosis and with positive estrogen and/or progesterone receptors received tamoxifen 20 mg/d for 3 years.

### Response evaluation

*Clinical.* Clinical evaluation was performed on day 1 of each cycle of chemotherapy and before local treatment. Complete clinical response was defined as the clinical complete disappearance of breast inflammation as well as of the underlying breast tumor mass. Partial response was at least a 50% decrease in tumor diameter with disappearance of inflammation.

*Pathologic.* Two independent pathologists performed pathologic evaluation using a blind study technique. Response in the breast was defined as described by Chevallier et al.[15]: grade 1, disappearance of all tumor both on macroscopic and microscopic examination; grade 2, presence of in situ carcinoma of the breast with no invasive tumor; grade 3, presence of invasive carcinoma with stromal alterations such as sclerosis or fibrosis; grade 4, no or few alterations in tumor appearance.

Lymph nodes were evaluated separately when available after chemotherapy and classified in two categories: involved or not involved.

### Statistical analysis

No interim analysis on efficacy was performed. However, it was planned to stop the study if the toxic death rate was too high. To keep the toxic death rate under 3% with a 5% $\alpha$ risk, only four or fewer toxic deaths among the first 30 patients were deemed acceptable.[16]

Medians are presented with their range and response rate with 95% confidence intervals. Percentage differentials were tested by application of the $\chi^2$ test. When a patient stopped her treatment, she was analyzed for received cycle toxicity, dose intensity, and pathologic response if mastectomy was performed before beginning another antineoplastic treatment and for follow-up.

## RESULTS

### Patients

Between December 1994 and September 1996, 100 patients from 17 participating centers entered the study.

Five patients were withdrawn from the study: four had metastatic IBC at diagnosis and were not eligible and one received another chemotherapy regimen before the first cycle. As a result, a total of 95 patients were considered valid for analysis.

**Table 1.** Tumor characteristics

| | |
|---|---|
| Extent of inflammatory signs | |
| Limited | 61% |
| Diffuse | 39% |
| Node involvement | |
| 0 | 20.2 % |
| 1 | 58.5% |
| 2 | 21.3% |
| Pathologic classification | |
| Ductal | 80.2% |
| Lobular | 5.5% |
| Other | 14.3% |
| SBR Grade | |
| I | 2% |
| II | 30.8% |
| III | 58.2% |
| Nonevaluable | 9% |
| Estrogen/progesterone receptors | |
| +/+ | 17% |
| +/2 or 2/+ | 18% |
| 2/2 | 42% |
| Unknown | 23% |

Median age of patients was 46 years (range 26–59); 83.2% were premenopausal at time of diagnosis. Initial characteristics of tumors are summarized in Table 1. Axillary dissection was initially performed in only 17 patients. Median number of involved nodes for these patients was eight (range 0–23), with eight patients having 10 or more involved nodes. Dermal lymphatic carcinomatosis was found in 43% of patients who had a skin biopsy.

## Stem cell collection and infusion

Ninety-seven percent of patients had a successful collection of $CD34^+$ cells after cycle 1 and/or cycle 2, and 93% of all patients after a single set of aphereses. Median number of collected $CD34^+$ cells was $14.75\times10^6$/kg (range 2.3 to >100), and a median of $6.05\times10^6$/kg (range 1.2 to >100) and $8.5\times10^6$/kg (range 1.2 to 59.1) $CD34^+$ cells were reinfused after cycles 3 and 4, respectively.

## Toxicity

*Nonhematologic.* Grade 3 or 4 vomiting occurred in 14% of cycles and grade 3 or 4 mucositis in 10% of cycles (4% in cycles 1 and 2, 15% in cycle 3 and 4,

**Table 2.** Neutropenia and febrile neutropenia

| | *Cycle 1* | *Cycle 2* | *Cycle 3* | *Cycle 4* |
|---|---|---|---|---|
| ANC $<0.1\times10^9$/L | | | | |
| Frequency | 64% | 41%* | 48% | 56% |
| Median duration in days (range) | 4 (1–16) | 3 (1–8) | 5 (1–10) | 4 (1–10) |
| ANC $<0.5\times10^9$/L | | | | |
| Frequency | 79% | 75% | 78% | 79% |
| Median duration in days (range) | 5 (1–16) | 4 (1–10) | 5 (1–10) | 5 (1–10) |
| Incidence of febrile neutropenia | 48% | 26%† | 48% | 51% |

ANC, absolute neutrophil count. *$P<0.05$; †$P<0.01$.

$P<0,01$). Grade 3 hepatic toxicity was seen in one patient in cycle 1 and in another patient in cycle 3. No other grade 3 or 4 toxicities were seen during the study. Monitoring of left ventricular ejection fraction showed no clinically significant diminution.

*Hematologic (Tables 2 and 3).* One patient died from the procedure. She was readmitted for febrile neutropenia after cycle 1 and died from septic shock with multiorgan failure. Grade 4 neutropenia occurred in between 75 and 79% of each cycle. Duration of neutropenia less than $0.5\times10^9$/L was a median 5 days per cycle. Febrile neutropenia was the most frequent reason for rehospitalization (85% of all rehospitalizations). Overall, there was no cumulative increase in frequency and duration of neutropenia or complications over the four cycles. However, cycle 2 was overall significantly associated with less toxicity.

**Table 3.** Thrombopenia and transfusions

| | *Cycle 1* | *Cycle 2* | *Cycle 3* | *Cycle 4* |
|---|---|---|---|---|
| Platelets $<20\times10^9$/L | | | | |
| Frequency | 41% | 26%* | 44% | 46% |
| Median duration in days (range) | 2(1,15) | 1(1,27) | 2(1,11) | 2(1,12) |
| Platelets $<50\times10^9$/L | | | | |
| Frequency | 63% | 56% | 70% | 69% |
| Median duration in days (range) | 4(1,15) | 3(1,33) | 5(1,27) | 5(1,14) |
| Platelet transfusions | | | | |
| Incidence | 43% | 29%** | 53% | 56% |
| Median no. transfusions (range) | 1(1,6) | 1(1,6) | 2(1,5) | 1(1,4) |
| Red blood cell transfusions | | | | |
| Incidence | 37% | 38% | 66% | 83% |
| Median no. transfusions (range) | 1(1,6) | 1(1,6) | 2(1,8) | 1(1,4) |

*$P<0.05$; †$P<0.01$.

**Table 4.** Response

| | *Number of evaluable patients* | *Percentage of responders* |
|---|---|---|
| Objective clinical response | 94 | 90 ± 6% |
| Pathologic response in breast | 87 | GR I and II: 32 ± 10% |
| | | GR III and IV: 68 ± 10% |

*GR I, disappearance of tumor on both macroscopic and microscopic examination; GR II, presence of in situ carcinoma of the breast, with no invasive tumor; GR III, presence of invasive carcinoma with stromal alterations such as sclerosis or fibrosis; GR IV, no or few alterations of tumoral appearance.*

Thrombocytopenia of less than $20\times10^9$/L occurred in 26 to 46% of cycles. Duration of thrombocytopenia less than $20\times10^9$/L was a median 2 days per cycle, with the same duration after each cycle. Platelet transfusions were needed in 29 to 56% of cycles, with administration of a median of one transfusion per cycle.

## Chemotherapy delivery

A total of 366 cycles of chemotherapy were administered to 95 evaluable patients. Median received dose intensity for cyclophosphamide and doxorubicin was 1211 mg/m$^2$/week (range 50–1305) and 24 mg/m$^2$/week (range 6–26) with respective relative dose intensity of 0.97 (range 0.40–1.04) and 0.96 (range 0.25–1.05).

## Anti-tumoral response (Table 4)

*Clinical.* Of the 94 evaluable patients, one had no clinical response after four cycles of chemotherapy (persistence of inflammatory signs). All other patients had a good clinical response to chemotherapy with complete disappearance of tumoral signs (clinical complete response) in 75 (80%). After the first cycle of chemotherapy, 34 patients (41%) had a complete disappearance of inflammatory signs.

*Pathological.* One patient in clinical failure did not undergo mastectomy and was evaluated as a pathologic failure. In eight other patients with good clinical responses, mastectomy was not performed at their request. Limited local treatment was given consisting of partial surgery and radiotherapy or radiotherapy alone. These eight patients were not evaluated for pathologic response.

Eighty-six patients underwent mastectomy performed a median of 3.5 months (range 3–9) after the first cycle of chemotherapy: 28 experienced complete disappearance of tumor cells (grade I) or persistence only of an intraductal component (grade II) (32 ± 10% of grade I or II pathologic response).

In 24 other patients, major changes in histology were found, such as tumor cell necrosis and stromal alteration showing partial efficacy of chemotherapy (grade III). However, an invasive component was persistent in these patients.

Finally, in 34 patients, despite good clinical response, no evidence of pathologic response to chemotherapy (grade IV) was seen.

Objective evaluation of response rate in breast and lymph nodes was difficult to assess since several patients had pathologic CR in the breast but had undergone previous axillary dissection. However, dissociation of response was clearly observed in some other patients, since eight patients had positive lymph node dissection despite pathologic complete response in the breast.

At present, with a follow up ranging from 12 to 34 months after inclusion, 31 patients have relapsed (local relapse or metastasis) and 14 have died.

## DISCUSSION

Acute toxicity related to chemotherapy mainly consisted of severe but reversible pancytopenia in all four cycles of chemotherapy. The second cycle of chemotherapy was less toxic, leading to fewer cases of severe neutropenia, febrile neutropenia, thrombocytopenia, and platelet transfusion. This difference was expected, since cycle 2 differed from cycle 1 in the cyclophosphamide dose (3 g/m$^2$ vs. 6 g/m$^2$) and from cycles 3 and 4 by the absence of 5FU.

The relatively short duration of neutropenia is probably related to use of rG-CSF. One can question the utility of peripheral blood stem cells in this study, in which there was no myeloablative chemotherapy. However, the incidence of severe neutropenia, thrombocytopenia, and febrile neutropenia did not increase from cycle 1 to cycle 4. In previously published studies of high-dose doxorubicin-cyclophosphamide regimens with rG-CSF but without stem cell transplantation,[17–19] thrombocytopenia is generally the dose-limiting toxicity and appears to be cumulative, increasing significantly between the first and last cycles.[17] Overall, peripheral blood stem cells appear to permit a safer and more regular increase of dose intensity in high-dose sequential chemotherapy regimens, as previously described.[20,21]

One of the risks in using peripheral blood stem cells is the mobilization, collection, and reinfusion of tumor cells.[22] This is a potential risk in our study in which most patients had the blood stem cell collection after the first cycle of chemotherapy. However, the significance of circulating tumor cells and their impact after reinfusion are not yet clearly established. Moreover, ex vivo therapy is not considered standard practice at present.

Nonhematologic toxicities were almost entirely mucositis, occurring more frequently after cycle 3 and 4 and probably related to administration of 5FU.

Relative dose intensity was 0.97 (range 0.4–1.04) for cyclophosphamide and 0.96 (range 0.25–1.05) for doxorubicin. Eighty-seven patients received four cycles

**Table 5.** Pathologic response rate with conventional chemotherapy

| *Reference* | *Patients (n)* | *Chemotherapy* | *Microscopic complete response rate* |
|---|---|---|---|
| Feldman[10] | 90 | 5FU-D-Cy | 7% |
| Israel[23] | 24 | 5FU-Cy | 0% |
| Maloisel[12] | 44 | D-5FU-Cy | 18% |
| Armstrong[13] | 24 | D-Cy-VC-MTX-L-5FU | 17% |
| Chevallier[15] | 97 | 5FU-Ep-Cy ± lenograstim | 22% |
| Colozza[24] | 31 | CDDP-D-Cy | 8% |

of chemotherapy, i.e., 15 g/m$^2$ of cyclophosphamide, 300 mg/m$^2$ of doxorubicin, and 5000 mg/m$^2$ of 5FU, in 9 weeks. This strategy of high-dose sequential chemotherapy with stem cell support allows the administration of a total dose of cyclophosphamide to be given that is approximately 7.5 times higher than given in four cycles of standard FAC[10] (5-fluorouracil, adriamycin, cyclophosphamide) and three times higher than in the 5-fluorouracil, epirubicin, cyclophosphamide (FEC) high-dose regimen described by Chevallier et al.[15] Our data show that such an increase in dose and dose intensity is achieved in 92% of patients with nonmetastatic inflammatory breast cancer with the use of rG-CSF on an outpatient schedule.

The second end point of our study was to evaluate the response rate of inflammatory breast cancer to a dose-intensified cyclophosphamide/doxorubicin/5FU regimen. The clinical response rate was high (overall response rate 90%), as generally described with other anthracycline-based regimens. When the pathologic response in the breast was considered in 87 evaluable patients, only 32% had total disappearance of invasive tumor cells. Pathologic response rates, comparably defined, have been previously published after conventional or moderately intensified systemic induction chemotherapy (Table 5). Feldman et al.,[10] using standard FAC, reported a 12% pathologic complete response rate in 90 patients. Chevallier et al.,[15] using an intensified FEC, reported a 22% pathologic complete response rate in 97 patients. In other studies with smaller numbers of patients (<50), complete pathologic response rates ranged from 0 to 18% after various intravenous chemotherapies.[12,13,23,24] Our results show a relatively higher pathologic response rate when compared with large series in the literature.

The achievement of such a pathologic response rate in our study, although encouraging, remains suboptimal and must be improved by designing new regimens that include other drugs, further escalation, and/or combinations.

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# High-Dose Chemotherapy With Autologous Blood Stem Cell Transplantation: Increasing Evidence for Efficacy in Patients With Metastatic Breast Cancer

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## ABSTRACT

High-dose chemotherapy (HDCT) with autologous blood stem cell transplantation has been increasingly used in patients with breast cancer and non-Hodgkin's lymphoma in Canada. Five ;evels of scientific evidence regarding therapeutic interventions (Sackett DL: Rules of evidence and clinical recommendations on the use of antithrombotic agents. *Chest* 95:2S–4S, 1989) have been identified. Level I evidence is strongest to support efficacy of a novel treatment and yet, for patients with metastatic breast cancer worldwide, no such evidence exists. In close collaboration with centers across Canada, we created level IV evidence indicating that patients who respond to initial chemotherapy have a median progression-free survival (PFS) of 8–12 months and median overall survival (OS) of 24–30 months. Recently, we have provided level III evidence from outcomes of 144 patients receiving HDCT over the time period of 1991–1995 compared with 135 contemporaneous controls. Multivariate analysis revealed a statistically significant difference in favor of HDCT for OS (relative risk [RR] 0.62; 95% CI 0.35–0.97; $P$=0.008) and PFS (RR 0.46; 95% CI 0.16–0.76; $P$<0.001). Level II evidence is available only from one small randomized trial from South Africa that suggests a PFS and OS benefit for HDCT compared with standard-dose CT. To establish level I evidence, the National Cancer Institute of Canada, Clinical Trials Group (NCIC-CTG), has recently commenced a Phase III study entitled, "A Randomized Trial of High-Dose Chemotherapy and Autologous Stem Cell Therapy Versus Standard Therapy in Women With Metastatic Breast Cancer Who Have Responded to Anthracycline or Taxane-Based Induction Chemotherapy (NCIC-CTG MA.16)." Approximately 400 patients will be enrolled in this study, and 200 will be randomized to each arm. To date, over 145 patients have been registered and the enrollment continues.

## INTRODUCTION

The use of HDCT and autoSCT for treatment of breast cancer has been steadily increasing over the last several years. More than 3500 cases were reported to the Autologous Blood and Marrow Transplant Registry (ABMTR) in 1995.[1] During the same time, the European Blood and Marrow Transplant Group (EBMT) registered 1300 breast cancer cases, resulting in the second leading indication for this treatment option in Europe.[2] For patients with metastatic breast cancer, the 100-day mortality has been decreasing to <5%, making HDCT and ABSCT a relatively safe therapeutic intervention.[1] Some centers have introduced an outpatient approach that certainly is the preferred option for patients and seems to be less expensive.[3] Nevertheless, the proof that HDCT with ABSCT is a superior treatment option for patients with metastatic cancer is still a matter of controversy. Five levels of scientific evidence regarding therapeutic interventions have been categorised (Table 1).[4] Although a large number of patients have been enrolled in clinical phase I and II studies, only one small randomized prospective phase III study has been published.[5] Peters et al.[6] reported data of a randomized phase III study, where immediate vs. delayed HDCT and ABSCT was tested in patients achieving complete remission to standard dose chemotherapy (SDCT). Large randomized prospective phase III studies, building level I evidence, are underway, but results are not expected within the next 2 years or so. Therefore, we report here on results of a series of phase I and II studies (level IV evidence) and a formal comparison with nonrandomized contemporaneous controls using our databases (level III evidence)[7] and describe a phase III study entitled, "A Randomized Trial of High-Dose Chemotherapy and Autologous Stem Cell Therapy Versus Standard Therapy in Women With Metastatic Breast Cancer Who Have Responded to Anthracycline or Taxane-Based Induction Chemotherapy," which was commenced recently by NCIC-CTG (MA.16) and will contribute to level I evidence.

## PHASE I STUDY

### Patients and treatments

Female patients age 18–55 years with metastatic breast cancer were eligible to enter this phase I study.[8] Major end-points of the study were to define the dose-limiting toxicity (DLT) and maximal tolerated dose (MTD). Minor end-points included response rates after induction chemotherapy (IDC) and after HDCT, ability to mobilize and collect sufficient numbers of blood stem cells for autotransplantation, and time to hematologic recovery. Progression-free and overall survival were also recorded. No previous chemotherapy for metastatic

**Table 1.** Levels of scientific evidence for therapeutic interventions

| *Evidence* | *Description* |
|---|---|
| Level V | Case series |
| Level IV | Formal comparison with historic controls |
| Level III | Formal comparison with nonrandomized contemporaneous controls |
| Level II | Randomized controlled trials that are too small |
| Level I | Randomized controlled trials that are big enough and meta-analyses |

disease was allowed. Adjuvant chemotherapy, if any, had to be at least 6 months before metastatic disease. Patients were excluded if they had exposure to taxanes. Karnofsky performance status was ≥60%, and cardiopulmonary and hematopoietic functions were normal. 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 100 mg/m$^2$ BSA, cyclophosphamide 750 mg/m$^2$ BSA, all delivered on day 1 as intravenous infusion. Starting on day 2, G-CSF (10 µg/kg subcutaneously) was administered daily for ~2 weeks until the total white blood cell (WBC) count reached 2.5/nL. On that day, standard apheresis procedure (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 $5\times10^8$ mononucleated cells per kilogram body weight and $2\times10^6$ CD34$^+$ cells/kg.[9] After the first cycle of IDC, the patients obtained a further two to three cycles of chemotherapy consisting of 5-FU 600 mg/m$^2$ BSA, epirubicin 60 mg/m$^2$ BSA, cyclophosphamide 600 mg/m$^2$ BSA, all administered intravenously. The treatment was delivered every 3 weeks if ANC was ≥1.5/nL and platelets ≥100/nL. After 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 cyclophosphamide 6 g/m$^2$ intravenously delivered on 3 consecutive days; MXT 70 mg/m$^2$ intravenously delivered on the same 3 consecutive days. On day 4, the paclitaxel dose starting at 250 mg/m$^2$ was delivered as a 3-hour infusion. The dose was increased by 50 mg/ m$^2$ + XL if three or more patients did not experience DCT. Usual premedication, including antiemetics and dexamethasone, was delivered on each day. After 24–48 hours of rest, the ABSC were reinfused and rhG-CSF was administered at the dose of 5 µg/kg daily until hematologic recovery (ANC ≥1.5/nL for 3 consecutive days). The ABSC were cryopreserved in 10% DMSO as previously described.[9] The four days of chemotherapy were delivered in the hospital. The patients were usually discharged on the day after HDCT just before the reinfusion of the autograft.[3]

## RESULTS

### Patient demographics

Between November 1994 and October 1996, 50 female patients with metastatic (M1) breast cancer were enrolled into the study. Forty patients who did not progress on IDC were eligible to be treated with HDCT and were fully evaluable. The median age was 46 years (29–55). Before developing metastatic disease, seven patients were diagnosed with stage I, 22 patients with stage II, and three patients with stage III breast cancer and eight patients presented initially with M1 disease. The time from diagnosis to PD ranged between 0 (stage IV) and 96 months, median receptor-negative. Twenty-two patients received adjuvant chemotherapy and eight of them received an anthracycline-containing regimen. Only 13 had adjuvant tamoxifen. Approximately one-third of our patients had three or more sites involved and represented a poor-prognosis population; 16 had lung and eight had liver metastases.

### Hematologic toxicity

Hematologic engraftment did not show any significant difference between cohorts; very short engraftment times of <2 weeks were observed, comparable to other published studies. No differences between cohorts were observed regarding red blood cell and platelet transfusion frequency and use of intravenous antibiotics for febrile neutropenia; on average, two units of PRBC (range 0–5) and two bags of single-donor platelets (range 1–5) were transfused per patient. Twenty-one patients required intravenous antibiotics for febrile neutropenia.

### Nonhematologic toxicities

Grade 3 mucositis (according to World Health Organization toxicity grading) was observed only at three dose levels: 300 mg/m$^2$ (one of five patients), cohort 3 350 mg/m$^2$ (three of 10 patients), and cohort 4a 400 mg/m$^2$ (two of nine patients), with no grade 4 mucositis documented. Nausea and vomiting, gastrointestinal, genitourinary, cardiac, and neurologic toxicities were moderate and infrequently observed. Very few patients experienced bone pain, fatigue, peripheral edema, redness at the site of rhG-CSF injection, insomnia, and occasional reaction to transfusion. Cardiotoxicity, defined as left ventricular ejection fraction (LVEF) dropping below normal values after HDCT, was observed in cohort 1 (two of seven patients), cohort 3 (two of 10 patients), cohort 4 (two of nine patients), and one patient in cohort 5. Three of these patients had clinically relevant but reversible cardiac symptoms. A fourth patient had pericardial involvement with metastatic disease (initially without clinical symptoms and with normal LVEF), and had

received adjuvant radiotherapy to the left chest as well as adjuvant chemotherapy with a total dose of 720 mg/m$^2$ epirubicin. She died 5 weeks after transplantation with clinical signs of cardiac failure. No other early or toxic deaths occurred on study. The DLT we observed was unexpected and not previously described. When reaching the dose level of 400 mg/m$^2$ paclitaxel, three of six patients experienced diaphoresis, bradycardia, mild hypotension, and diarrhoea approximately 20–40 minutes after completion of infusion; two of these three patients lost consciousness for a few minutes. Therefore, another four patients were treated again at the paclitaxel dose level of 350 mg/m$^2$, infused over 3 hours, for a total of 10 patients at this dose level. Since these side effects were not observed, we decided to increase the 350 mg/m$^2$ paclitaxel infusion time to 6 h; with the extended infusion time, we were able to increase the dose to 400 mg/m$^2$ paclitaxel in the next six patients. At the next level of paclitaxel, 450 mg/m$^2$ infused over 6 h, the same complication with a few minutes' loss of consciousness occurred in one patient. Therefore, further accrual was terminated and paclitaxel 450 mg/m$^2$ was defined as DLT.

### Response to treatment

Table 2 summarizes the response rates for all 50 patients enrolled into our phase I study. Nine were taken off study because of PD, and one patient withdrew her consent. Twenty-one patients achieved a PR and nine additional patients CR with no apparent differences in each cohort. Forty patients actually obtained the HDCT with ABSC. In Table 3, the response rates of those 40 patients completing the whole protocol are shown. Although another three patients progressed quickly within 4 weeks of evaluation, 32 of 40 had an objective response, with 13 patients reaching a CR. We observed a conversion from PR to CR in two patients in cohort

**Table 2.** Response rates after induction therapy just prior to high dose-chemotherapy for each paclitaxel (TXL) dose

| *TXL dose and infusion time* | *Patients (n)* | *Progression of disease* | *Stable disease* | *Partial response* | *Complete response* |
|---|---|---|---|---|---|
| 250 (3 h) | 7 | 0 | 1 | 5 | 1 |
| 300 (3 h) | 5 | 0 | 0 | 3 | 2 |
| 350 (3 h) | 10 | 0 | 2 | 7 | 1 |
| 350 (6 h) | 2 | 0 | 0 | 1 | 1 |
| 400 (3 h) | 6 | 0 | 2 | 4 | 0 |
| 400 (6 h) | 9 | 0 | 5 | 1 | 3 |
| 450 (6 h) | 1 | 0 | 1 | 0 | 0 |
| Off study | 10 | 9 | 0 | 0 | 1 |
| Total | 50 | 9 | 11 | 21 | 9 |

**Table 3.** Response rates 4–6 weeks after high dose chemotherapy for each group; patients who were removed from the study prior to high-dose chemotherapy are excluded from response evaluation

| *TXL dose and infusion time* | *Patients (n)* | *Progression of disease* | *Stable disease* | *Partial response* | *Complete response* |
|---|---|---|---|---|---|
| 250 (3 h) | 7 | 1 | 0 | 5 | 1 |
| 300 (3 h) | 5 | 0 | 0 | 1 | 4 |
| 350 (3 h) | 10 | 0 | 2 | 5 | 3 |
| 350 (6 h) | 2 | 0 | 0 | 1 | 1 |
| 400 (3 h) | 6 | 1 | 0 | 5 | 0 |
| 400 (6 h) | 9 | 0 | 3 | 2 | 4 |
| 450 (6 h) | 1 | 1 | 0 | 0 | 0 |
| Total | 40 | 3 | 5 | 19 | 13 |

2, two patients in cohort 3, and one patient in cohort 4a, for a total of five patients; increasing the number of CR from eight to 13 in the HDCT group.

## PHASE II STUDIES

### Patients and treatments

Seventy-four patients were recruited between November 1991 and October 1994. The eligibility criteria were the same as the phase I study.[10] All patients underwent two cycles of the following IDC: cyclophosphamide 500 mg/m$^2$ intravenously, day 1; epirubicin 60 mg/m$^2$ intravenously, day 1; 5-FU 500 mg/m$^2$ intravenously, day 1. The regimen was repeated once. All responding patients were then treated with augmented doses and hematopoietic growth factor support to ameliorate neutropenia and effectively mobilize hematopoietic stem cells: CTX 2000 mg/m$^2$ intravenously;epirubicin 60 mg/m$^2$ intravenously; 5-FU 500 mg/m$^2$ intravenously, all drugs on day 1. Starting on day 2 of each treatment cycle, human recombinant GM-CSF (Sandoz Canada, Montreal, and Schering-Canada), or human recombinant G-CSF (Amgen, Thousand Oaks, CA) was administered subcutaneously at a daily dose of 5 µg/kg until the AP were complete. The procedure was same as described in the phase I study.

### High-dose chemotherapy regimen

The HDCT regimen consisted of carboplatin 800 mg/m$^2$ or VBL 12 mg/m$^2$, methotrexate 50–70 mg/m$^2$, cyclophosphamide 6 g/m$^2$, all administered intravenously over 4 days (days –5 through –2) in four equal doses.[10] After one day

of rest (day –1), ABSC were reinfused on day 0. Starting within 24 hours, rhG-CSF at the dose of 5 µg/kg was administered subcutaneously until hematologic recovery.

## RESULTS

Ninety-two patients were enrolled into the study. Eighteen patients were withdrawn from the study after completion of the induction regimen because of PD or other reasons (increasing renal insufficiency, one patient; diagnosis of a second cancer, one patient; withdrawal of consent, one patient). Thus far, 74 have undergone the complete treatment and are evaluable for toxicity and efficacy. In general, treatment-related toxicity was rather rare and mild, except for hematotoxicity. None of our patients died from acute toxic complications within the first 6 weeks during and after transplantation. Transfusion frequency for packed red blood cells after the high-dose chemotherapy ranged between 0 and 26 units for a median of only two units. There were between 1 and 29 platelet transfusion encounters, for median of only two encounters. Median time of hematologic recovery to ANC ≥0.5/nL was 11.5 days (range 8–17), days to platelet recovery of ≥20/nL was 12 (range 0–17). The patients' age was 42 years (19–55). Those who did not fulfill the criteria of response and did not obtain the HDCT had a median survival of only 8 months. Patients who underwent HDCT and autologous BSC showed a median survival of 28.5 months. This is longer than the average for historical controls treated with standard-dose chemotherapy (SDCT). We performed univariate analysis. From all patients enrolled into the study (intent-to-treat analysis), the median survival was 28 months if liver was not involved by metastatic disease, and only 8 months with liver involvement. The hazard ratio of dying with metastatic breast cancer to the liver was 3.25. Adjuvant chemotherapy predicted a statistically significant difference in survival: median survival for patients without adjuvant chemotherapy is 28 months. Patients developing metastasis after adjuvant chemotherapy have a median survival probability of 13.5 months (difference significant; $P$=0.049, log-rank test; hazard ratio 0.54). Prior doxorubicin, bone marrow involvement, and age did not reveal a significant difference. Figure 1 shows the probability of survival of all 74 transplanted patients as estimated by the Kapler-Meier method showing differences in survival in patients with bone metastases and visceral metastases, respectively ($P$<0.001, log-rank test).

## FORMAL COMPARATIVE RETROSPECTIVE COHORT ANALYSIS

This study was designed as a comparative (retrospective cohort) analysis of two groups of patients with metastatic breast cancer, one treated with high-dose chemotherapy/stem cell transplantation (the experimental therapy group), and the other given modern conventional-dose chemotherapy regimens without stem cell support (control group).[11] The study period was January 1991 to December 1995

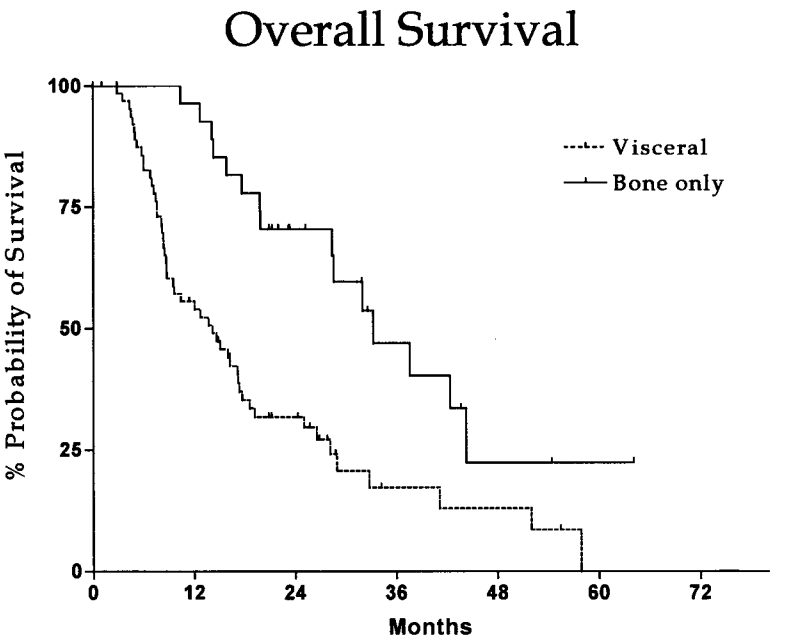


**Figure 1**

with a minimum follow-up of 2 years for all patients in both groups. Patient eligibility was the same as for our previous phase I and II studies.

## SOURCE OF SUBJECTS

### Experimental patients

The experimental group was compiled from two separate datasets. The first experimental dataset included patients who received high-dose chemotherapy/stem cell transplantation as part of a prospective phase II clinical trial at the University of Nebraska Medical Centre (UNMC). The principal investigators for this study were Drs. Elizabeth Reed and Stefano Tarantolo. The second experimental dataset consisted of information on patients who received high-dose therapy/stem cell transplantation in phase II trials at the Northeastern Ontario Regional Cancer Centre (NEORCC). The principal investigator for these trials was Dr. Stefan Glück.

### Control patients

The Ottawa Regional Cancer Centre (ORCC) has a wide referral base of ~1.5 million people and sees ~800 new cases of breast cancer annually (internal data). After

obtaining appropriate internal institutional consent to collect ORCC patient-related information, the population from which the control group would be selected was identified. This population was defined as all patients referred to the ORCC with a diagnosis of metastatic breast cancer treated with chemotherapy for metastatic disease at some point during the study period. This population was generated using a computerized search strategy within the Oncology Patient Information System (OPIS). This population was then sampled and individual patient charts reviewed to determine the patients deemed eligible for high-dose chemotherapy/stem cell transplantation, but who received conventional chemotherapy for their illness (the control group). The inclusion criteria were designed to approximate as closely as possible the same physiologic/biological characteristics as was represented by the experimental group to minimize the effects of selection bias. Also, to minimize the introduction of any possible bias incurred by a temporal or sequenced selection of patients, chart numbers were selected at random using numbers generated from a random number table (generated using the random number function in Microsoft Excel).

## TREATMENT PROTOCOLS

### Standard-dose chemotherapy

For patients who received adjuvant chemotherapy, CMF, FAC, and FEC accounted for the vast majority of chemotherapy regimens. If a patient had not received prior adjuvant therapy, the first-line treatment for metastatic disease was most often anthracycline-based (FEC or FAC), although a few patients were treated with CMF. For patients who had received anthracycline-based therapy in the adjuvant setting, the first-line chemotherapy was most often single-agent paclitaxel or docetaxel. Other chemotherapy regimens included vinorelbine (generally as a single agent), 5-FU/folinic acid, 5-FU/folinic acid/mitoxantrone, etoposide/cisplatin, mitomycin-C, and vinblastine. Only the response and response duration of the first chemotherapy for metastatic disease were used to calculate the failure-free survival. The duration of chemotherapy (number of cycles) was at the treating physician's discretion and generally depended on tolerance of therapy as well as ongoing response.

### High-dose chemotherapy

At UNMC, the high-dose chemotherapy regimen was as follows: cyclophosphamide 1.5 g/m$^2$/day intravenously as a continuous infusion for 4 days; thiotepa 150 mg/m$^2$/day intravenously as a continuous infusion for 4 days; hydroxyurea 1.5 g/m$^2$ orally q 6 hours for 12 doses; and stem cell infusion 72 hours after the last doses of thiotepa and cyclophosphamide.

Sudbury patients were treated on one of the two high-dose chemotherapy regimens. Regimen 1: cyclophosphamide 3 g/m$^2$/day intravenously for 2 days; mitoxantrone 23 mg/m$^2$/day intravenously for 3 days; vinblastine 12 mg/m$^2$ intravenously as continuous infusion over 5 days. Regimen 2: cyclophosphamide 3 g/m$^2$/day intravenously for 2 days; mitoxantrone 23 mg/m$^2$/day intravenously for 3 days; carboplatin 800 mg/m$^2$ intravenously for 1 day; and stem cell infusion 48–72 hours postchemotherapy.

## RESULTS

### Patients

The datasets consisted of patients treated between 1 January 1991 and 31 December 1995. The UNMC high-dose chemotherapy dataset contained 77 eligible evaluable UNMC patients. The NEORCC high-dose dataset consisted of 77 eligible patients, for a total of 154 patients. The ORCC standard-dose chemotherapy database consisted of 154 patients.

### Overall survival outcomes

The median survival of all patients after the development of overt recurrent/metastatic disease was 27.4 months (95% CI 25.6–33.1). When grouped by treatment, the median survival of patients treated with standard chemotherapy was 25.6 months (95% CI 23.1–33.2), and for patients treated with high-dose chemotherapy, 28.1 months (95% CI 26.4–36.1). This difference was not statistically significant by univariate testing (unadjusted $P$=0.39 by log-rank, $P$=0.43 by Tarone-Ware). Using the Cox proportional hazards model with stepwise regression analysis to adjust for the effects of multiple potential confounding variables, a highly significant treatment effect was found in favor of high-dose therapy ($P$=0.0076, hazard ratio 0.62, 95% CI 0.27–0.97).

### Failure-free survival; all patients

The median duration of failure-free survival for all patients after chemotherapy was 12.4 months (95% CI 11.2–14.3). Using the Cox proportional hazards model and stepwise regression analysis to adjust for the effects of multiple potential confounding variables (excluding type of treatment), factors that had an independent effect on failure-free survival were determined. When grouped by type of treatment, the median failure-free survival of patients treated with standard chemotherapy was 9.8 months (95% CI 8.8–11.4), and for patients treated with

high-dose chemotherapy, 15.6 months (95% CI 13.3–19.7). This difference was statistically significant in univariate testing (unadjusted $P$=0.0048 by log-rank, $P$<0.0001 by Tarone-Ware). The variable "treatment" was then added to the analysis, and again the Cox proportional hazards model was used to determine whether the apparent effect of treatment remained statistically significant in the multivariate model. This analysis revealed that a highly significant treatment effect in favor of high-dose therapy remained (hazard ratio 0.54, 95% CI 0.24–0.84, $P$=0.0001).

## LEVEL II EVIDENCE

In 1995, Bezwoda and his group[5] published a prospective randomized phase III study, but because of the rather low number of patients (45 in each arm), it represents only level II evidence. In this paper, the authors compared the results of high-dose vs. conventional-dose chemotherapy as first-line treatment for metastatic breast cancer. The comparison included complete response rate, duration of response, and duration of survival. Ninety patients were entered in the study, which compared two cycles of the following high-dose regimen: cyclophosphamide 2.4 g/m$^2$, mitoxantrone 35–45 mg/m$^2$, etoposide 2.5 g/m$^2$. The standard-dose arm consisted of six to eight cycles of the following treatment: cyclophosphamide 600 mg/m$^2$, methotrexate 12 mg/m$^2$, and vincristine 1.4 mg/m$^2$. The high-dose regimen included either autologous bone marrow or peripheral blood stem cell rescue. All 90 patients who were randomized were assessable for all objectives.

The group found that response rates, duration of response, and duration of survival were significantly longer for patients who received high dose CNV. Toxicity overall was acceptable, and hematologic recovery was also acceptable. In conclusion, the authors concluded that high-dose CNV appeared to be a promising regimen if delivered twice to patients with metastatic breast cancer.

Because the study had only 90 patients randomized (45 patients each arm) and some imbalance regarding further tamoxifen treatment after achieving response, it remains controversial. Nevertheless, in the absence of published, large, prospective randomized trials, this study provides the best available evidence.

## LEVEL I EVIDENCE

The data obtained through studies described above and published elsewhere do not provide any level I evidence to support the superiority of high-dose chemotherapy with autologous blood and marrow stem cell transplantation in patients with metastatic breast cancer. Therefore, the NCIC-CTG recently designed a study entitled, "A randomized trial of high dose chemotherapy and autologous stem cell therapy versus standard therapy in women with metastatic breast cancer who have responded to

anthracycline or taxane based induction chemotherapy".[12] Women with evidence of metastatic breast cancer who have not received chemotherapy for their metastatic/recurrent disease are eligible for the study. Women must have complete or partial responses to induction chemotherapy or have no evaluable disease. Each patient will receive either four cycles of anthracycline-based chemotherapy or, for patients who have prior anthracycline exposure, four cycles of paclitaxel- or docetaxel-containing regimen. After four cycles of induction chemotherapy, patients will be evaluated and the responders will be randomized to either high-dose chemotherapy and autologous peripheral blood stem cell therapy or standard therapy. The standard arm includes at least two cycles of the same induction chemotherapy followed by maintenance therapy at the discretion of the investigator. The high-dose arm consists of a further two cycles of induction chemotherapy in conjunction with hematopoietic growth factors to harvest sufficient numbers of blood stem cells. If the response is confirmed after these two additional cycles, high-dose chemotherapy consisting of 6 g/m$^2$ cyclophosphamide, 70 g/m$^2$ mitoxantrone, 1800 mg/m$^2$ CBCDA will be delivered in four equal doses on four consecutive days. Forty-eight hours later, the previously harvested stem cell products will be reinfused into the patient. The patients on this arm will not receive further chemotherapy. Hormonal intervention is left up to the discretion of the primary physician. The objectives of this trial are to compare overall survival, the final response rates, and treatment toxicity. Further objectives include a comparison of health-related quality of life. It is anticipated that 400 patients will be enrolled within 4 years from the date of activation, ~200 of them randomized. The study was activated in more than 25 centers across Canada and, as of December 1998, more than 200 patients have been enrolled.

## ACKNOWLEDGMENTS

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# Repetitive High-Dose Therapy With Peripheral Blood Progenitor Cell Support for Metastatic and Locally Advanced Breast Cancer

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## ABSTRACT

We have treated 42 patients as part of phase I or II studies using three cycles of high-dose chemotherapy delivered every 28 days, with each cycle supported by peripheral blood progenitor cells (PBPCs) and granulocyte colony-stimulating factor (G-CSF). Sufficient PBPCs were collected before the first cycle of high-dose therapy and equally divided to support the consecutive cycles. The purpose of these studies was to develop a suitable outpatient regimen for a subsequent phase III study. Patients were prospectively entered into consecutive studies: trial 1 phase I ITP study ($n$=23), consisting of three cycles of high-dose ifosfamide (7.5–12.5 g/m$^2$/cycle), thiotepa (200–350 mg/m$^2$/cycle), and paclitaxel (175 mg/m$^2$/cycle). The dose-limiting toxicities were renal tubular acidosis (grade 3) and mucositis (grade 4). Other relevant but non-dose-limiting toxicities were ifosfamide encephalopathy (grade 3 and 4), enterocolitis (grade 4), and reversible interstitial pneumonitis (grade 2). The recommended dose for phase II studies is ifosfamide 10 g/m$^2$, thiotepa 350 mg/m$^2$, and paclitaxel 175 mg/m$^2$. In trial 2 phase II Isolex300i CD34-selection study, at the doses identified in the phase I study and using Isolex300i CD34-selection, eight patients have undergone repetitive high-dose therapy (total of 19 cycles completed to date). Recovery was compared with that of patients infused with unselected cells. CD34-selected patients had a moderate delay in recovery to absolute neutrophil count (ANC) >0.5×10$^9$/L ($P$=0.0387) and platelets >20×10$^9$/L ($P$=0.0305) and >50×10$^9$/L ($P$=0.0421). In trial 3, CTP study, patients were treated immediately after determining the maximum tolerated dose (MTD) of the ITP study. Cyclophosphamide (4 g/m$^2$) replaced ifosfamide, since the major toxicities observed in the ITP study were ifosfamide-related. For the entire cohort of 42 patients (all studies combined), 109 of the planned 126 treatment cycles have been delivered. The median time to ANC >1.0×10$^9$/L was 10 days (range 8–28), with no significant slowing in neutrophil recovery over the

three consecutive cycles of treatment ($P$=0.1081). Febrile neutropenia occurred after 67% of cycles and lasted for a median of 3 days. For all 109 cycles, the median time to platelets $>20\times10^9$/L, $50\times10^9$/L, and $100\times10^9$/L was 13 (range 8–38), 19 (range 10–41) and 25 days (range 10–152), respectively. There was significant slowing in platelet recovery between the three consecutive cycles of treatment ($P$=0.0123); however, in patients whose autograft CD34 content exceeded $3\times10^6$/kg there was no slowing of platelet recovery over the three cycles ($P$=0.1315). The majority of patients had metastatic disease ($n$=38) with a median of two sites involved. 38% of patients were refractory to, or had relapsed within 12 months of, their adjuvant therapy, and 33% were refractory to the conventional-dose chemotherapy immediately before transplant. The response rate (RR) to high-dose therapy was 88% and at a median follow-up of 8 months (range 4–21), the median progression-free survival (PFS) is 11.3 months, and the median survival has not been reached. We conclude that repetitive high-dose therapy can be delivered in the majority of patients and achieves promising response rates. These studies provide important findings relating to hematopoietic recovery and will form the basis of a subsequent phase III study in metastatic breast cancer.

## INTRODUCTION

The value of dose-intensive chemotherapy in metastatic breast cancer (MBC) continues to be debated,[1–4] and although the results of phase II, phase III studies and registry data examining high-dose therapy (HDT) with stem cell support are promising, ~80% or more of these highly-selected patients relapse.[5–8]

Tumor growth models predict that the delivery of multiple cycles of intensive therapy rather than a single cycle may be a more effective way of further increasing chemotherapy dose-intensity. Indeed, the Gompertzian model of breast cancer growth predicts that the effect of chemotherapy should be maximum during the period of rapid regrowth immediately after a cycle of chemotherapy. Repetitive high-dose therapy uses this model by attempting to administer high doses of chemotherapy during this phase of rapid regrowth.[9–15]

We therefore initiated a phase I study examining the tolerability and efficacy of repetitive administration of a novel combination of high-dose chemotherapy as an outpatient regimen for patients with MBC.

In a subsequent study, we also examined the use of CD34-selected cells to support multiple cycles of high-dose therapy. CD34-selected cells may be advantageous over unmanipulated cells, as there is less dimethylsulfoxide (DMSO) infused into the patient, there is potential to purge the autograft of contaminating tumor cells, and of particular interest for our group, this purified population can be subsequently used for protocols involving cell expansion and gene transfer. Although other groups have demonstrated that CD34-selected cells obtained by

paramagnetic separation can support high-dose therapy,[16] the ability of CD34 selection to support multiple cycles of such therapy has not been examined.

To date, we have treated 42 patients with metastatic or locally advanced breast cancer as part of phase I or II studies using three cycles of high-dose chemotherapy delivered every 28 days, with each cycle supported by PBPCs and G-CSF. The primary purpose of these studies was to develop a suitable outpatient regimen for a subsequent phase III study. The results of these studies are presented here.

## METHODS

### Eligibility criteria

Patients with histologically proven metastatic or locally advanced breast cancer were prospectively entered into four consecutive studies using three cycles of high-dose chemotherapy with each cycle being supported with PBPCs and G-CSF (Table 1). Sufficient PBPCs to support these three cycles of high-dose therapy were collected before the first cycle of high-dose therapy. Patients were excluded if they had significant cardiac, hepatic, or renal impairment, an Eastern Cooperative Oncology Group (ECOG) performance status >2, ANC $<1.5\times10^9$/L, or platelet count $<100\times10^9$/L.

### Mobilization chemotherapy

The majority of patients had received at least one cycle of conventional-dose therapy immediately before study entry. Patients subsequently underwent mobilization of PBPCs according to the study protocol into which they were enrolled. The mobilization regimens are summarized in Table 3.

### Disease assessment

Patients had disease assessment immediately before their first cycle of high-dose therapy and were classified as sensitive or resistant to the conventional-dose treatment they received (including mobilization chemotherapy) immediately preceding the high-dose therapy. Patients who entered the transplant phase in a complete response (CR) or partial response (PR) were defined as having chemotherapy-sensitive disease. Patients with progressive or stable disease were defined as having resistant disease.

Response to high-dose therapy was assessed six to eight weeks after completion of the third cycle of high-dose therapy, and patients were classified using standard criteria CR, PR, progressive disease (PD), or stable disease (SD). In addition,

patients who had a complete response in all visceral and/or nodal sites but had residual bone scan abnormalities with a negative positron emission tomography (PET) scan were defined as CR (+bone scan).

## Apheresis procedure

The apheresis procedure has been previously described.[17] Briefly, apheresis was initiated when the PB CD34 count exceeded $5\times10^6$/L. By employing this trigger point for initiating apheresis, patients were harvested 8–16 days after mobilization. For patients mobilized with G-CSF alone, apheresis was commenced on day 5.

Apheresis was continued until sufficient cells to support three separate cycles of high-dose therapy had been collected, i.e., a minimum target of $4.5\times10^6$/kg (three cycles of high-dose therapy, each with a minimum of $1.5\times10^6$/kg $CD34^+$ cells). Repeat PBPC collection was performed if the initial set of collections was deemed inadequate (i.e., initial total collection $<4.5\times10^6$/kg). For patients with a borderline collection according to CD34 criteria (i.e., $3.0–4.5\times10^6$/kg), an autograft was deemed acceptable if the total colony-forming units granulocyte-macrophage (CFU-GM) exceeded a total of $20\times10^4$/kg. The apheresis and cryopreservation protocol for the Isolex300i CD34-selection protocol is detailed below. To ensure uniformity, each collection was divided into three separate bags, so that each reinfusion contained PBPCs collected from each day of apheresis. Cells were cryopreserved in 10% DMSO and stored in vapor-phase liquid nitrogen.

## Autograft assays

The cell count, $CD34^+$ cell count, and colony assays methodology has been described previously.[17] The CD34 enumeration methodology has been verified against the International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines.[18]

## High-dose therapy

*Phase I ITP study (n=23).* The planned treatment schedule was three cycles of high-dose ifosfamide (7.5–12.5 g/m$^2$/cycle), thiotepa (200–350 mg/m$^2$/cycle), and conventional-dose paclitaxel (175 mg/m$^2$/cycle). Cohorts of four patients were enrolled at sequential dose levels (Table 1). Additional patients were accrued to a given dose level if a patient developed toxicity and did not complete all three cycles.

High-dose therapy was administered according to the following schedule; on day −4, patients received mesna (20% wt/wt of the ifosfamide dose) over 30 minutes followed immediately by a 24-hour infusion of ifosfamide with an equivalent dose of mesna administered simultaneously. Immediately after the

**Table 1.** High-dose regimens (planned three cycles)

| *Regimens* | *Dose/cycle* | *Patients (n)* | *Total cycles delivered* |
|---|---|---|---|
| MT | | | |
| Melphalan | 100 mg/m$^2$ | 2 | 6 |
| Thiotepa | 200 mg/m$^2$ | | |
| ITP (Phase I study) | | | |
| Ifosfamide | 10–12.5 g/m$^2$ | 23 | 59 |
| Thiotepa | 200–350 mg/m$^2$ | | |
| Paclitaxel | 175 mg/m$^2$ | | |
| ITP with Isolex300i CD34 selection | | | |
| Ifosfamide | 10 g/m$^2$ | 14* | 36 |
| Thiotepa | 300 mg/m$^2$ | | |
| Paclitaxel | 175 mg/m$^2$ | | |
| CTP | | | |
| Cyclophosphamide | 4 g/m$^2$ | 3 | 8 |
| Thiotepa | 350 mg/m$^2$ | | |
| Paclitaxel | 175 mg/m$^2$ | | |
| Total | | 42 | 109 |

**Eight underwent CD34 selection.*

ifosfamide/mesna infusion, mesna (20% wt/wt of the ifosfamide dose) was administered intravenously over 8 hours. Thiotepa was administered in equally divided doses on days –4, –3, and –2. Paclitaxel (175 mg/m$^2$) was administered as a 3-hour infusion on day –2. Cryopreserved PBPC were thawed and infused on day 0. All patients received recombinant human (rh)G-CSF (filgrastim; Neupogen, Amgen Australasia, Sydney, Australia) (5 µg/kg/d) subcutaneously from day 1 until the ANC was $>1.5\times10^9$/L for 3 consecutive days. All patients received prophylactic ciprofloxacin, acyclovir, and ranitidine. The MTD was defined when two or more patients developed National Cancer Institute (NCI) grade 4 nonhematologic toxicity (excluding alopecia) or grade 3 neurologic, cardiac, or renal toxicity or when two or more patients were unable to receive further high-dose therapy because of hematologic toxicity.

*Phase II Isolex300i CD34-selection study (n=14).* To date, 14 patients have been enrolled in a phase II study involving CD34-selection of PBPCs. Briefly, in patients from whom $>12\times10^6$/kg CD34$^+$ cells were obtained on a single day or on two consecutive days of collections ($n$=8), cells underwent CD34$^+$ paramagnetic selection using the Isolex300i (Baxter Healthcare, Irvine, CA). After selection, the cells were divided and cryopreserved in the same way as described above. The total volume of CD34-selected cells infused following each cycle was 20 mL. A

separate back-up collection was also obtained and cryopreserved unmanipulated (i.e., three bags with $>2\times10^6$/kg $CD34^+$ cells in each). In this study, patients received ifosfamide (10 g/m$^2$/cycle), thiotepa (300 mg/m$^2$/cycle), and paclitaxel (175 mg/m$^2$/cycle).

*CTP study (n=3).* This cohort of patients was treated immediately after determining the MTD of the ITP study. As the major toxicities observed in the ITP study were ifosfamide-related, cyclophosphamide was substituted for ifosfamide; it was anticipated that this combination would be more acceptable in terms of toxicity for the subsequent phase III multi-institutional study.

### Consolidative radiotherapy

Indications for radiotherapy followed published practice for radiotherapy after high-dose therapy.[19–21]

### Statistical analysis

To compare ANC and platelet recovery between the three cycles of high-dose therapy (individual patient data sets), repeated measures analysis of variance (ANOVA) was used. Kaplan-Meier estimates of platelet and ANC recovery were performed, and recovery of the consecutive cycles was compared using the log-rank test. Spearman correlation was used to compare autograft $CD34^+$ cell content and platelet and ANC recovery. All results are expressed as two-sided *P* values. Statistical analysis was performed using GraphPad Prism version 2.01 and GraphPad StatMate version 1.00 for Windows 3.1 (GraphPad Software, San Diego, CA).

## RESULTS

### Patient characteristics

The characteristics of the 42 patients are summarized in Table 2. The median age was 45 years, and the median time from initial diagnosis of breast cancer to transplant was 15 months (range 2.6–53). The majority of patients had metastatic disease ($n$=38) with a median of two sites involved. Ten patients underwent high-dose therapy as part of primary therapy for metastatic disease, and 32 patients had relapsed/progressed after prior adjuvant chemotherapy. Of these, 16 (38%) were refractory to, or had relapsed within 12 months of, their adjuvant therapy. Most patients were heavily pretreated, and 14 (33%) were resistant to conventional-dose chemotherapy. A third of patients had received a taxane-containing regimen before entry into the study.

**Table 2.** Patient characteristics ($n$=42)

| *Characteristic* | *n (%)* |
|---|---|
| Age | 45 years (range 31–59) |
| Status at trial entry | |
| Primary metastatic | 10 (24%) |
| Progressed during adjuvant therapy | 2 (4%) |
| Relapsed < 12 mo post adjuvant chemotherapy | 11 (26%) |
| Relapsed > 12 mo post adjuvant chemotherapy | 15 (36%) |
| Locally advanced and refractory chemotherapy | 3 (7%) |
| High-risk adjuvant (>10 nodes) | 1 (2%) |
| Pretransplant status | |
| Sensitive | 25 (60%) |
| Resistant | 14 (33%) |
| Not evaluable | 3 (7%) |
| Disease sites ($n$=42) | |
| High-risk adjuvant (>10 nodes) | 1 |
| Locally advanced | 3 |
| Metastatic | 38 |
| Median number of metastatic site | 2 (1–4) |
| Metastatic Sites ($n$=38) | |
| Nodal/soft tissue only | 4 |
| Bone + nodal | 7 |
| Visceral ± bone ± nodal | 27 |
| Prior treatment | |
| Median number prior regimens | 2 (1–5) |
| Median number prior chemotherapy cycles | 8 (1–26) |
| Prior anthracyclines | 29 (70%) |
| Prior taxanes | 14 (33%) |
| Prior cyclophosphamide | 34 (81%) |
| Prior radiotherapy for bone metastases | 4 (17%) |

## Apheresis collections

Mobilization strategies and autograft parameters are detailed in Table 3. Four patients had an additional mobilization and collection with either the same regimen or G-CSF alone.

## Dose intensity

Of the 42 patients undergoing high-dose therapy, 109 of the planned 126 treatment cycles have been delivered. Twenty-nine patients completed all three cycles (total cycles 87), nine completed two cycles (total cycles 18), and four

**Table 3.** Mobilization characteristics

| *Regimen* | *n (range)* |
|---|---|
| Total number of collection sets | 46* |
| Ifosfamide + paclitaxel | 13 |
| Ifosfamide + doxorubicin | 23 |
| Cyclophosphamide + paclitaxel | 1 |
| Cyclophosphamide + doxorubicin | 3 |
| G-CSF alone | 6 |
| CD34 dose per cycle x $10^6$/kg | 2.82 (0.41–9.6) |
| MNC dose per cycle x $10^8$/kg | 2.7 (0.69–9.7) |
| CFU-GM dose per cycle x $10^4$/kg | 12.7 (2.2–82.5) |

**Four patients were collected twice.*

completed only one cycle (total cycles 4). Three patients are yet to complete their planned therapy (two patients completed one of three and one patient two of three3). The planned treatment interval was 28 days, and since rapid hematopoietic recovery was observed after most cycles (see below), the subsequent cycle of high-dose treatment was usually delivered 24 days following infusion of the PBPCs (range 24–38).

## Nonhematopoietic toxicity

Nonhematopoietic toxicity was closely examined in the Phase I study of ITP and has previously been presented.[22] The dose-limiting toxicities were renal tubular acidosis (grade 3) and mucositis (grade 4). There was one treatment-related death due to intractable gastrointestinal hemorrhage. Other relevant but non-dose-limiting toxicities were ifosfamide encephalopathy (grade 4) and grade 2 reversible interstitial pneumonitis (IP). Both patients with IP responded to steroid treatment and were retreated with high-dose therapy. The recommended dose for phase II studies is ifosfamide (10 g/m$^2$), thiotepa (350 mg/m$^2$), and paclitaxel (175 mg/m$^2$).

## Hematopoietic recovery

*Neutrophil recovery.* Hematopoietic recovery after the high-dose treatment is summarized in Table 4. For all 109 cycles, the median time to ANC $>0.5\times10^9$/L and $1.0\times10^9$/L was 10 days (range 7–26) and 10 days (range 8–28), respectively. There were no significant differences in neutrophil recovery over consecutive cycles when comparing either recovery of consecutive cycles for individual patients (i.e., for ANC >0.5; $P$=0.1081; repeated measures ANOVA) or mean recoveries for the entire cohort (i.e., for ANC >0.5; $P$=0.5233; log-rank test) (Table

**Table 4.** Hematopoietic recovery following high-dose therapy

| | *All cycles combined* | *Cycle 1* | *Cycle 2* | *Cycle 3* | *P value** | *P value†* |
|---|---|---|---|---|---|---|
| *n* | 109 | 42 | 37 | 28 | | |
| ANC | | | | | | |
| $>0.5\times10^9$/L | 10 (7–26) | 10 (8–19) | 10 (8–26) | 10 (7–14) | 0.1081 | 0.5233 |
| $>1.0\times10^9$/L | 10 (8–28) | 10 (8–20) | 10 (9–28) | 10 (8–15) | 0.2027 | 0.5545 |
| Platelets | | | | | | |
| $>20\times10^9$/L | 13 (8–38) | 13 (8–25) | 13 (9–38) | 14 (10–21) | 0.0644 | 0.4496 |
| $>50\times10^9$/L | 19 (10–41) | 17 (10–32) | 20 (11–40) | 21 (12–41) | 0.0013 | 0.0887 |
| $>100\times10^9$/L | 25 (10–152) | 23 (10–45) | 27 (13–124) | 29 (14–152) | 0.0123 | 0.0731 |

**Repeated-measures ANOVA for patients undergoing all three cycles; †log rank test of recovery (see Figs. 1 and 2).*

4 and Fig. 1). G-CSF was required for a median of 11 days (range 6–32). Febrile neutropenia occurred after 67% of all cycles and, when it occurred, lasted for a median of 3 days (range 1–16). Admission to hospital was required after 78% of cycles, with a median stay in hospital of 5 days (range 2 –20). The reason for admission into hospital was febrile neutropenia in 84% of cases.

*Platelet recovery.* The median time to platelets $>20\times10^9$/L, $50\times10^9$/L, and $100\times10^9$/L was 13 (range 8–38), 19 (range 10–41), and 25 days (range 10–152), respectively (Table 4 and Fig. 1). A median of two platelet transfusions and two packed red blood cell transfusions were required after each cycle of treatment.

There was significant slowing in platelet recovery between cycles. Analysis comparing time to recovery of platelets after each consecutive cycle of high-dose therapy (repeated-measures ANOVA), demonstrated that although recovery of platelets to $>20\times10^9$/L was not significantly slowed ($P$=0.0644), recovery to $>50\times10^9$/L ($P$=0.0013) and $>100\times10^9$/L ($P$=0.0123) was slowed (Table 4 and Fig. 2).

*Autograft CD34 content and ANC hematopoietic recovery.* The range of autograft $CD34^+$ cells infused was 0.41 to $9.61\times10^9$/kg per cycle. There was a correlation between $CD34^+$ cell count and ANC recovery to $>0.5\times10^9$/L (Spearman correlation: $r$=0.4904, $P$<0.0001) and $>1.0\times10^9$/L ($r$=0.5015, $P$<0.0001) (Fig. 3).

Furthermore, when a threshold $CD34^+$ cell content of $5\times10^6$/kg was used, a significant difference in ANC recovery to $1.0\times10^9$/L was observed (log-rank test: $P$<0.0001) (Fig. 3). If this threshold was lowered further to $3\times10^6$/kg, the difference remained significant ($P$=0.0184) (Fig. 3). Although there was an influence of autograft CD34 content on neutrophil recovery for all 109 cycles, the

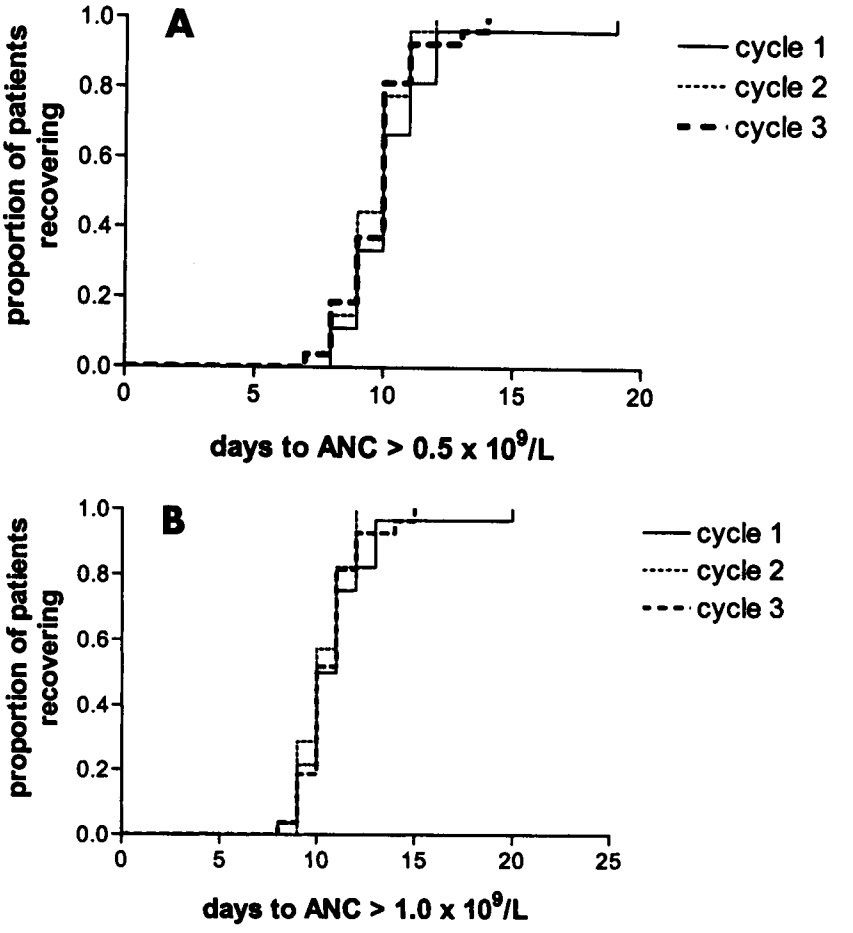


**Figure 1.** *ANC recovery over consecutive cycles of high-dose treatment. Kaplan Meier plots of time to (A) ANC recovery >0.5310⁹/L (log rank P=0.5233, RM ANOVA P=0.1081); (B) ANC recovery >1.0310⁹/L (log rank P=0.5545, RM ANOVA P=0.2027).*

CD34 count did not appear to predict for slowing over consecutive cycles. Indeed, even if patients had an autograft CD34 content of less than $3\times10^6$/kg, neutrophil recovery did not appear to slow over consecutive cycles (repeated measures ANOVA: $P$=0.1479, log-rank test: $P$=0.4360) (Fig. 3).

*Autograft CD34 content and platelet recovery.* There was a correlation between $CD34^+$ cell count and platelet recovery to $50\times10^9$/L ($r$=0.3475, $P$=0.0002) (Fig. 4). Again, using a threshold of $3\times10^6$/kg, platelet recovery to $50\times10^9$/L was faster in

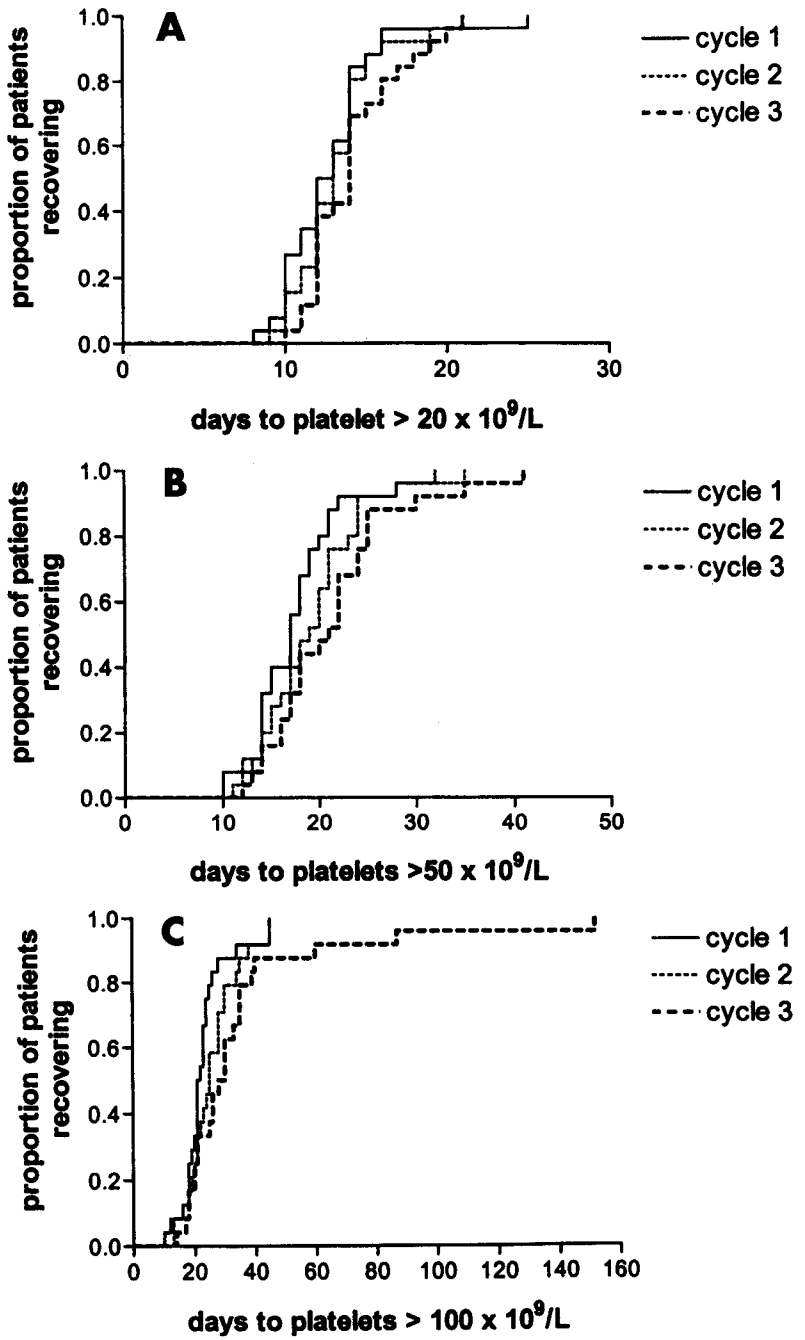


**Figure 2.** *Platelet recovery over consecutive cycles of high-dose treatment. Kaplan Meier plots of time to (A) platelet recovery >20310⁹/L (log rank P=0.4496, RM ANOVA P=0.0.0644); (B) platelet recovery >50310⁹/L (log rank P=0.0.0877, RM ANOVA P=0.0013); (C) platelet recovery > 100310⁹/L (log rank P=0.0.0731, RM ANOVA P=0.0123).*

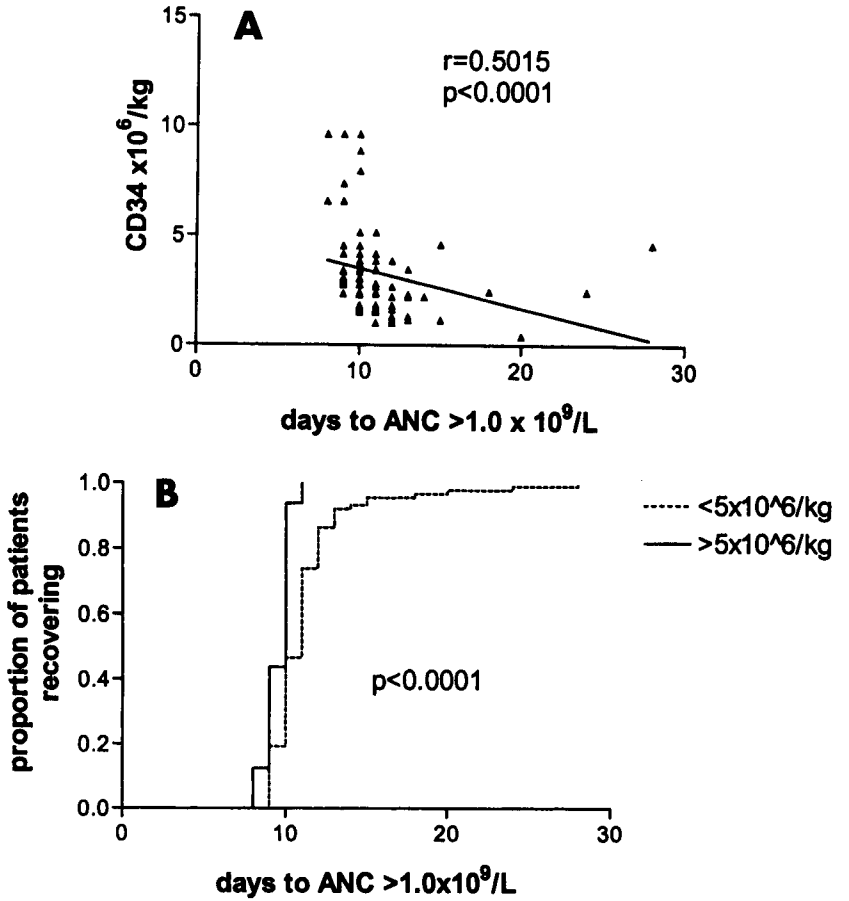


**Figure 3.** *Relationship between autograft CD34⁺ cell content and ANC recovery. A: Spearman correlation; B: Kaplan Meier plots of time to ANC recovery according to autograft CD34⁺ cell content threshold of 5×10⁶/kg (all 109 cycles). No statistical difference was observed (log rank P=0.4360, RM ANOVA P=0.1479)*

those who exceeded this threshold, with a median time to platelet recovery of 17 vs. 20 days as well as more patients with prolonged platelet recovery ($P$=0.0071). The Kaplan-Meier estimates of this recovery are presented in Fig. 4. The correlation with CD34⁺ cell content was also observed for platelet recovery to >100×10⁹/L ($r$=0.3661, $P$<0.0001) (Fig. 4). Again, when a threshold CD34⁺ cell content of 3×10⁶/kg was used, a significant difference in platelet recovery was observed ($P$=0.0199), with a median time to platelet recovery of 21 vs. 28 days.

This phenomenon of progressive slowing in platelet recovery over consecutive cycles was further explored by examining platelet recovery over three cycles in

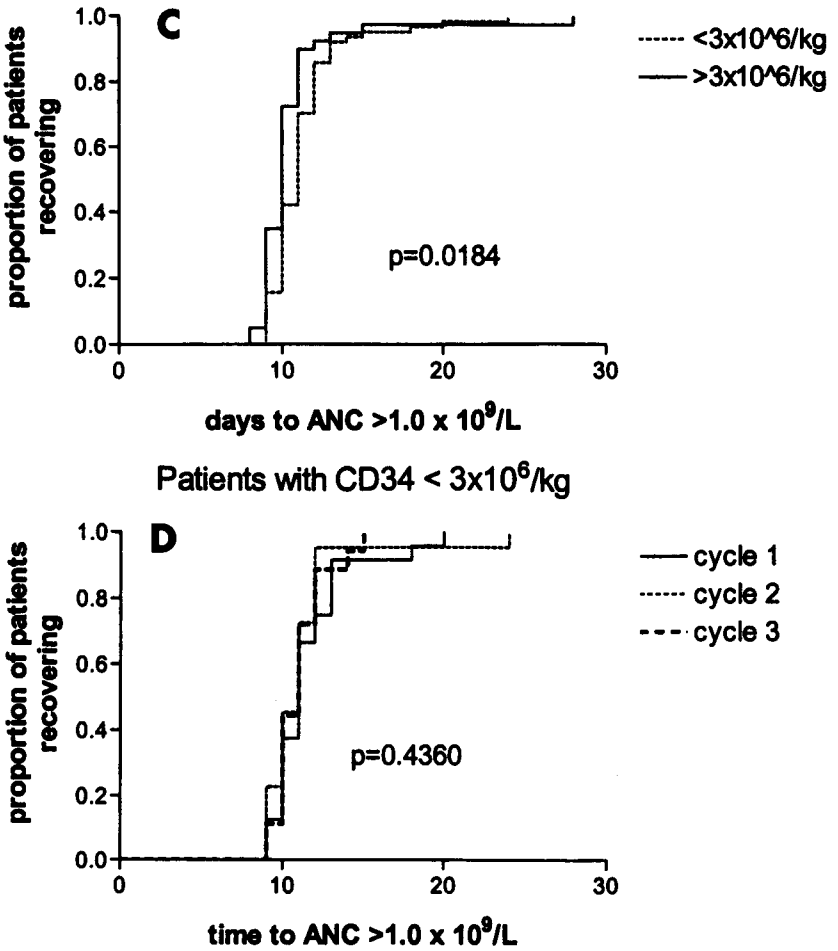


**Figure 3, continued.** *Relationship between autograft CD34⁺ cell content and ANC recovery. C: Kaplan Meier plots of time to ANC recovery according to autograft CD34⁺ cell content threshold of $3\times10^6$/kg (all 109 cycles). A statistically significant difference was observed (P=0.0184); D: Kaplan Meier plots of time to ANC recovery over consecutive cycles in patients with a autograft CD34⁺ cell content $<3\times10^6$/kg (n=66 cycles). No statistical difference was observed (log rank P=0.4360, RM ANOVA P=0.1479)*

patients whose autograft CD34 content of each cycle exceeded the threshold of $3\times10^6$/kg. In such patients there was no statistical prolongation of platelet recovery to $100\times10^9$/L over the three cycles (repeated measures ANOVA, $P$=0.1315) although the median time to platelet recovery ranged from 19 to 30 days (Fig. 4). In comparison, patients whose autograft CD34 content was less than $3\times10^6$/kg had

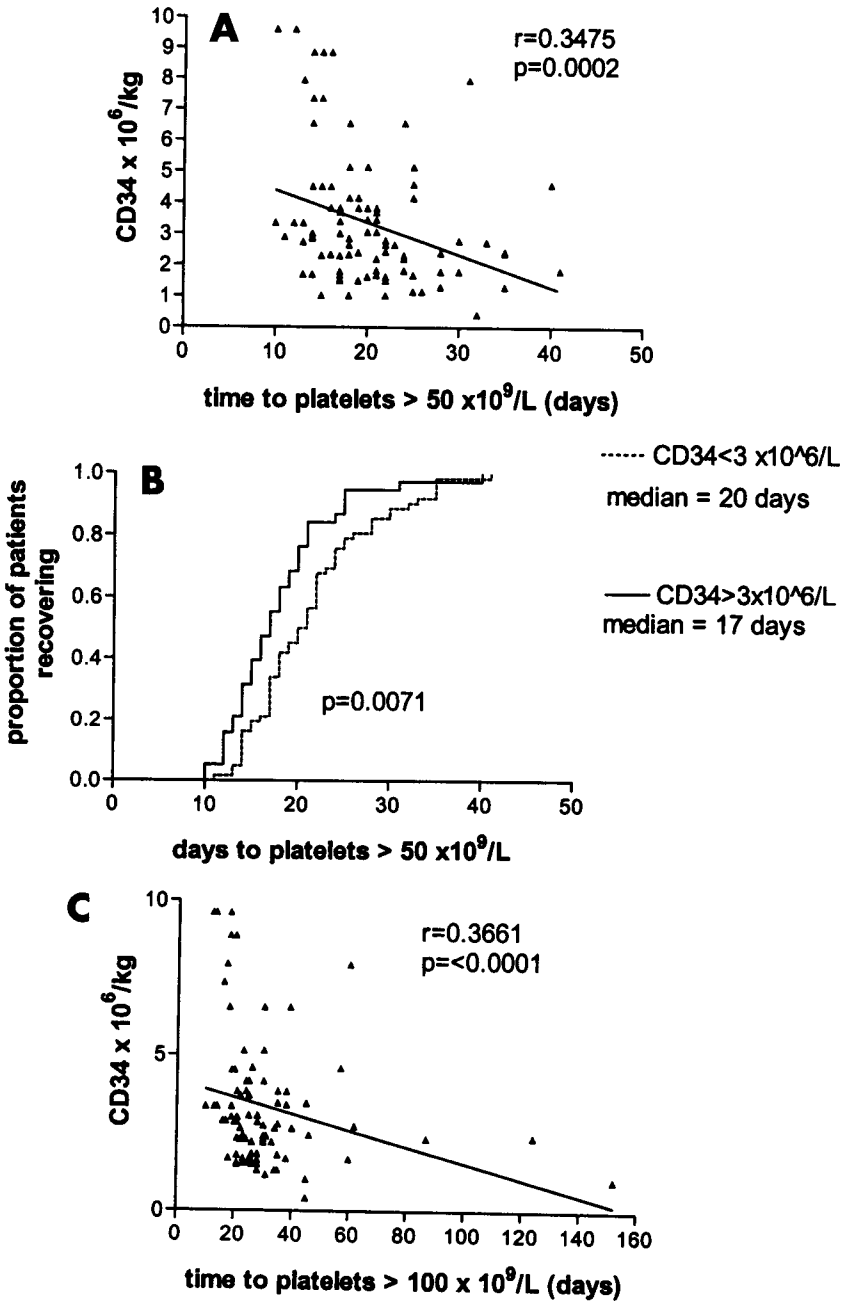


**Figure 4.** *Relationship between autograft CD34⁺ cell content and platelet recovery. A: Spearman correlation (for platelet recovery to 50×10⁹/L); B: Kaplan Meier plots of time to platelet recovery to 50×10⁹/L according to autograft CD34⁺ cell content threshold of 3×10⁶/kg (all 109 cycles); C: Spearman correlation (for platelet recovery to 100×10⁹/L). No statistical difference was observed (log rank P=0.2155, RM ANOVA P=0.1315).*

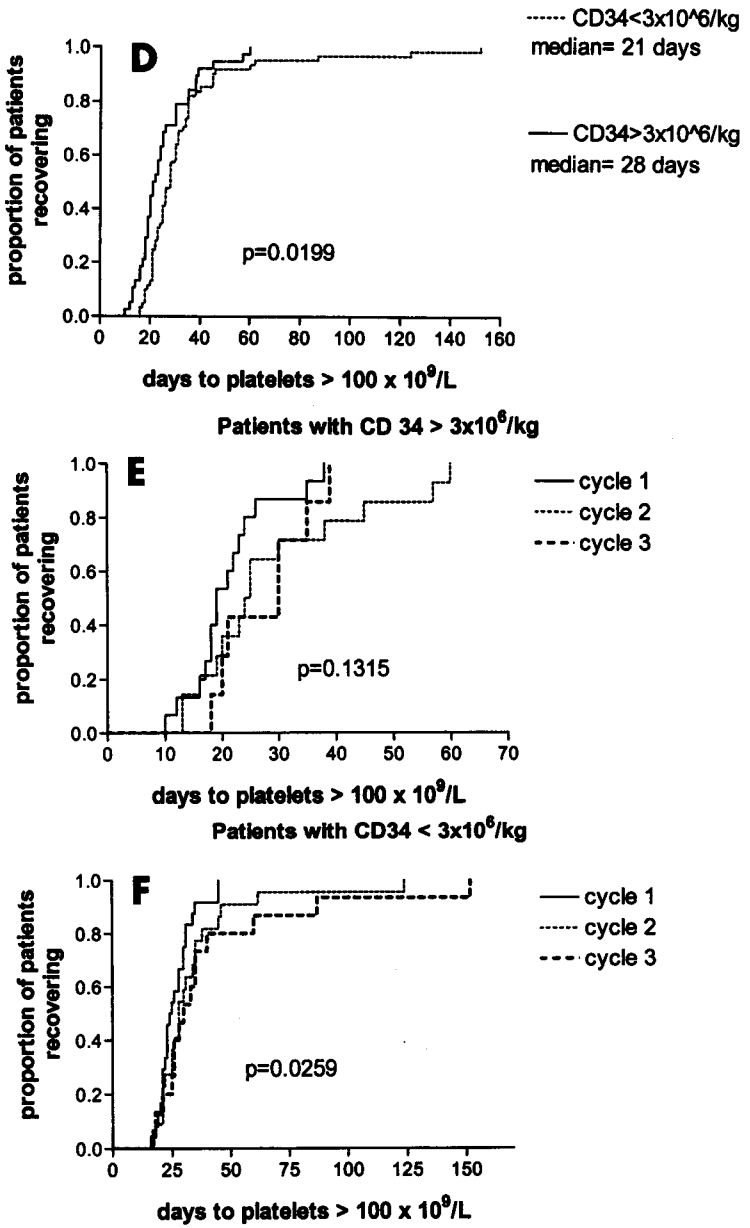


**Figure 4, continued.** *Relationship between autograft CD34⁺ cell content and platelet recovery. D: Kaplan Meier plots of time to platelet recovery to 100×10⁹/L according to autograft CD34⁺ cell content threshold of 3×10⁶/kg (all 109 cycles); E: Kaplan Meier plots of time to platelet recovery over consecutive cycles in patients with a autograft CD34⁺ cell content >3×10⁶/kg (n=43 cycles). No statistical difference was observed (log rank P=0.2155, RM ANOVA P=0.1315); F: Kaplan Meier plots of time to platelet recovery over consecutive cycles in patients with a autograft CD34⁺ cell content <3×10⁶/kg (n=66 cycles). A statistical difference was observed (log rank P=0.0623, RM ANOVA P=0.0259).*

progressive slowing of platelet recovery ($P$=0.0259), with median times to platelet recovery from 25 to 30 days. Indeed, a more important observation, which we believe is of substantial clinical importance, is the increased incidence of delayed platelet engraftment beyond 30 days in this group (Fig. 4).

### Isolex 300i CD34-selection procedure

To date, 14 patients entered on this study have undergone at least one high-dose cycle. Eight patients have had their PBPCs undergo CD34-selection, with 19 cycles completed to date. The results of the CD34-selection process are summarized in Table 5. Of the 19 cycles completed, the median time to ANC >0.5 and $1.0\times10^9$/L is 10 (range 9–19) and 11 days (range 9–20), respectively. Time to platelets >20, 50, and $100\times10^9$/L is 14 (range 12–25), 20 (range 14–22), and 26 days (range 19–45), respectively.

To analyze hematopoietic recovery, these 19 cycles from eight patients were compared to 29 cycles from 11 other patients who received the same high-dose regimen (six patients enrolled in the concurrent study whose PBPCs had not undergone CD34-selection and five patients from the phase I study who received the same drug dosages). In the cohort who had their PBPCs undergo CD34-selection, there was a moderate delay in recovery to ANC $>0.5\times10^9$/L ($P$=0.0387) and platelets $>20\times10^9$/L ($P$= 0.0305) and $>50\times10^9$/L ($P$=0.0421). No significant differences in platelet recovery to $>100\times10^9$/L were observed ($P$=0.25) (Fig. 5).

### Responses to therapy

Thirty-three patients were evaluable for response (Table 6). The response rate for these 33 patients was 88% (29), with 6% (two) having stable disease and 3% (one) progressive disease. All 22 patients with evaluable chemotherapy-sensitive disease before high-dose chemotherapy had a further response (17 CRs, five PRs). Of the 14 patients who had disease resistant to conventional-dose chemotherapy before high-dose therapy (four patients with nonevaluable disease), there was a 70% (seven of 10) response rate with two (20%) having stable disease and one (10%) with progressive disease. At a median follow-up for all alive patients of 8 months (range 4–21), the median progression-free survival is 11.3 months and the median survival has not been reached (Fig. 6).

## DISCUSSION

### Previous studies of repetitive or sequential high-dose therapy in MBC

A number of other investigators have explored repetitive or sequential high-dose therapy for breast cancer, and selected studies are summarized in Table 7. The

**Table 5.** Results of Isolex 300i CD34 selection

| | *Preselection* | | *Postselection* | | | | *Hematopoietic recovery (cycle 1)* | |
|---|---|---|---|---|---|---|---|---|
| | *CD34 content of apheresis product* | | *CD34 purity* | *Recovery* | *CD34 content* | *Infused/cycle (i.e 1/3 total) (CD34* | *ANC >0.5×10⁹/L* | *Platelets >50×10⁹/L* |
| *ID* | *%* | *×10⁶/kg* | *(%)* | *(%)* | *(×10⁶/kg)* | *×10⁶/kg)* | *(days)* | *(days)* |
| 1617 | 3.22 | 3.04* | 27.2 | 13.6 | 0.41 | 0.14 | 19‡ | 32‡ |
| 0118 | 2.33 | 12.8 | 28 | 1 | 0.13 | 0.04 | 10 | 19 |
| 4026 | 1.04 | 8.84 | 81.3 | 39.3 | 3.46 | 1.15 | 10 | 17 |
| 4230 | 3.63 | 23.82 | 87.4 | 35.4 | 8.43 | — | — | — |
| 0637† | 2.11 | 17.55 | 85.5 | 17.1 | 3.01 | | | |
| | 0.85 | 4.4 | 81.1 | 34.3 | 1.51 | 1.5 | 10 | 17 |
| 1050 | 0.23 | 15.3 | 90.4 | 60 | 9.01 | 3.01 | 9 | 14 |
| 1506 | 1.61 | 14.1 | 92.9 | 34.4 | 4.85 | 1.62 | 11 | 22 |
| 0220 | 3.1 | 17.43 | 91.3 | 37.4 | 6.9 | 2.3 | 12 | 15 |
| 1603 | 0.32 | 26.88 | 75.1 | 16.7 | 4.5 | 1.5 | 11 | 17 |
| Mean | 1.84 | 14.3 | 74.0 | 29.0 | 4.22 | 1.27 | 10 | 17 |

*Thawed before undergoing CD34 selection; CD34 count represents postthaw specimen; †separate separations on two consecutive days; ‡delayed neutrophil and platelet engraftment; unselected "back-up" cells used for next two cycles.

most common approach used to formulate these repetitive regimens has been to repeat or combine recognized "single-transplant" regimens or modify existing regimens by selective reductions in drug doses. Not unexpectedly, this has resulted in a wide variation in toxicities observed. Conversely, arbitrary modifications of drug dose may lead to ineffective regimens. For this reason, we decided to formally explore the ITP drug combination in a phase I study.

Although no formal phase I studies of repetitive high-dose therapy have been published in full, the best investigated regimen to date has been modifications of CTCb (cyclophosphamide, thiotepa, carboplatin). Rodenhuis et al.,[23] in a phase II study, attempted to deliver three cycles of full dose CTCb (cyclophosphamide 6 g/m$^2$, thiotepa 480 mg/m$^2$, carboplatin 1600 mg/m$^2$) with only 10 of the 35 patients receiving three cycles of the planned therapy. Of the 10, only four could be administered at full dose as planned. Of these four, two died of toxicity. The same investigators administered the same drugs for three cycles at an arbitrarily selected two-thirds dose. Fourteen of the 23 patients received all three cycles, and the treatment was associated with mild to moderate nonhematopoietic toxicity.[24] Shapiro et al.[25] administered even lower doses of CTCb (cyclophosphamide 1.5 g/m$^2$, thiotepa 125 mg/m$^2$, carboplatin 200

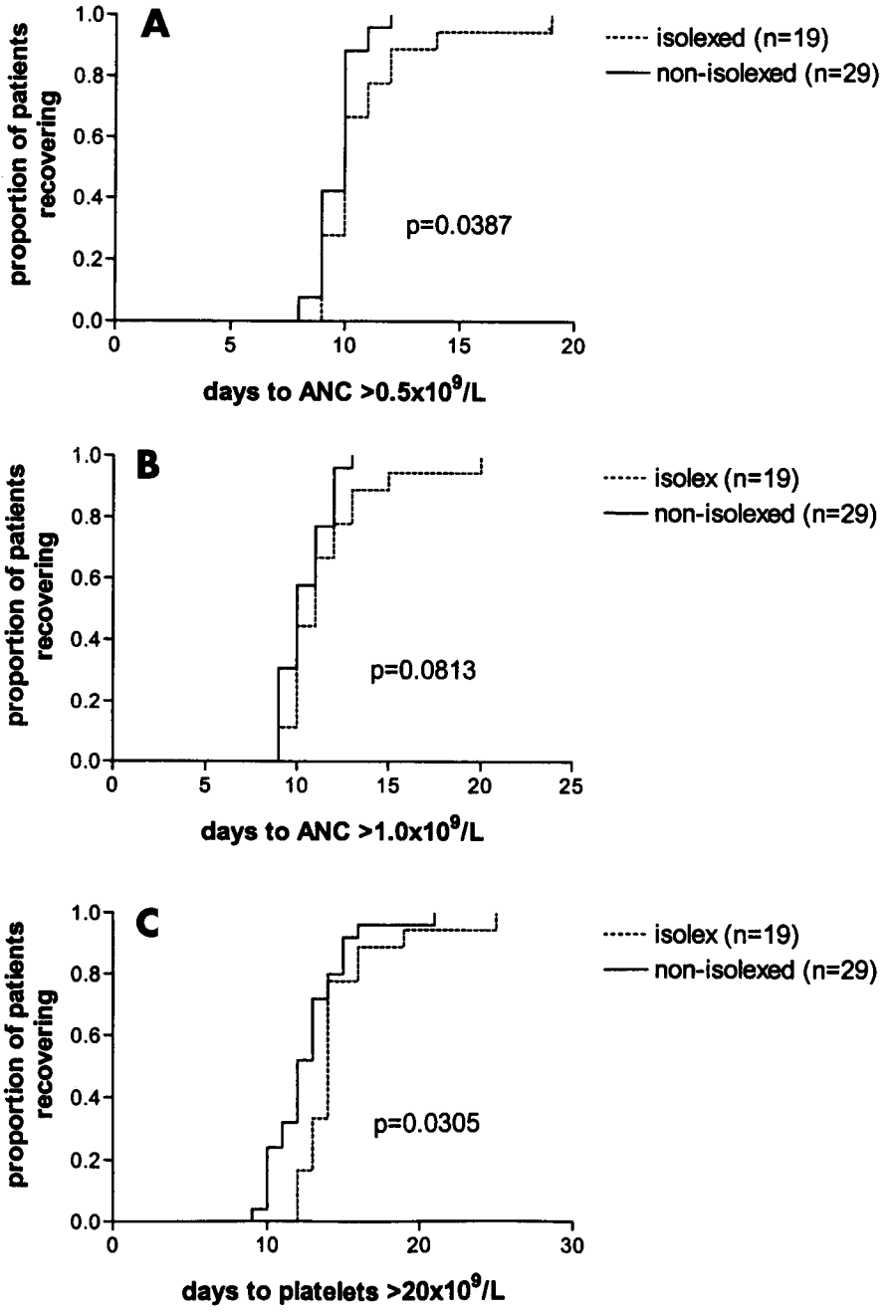


**Figure 5.** *Kaplan Meier plots of time to hematopoietic recovery according to infusion of either Isolex 300i CD34-selected cells or unmanipulated cells. A: ANC recovery to >0.5×10⁹/L; B: ANC recovery to >1.0×10⁹/L; C: Platelet recovery to 20×10⁹/L.*

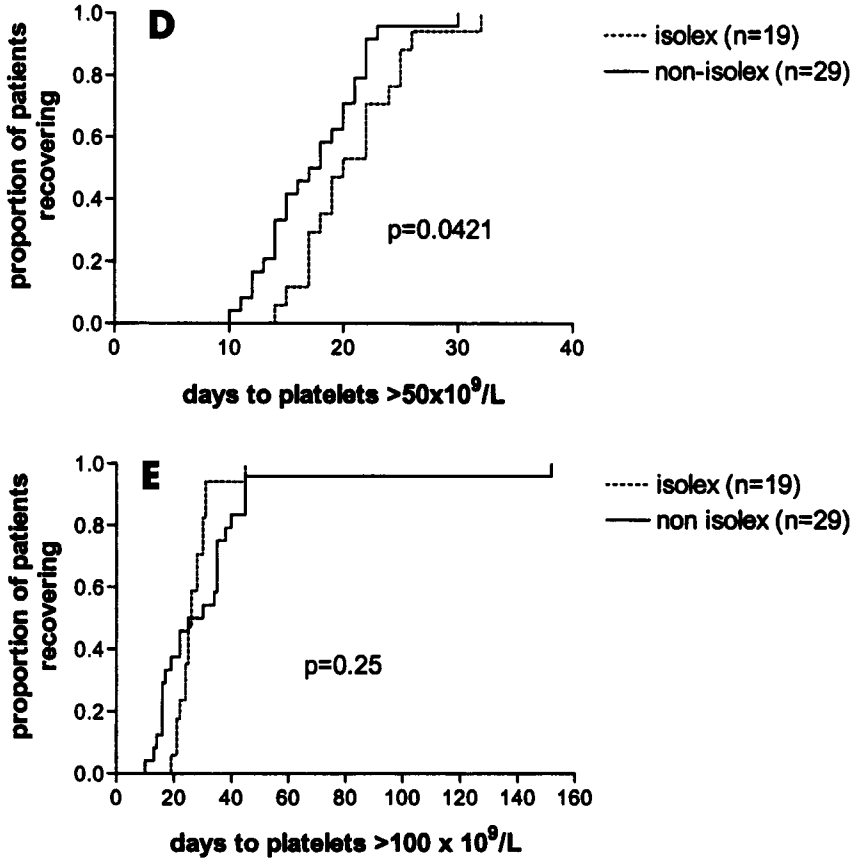


**Figure 5, continued.** *Kaplan Meier plots of time to hematopoietic recovery according to infusion of either Isolex 300iCD34-selected cells or unmanipulated cells. D: Platelet recovery to 50310⁹/L; E: Platelet recovery to 100310⁹/L.very to 20310⁹/L*

mg/m$^2$) for four planned cycles every 21–42 days. Of the 18 patients, 67% completed all four cycles of CTCb treatment and not surprisingly, the toxicity was primarily hematologic. These observations emphasize the need for careful dose-escalation studies of repetitive high-dose therapy.

## ITP drug combination

The most commonly used drugs in published repetitive high-dose therapy regimens have been the alkylating agents, carboplatin, etoposide, and the anthracyclines. Although the results of studies using dose-escalated anthracyclines are

**Table 6.** Response to high-dose therapy

| *Pretransplant response to conventional-dose chemotherapy* | | *Evaluable for response after transplant* | *Posttransplant status* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *CR* | *CR (+bone scan)* | *PR* | *RR* | *Stable* | *Prog* | *NE* |
| Sensitive | $n$=25 | 22 | 10 | 7 | 5 | 22/22 (100%) | | | 3* |
| Resistant | $n$=14 | 10 | 1 | 3 | 3 | 7/10 (70%) | 2 | 1 | 4† |
| NE | $n$=3 | 1 | | 1‡ | | | | | 2 |
| Total | $n$=42 | 33 | 11/33 (33%) | 10/33 (30%) | 9/33 (27%) | 29/33 (88%) | 2/33 (6%) | 1/33 (3%) | |

**Three yet to complete planned treatment; †three patients had locally advanced disease, were refractory to conventional-dose chemotherapy, and had disease surgically resected before high-dose therapy; one patient died of transplant-related toxicity and was not evaluable for response; ‡patient had measurable disease before transplant, had mobilization chemotherapy, and went directly to transplant (i.e., sensitivity to conventional-dose therapy was not assessed).*

promising,[26–28] our protocol did not incorporate these agents since we predicted that the population of patients in our study were likely to have failed an anthracycline-based regimen. Indeed this was the case, with 70% having received a prior anthracycline. Instead, we designed a protocol incorporating conventional doses of paclitaxel while focusing on the dose-escalation of the alkylating agents.

*Ifosfamide dose-escalation.* We selected two alkylating agents for our regimen; ifosfamide and thiotepa. Ifosfamide has efficacy in advanced breast carcinoma,[29–33] and we anticipated that a number of patients eligible for this study would have failed prior cyclophosphamide treatment. Although there is to date no conclusive evidence that ifosfamide is superior to cyclophosphamide in the treatment of breast cancer, preclinical data suggest it has potential advantages.[31,34–37] Taken together, we believed it rational to use ifosfamide in our cohort of heavily pretreated patients. High-dose ifosfamide has been used extensively as part of high-dose conditioning regimens (usually with etoposide and carboplatin in the ICE regimen) for the treatment of various malignancies. In these regimens, doses of 12.5–21 g/m$^2$ have been administered as part of a single transplant regimen, with doses of 10–12.5 g/m$^2$ used in double-transplant procedures. A major toxicity is the cortical neurotoxicity (or ifosfamide-encephalopathy) which is caused by the metabolic product of ifosfamide, chloracetaldehyde. Other toxicities include hemorrhagic cystitis and renal tubular acidosis.

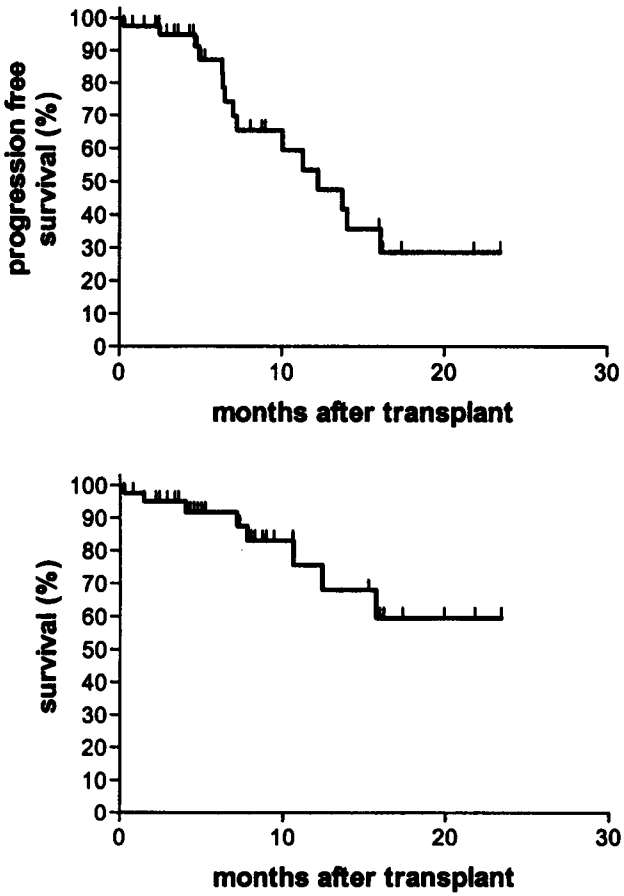


**Figure 6.** *Kaplan Meier estimates of (A) progression-free survival and (B) survival from time of first cycle of high-dose therapy (n=42)*

In our phase I study of ITP, nonhematopoietic toxicity determined the MTD (10 g/m$^2$ for ifosfamide) with the dose-limiting toxicities of renal-tubular acidosis and mucositis being observed at the dose of 12.5 g/m$^2$. The recommended doses for phase II and III studies are ifosfamide 10 g/m$^2$, thiotepa 350 mg/m$^2$, and paclitaxel 175 mg/m$^2$. Hemorrhagic cystitis was not observed in any of the patients treated. Another significant but non-dose-limiting toxicity was ifosfamide-induced encephalopathy. Despite maintaining an adequate albumin at the time of infusion, the use of aggressive bicarbonate replacement, and methylene blue for the treatment of encephalopathy, the renal and cerebral toxicities were still observed. Because we were developing these protocols with the intention of using the protocol in a multi-institutional phase III study, we felt that the ifosfamide-related cortical and renal toxicities were too

**Table 7.** Repetitive HDT regimens for locally advanced and MBC

| *Reference* | *Phase* | *n* | *Regimen* | *No. HDT cycles* | *Stage* | *Follow-up (months)* | *RR (%)* | *PFS (%)* |
|---|---|---|---|---|---|---|---|---|
| Rodenhuis[23] | II | 35 | Full CTCb: C (6), TT(480), Cb(1600) | 1=35<br>2=28<br>3=10 | III/IV | | | |
| Rodenhuis[24] | II | 23 | Mini-CTCb (67%): C (4),TT(320), Cb (1100) | 1=21<br>2=19<br>3=14 | III/IV | | | |
| Shapiro et al.[25] | II | 20 | Mini-CTCb: C (1.5), TT(125),Cb (200) | 4 | III/IV | | 55 | |
| Ayash et al.[47] | II | 67 | L-PAM (140)+PBPC then CTCb:C (6), TT (500), Cb (800) | 2 | IV | 16 | | 34* |
| Ghalie et al.[48] | II | 44 | #1: Full CTCb:C (6), TT(600), Cb (800)<br>#2: Bu (16)+VP16(60) | 1=39<br>2=32 | IV | 22 | 43<br>32 CR | 24† |
| Crown et al.[49] | II | 20 | #1: L-PAM (180)<br>#2=TT (700) | 2 | IV | | 89 CR | |
| Crown et al.[50] | I/II | 42 | #1=TT (400–700)<br>#2=TT (400–700) | 2 | IV | | 42 CR | |
| Murphy et al.[41] | I/II | 44 | C (3)+P(250) x2 then TT(700)+PBPC x2 | 4 | IV | | 36 CR | 61‡ |
| Bitran et al.[51] | II | 27 | C (7.5)+TT (750)+ PBPC then L-PAM (140)+PBPC | 2 | IV | 24 | 67 | 56† |
| Prince et al.[52] | II | 10 | L-Pam (100),TT (150) | 4 | III/IV | | | |
| Gianni et al.[53] | II | 67 | C,VCR,MTX,CDD then LPAM(200) +PBPC | 1 | II/III | 49 | | 57 |
| Frick et al.[54] | II/III | 36 | C (3), Epi (90), TT (400) | 2 | II/III | | | |
| Anelli et al.[42] | I/II | 10 | Cb (1200)+P(550) | 2 | IV | 10 | 66 CR | 42† |
| Mehra et al.[43] | I/II | 53 | C (4.5), Cb (AUC=13), P (250) | 4 | IV | | 70 RR<br>49 CR | |
| Broun et al.[44] | I/II | 21 | C (6), Cb (1800), P (300) | 1 | II/III | | | |

**At 16 months; †at 2 years; ‡at 12 months.*

excessive to be used in such a setting. To this end, we modified the protocol by substituting ifosfamide with cyclophosphamide (4 g/m$^2$/cycle). In this cohort, tolerable grade 3 mucositis in two patients was the only major toxicity observed.

*Thiotepa toxicity.* Standard doses of thiotepa are 10–30 mg/m$^2$ but can be escalated beyond 170 mg/m$^2$ when supported by autologous stem cells.[38,39] The MTD is 900–1,125 mg/m$^2$ when administered alone, and doses of 350–550 mg/m$^2$ have been used as part of combination high-dose chemotherapy regimens, although the MTD in these latter regimens has not been formally determined. Toxicities include stomatitis, enterocolitis (at doses >720 mg/m$^2$), dermatitis, hepatotoxicity, interstitial pneumonitis, with severe neurotoxicity being the major dose-limiting toxicity. The latter toxicity is manifested by inappropriate behavior, forgetfulness, confusion, and somnolence at an incidence >15% when doses exceed 900 mg/m$^2$.[38,40] The above dose-finding studies were performed after single doses of thiotepa; however, limited information is known about the cumulative toxic effects of repetitive administration of high-dose thiotepa, i.e., cumulative doses >900 mg/m$^2$. Thus in our phase I study, we planned for careful dose-escalation of thiotepa. Indeed, there was an additional concern that the neurotoxicity would be increased because of the combination with ifosfamide.

The major thiotepa-related toxicities observed in our phase I study were mucositis, interstitial pneumonitis, and skin rash. Importantly, although the maximum cumulative dose of thiotepa in this study was 1050 mg/m$^2$, the typical neurologic toxicity of a dementia-like syndrome was not observed. However, formal neuropsychiatric testing was not performed, and subtle defects may not have been detected. Reversible grade 2 interstitial pneumonitis and skin rash were also observed and probably related to thiotepa.[40]

*Paclitaxel toxicity.* We selected paclitaxel in combination with the two alkylating agents because we anticipated that a number of patients in this prospective study would previously had received considerable prior anthracycline therapy or would have recently failed an anthracycline, and paclitaxel had little known additive toxicity with the selected alkylating agents. Other investigators have escalated the dose of paclitaxel in their high-dose regimens,[41–44] with the maximum dose of paclitaxel delivered being 550 mg/m$^2$ when combined with carboplatin.[42] We elected not to escalate the dose of paclitaxel beyond the conventional-dose range, as it allowed us to focus on the dose-escalation of the alkylating agents. Indeed, there is little evidence that there is a meaningful dose-response relationship for paclitaxel above the dose of 175 mg/m$^2$.[45] In our phase I study, only grade 2 paclitaxel-associated peripheral neuropathy was observed. Not surprisingly, as the doses of paclitaxel were not above those in the conventional-dose range, no unexpected toxicity was observed.

## An outpatient regimen

The design of our protocols has focused on the development of an acceptable outpatient regimen. Unlike other high-dose regimens in which mucositis and enterocolitis requiring parenteral nutrition or opioid analgesics are acceptable, we designed these protocols such that severe mucositis and enterocolitis would be dose-limiting toxicities so that it remained a suitable outpatient regimen. Although the readmission rate was relatively high (78%), the length of stay in hospital was relatively short at 5 days, and patients treated at level 4 or below did not require opiate analgesia or parenteral nutrition for mucositis. This is of some importance, as the regimen achieves substantial dose intensity (delivered every 28 days in 71% of patients) but remains very much an outpatient therapy.

## Hematopoietic recovery

We demonstrate that recovery of neutrophils to $1.0\times10^9$/L and platelets to $50\times10^9$/L is rapid and does not slow over the three cycles of therapy. However, platelet recovery to $100\times10^9$/L is progressively slowed. Basser et al.[46] have also demonstrated that neutrophil and platelet recovery slows over three consecutive cycles of high-dose epirubicin and cyclophosphamide. In their study, there was no observable relationship between the autograft CFU-GM content and platelet and neutrophil recovery. We, however, were able to demonstrate a correlation between autograft $CD34^+$ cell content and neutrophil and platelet recovery. Furthermore, this relationship (in terms of platelet recovery) is most evident when the autograft has a low CD34 content (i.e., $<3\times10^6$/kg).

The observation that this slowing in platelet recovery can be overcome with infusion of high numbers of $CD34^+$ cells raises important biological questions. This finding questions the concept that cumulative stromal damage is the only reason for progressive slowing in platelet recovery over consecutive cycles of high-dose therapy. Indeed, if stromal damage was the sole cause of this progressive delay in recovery, this should occur independent of the infused CD34 dose. One explanation is that there are other components in the infused autograft that are important for engraftment into marrow stroma. Such cells may be the hematopoietic progenitors themselves (as reflected by the $CD34^+$ cell count) or possibly accessory cells in the autograft. The absolute numbers of these cells may be a critical component in the balance between stroma and the infused product. However, there is an alternative explanation: patients in whom we were able to mobilize and collect large quantities of $CD34^+$ cells may have a more sturdy marrow microenvironment that has a better capacity to withstand the repetitive cycles of high-dose therapy. This latter postulate is in part supported by the results of the hematopoietic recovery in patients who received CD34-selected cells. These

eight patients had high apheresis yields (perhaps reflecting a good marrow microenvironment) but because of the relatively low $CD34^+$ cell recovery after the selection process (29%), the actual infused number of cells per cycle was low (mean $1.27 \times 10^6$/kg). Despite the low $CD34^+$ cells infused in these patients, they engrafted well, with only slightly delayed hematopoietic recovery compared with the recovery of patients who received unselected cells. These findings are of considerable importance for the design of subsequent trials of repetitive high-dose therapy, and further studies are required to resolve some of the issues raised.

### Response rates and survival

The response rates in this study were high given the poor prognostic characteristics of this cohort. Indeed, responses were observed in chemotherapy-resistant patients, with only one patient having progressive disease during treatment. At a median follow up of only 8 months, the median survival has not been reached, with a median progression-free survival of 11.3 months.

## CONCLUSION

We conclude that repeated cycles of high-dose therapy can be delivered in the majority of patients and achieve promising response rates. These studies provide important information relating to hematopoietic recovery after repetitive high-dose therapy and have implications for the design of subsequent repetitive high-dose studies.

## ACKNOWLEDGMENTS

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# An Aggressive Approach to Metastatic Breast Cancer Is Warranted

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## INTRODUCTION

The prognosis of metastatic breast cancer with conventional chemotherapy is poor; a median survival of 19 months[1,2] and a <5% 5-year survival have been reported. The use of higher doses of chemotherapy has increased response rates; survival, however, has hardly been influenced.[3] Since the mid-1980s, several intensive-dose chemotherapy protocols with hematopoietic stem cell rescues have been initiated; 5-year disease survival rates of 20% were reported, whereas the median survival did not change significantly.[4] In those series, the relapse rate was up to 80%, of which the majority occurred within 2 years after high-dose therapy. We initiated 5 years ago a program in metastatic disease which consisted of aggressive normal-dose chemotherapy of three different non-crossresistant chemotherapy protocols followed by intensive chemotherapy consisting of three different protocols over a duration of 2 years. The results of 78 patients are presented in this chapter.

## MATERIALS AND METHODS

Informed consent of protocols were signed. Protocols were approved by our institutional review board. Seventy-eight patients with metastatic breast cancer were treated with normal-dose chemotherapy consisting of three consecutive non-crossresistant protocols: three to six courses of CAVe (cyclophosphamide, adriamycin, and VP-16), three to six courses of adria/velban, and two to four courses of FuMEP (5FU, mitomycin, VP-16, platinol) with a median duration of 12 months. After that, two schemas of treatment were used as has been documented in Fig. 1. Schema A consisted of a 2-year intensive program with CVP (cytoxan 2 g/m$^2$, VP-16 600 mg/m$^2$, and platinol 90 mg/m$^2$) alternated with TIP (taxol 300 mg/m$^2$, ifosfamide 7.5 g/m$^2$, and platinol 90 mg/m$^2$) with a 5-week interval between the first two courses, followed by a 2- to 3-month interval for 2 years. The last course was followed by MTB (mitoxantrone 30 mg/m$^2$, thiotepa 300 mg/m$^2$, and BCNU 300

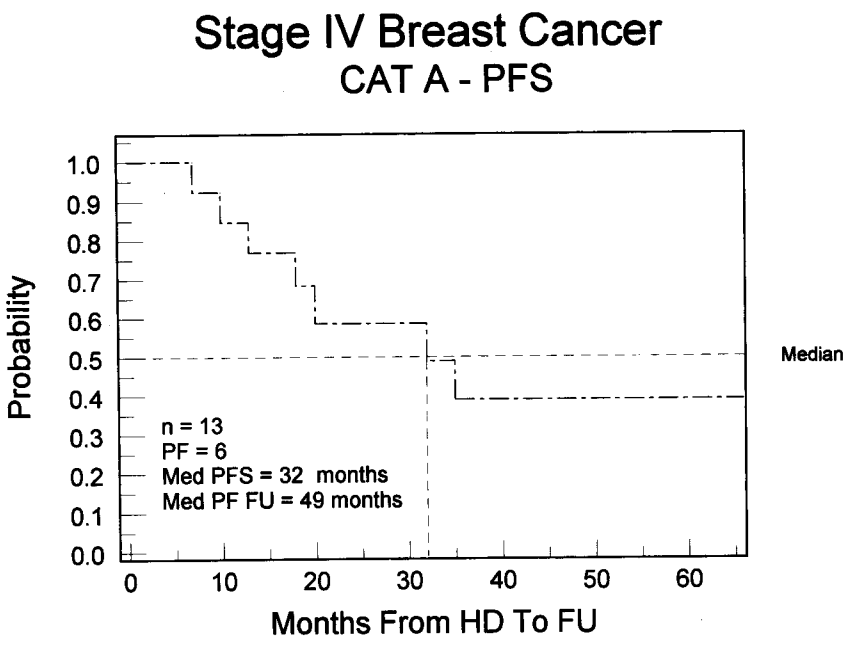


**Figure 1.** *Progression-free survival (PFS) in category A metastatic breast cancer. Kaplan-Meier estimate. On horizontal axis, months from high dose (HD) to last follow-up (FU) has been plotted. N, number; PF, progression-free; Med PF FU, median follow-up of progression-free patients; Med PFS, median progression-free survival.*

mg/m$^2$) with bone marrow support. Later on, schema B was initiated and consisted of one course of CVP and TIP followed by one course of MTB with peripheral stem cell support; the interval between courses was 5–6 weeks. After that, a 2-year maintenance chemotherapy program was used consisting of normal-dose chemotherapy. The interval between courses was 6–8 weeks. In schema A, bone marrow support was used at the end of the program since hematopoietic recovery was relatively slow and often incomplete. In contrast, with the availability of mobilized peripheral blood cells, after which hematopoietic recovery was fast and complete, patients could sustain further chemotherapy; therefore, the MTB protocol was used earlier and followed by maintenance chemotherapy. Due to the small number of patients, results of schemas A and B are reported. The program is done on an outpatient basis; when white blood cells (WBC) are <2000/mm$^3$, prophylactic intravenous antibiotics were started and continued until counts are >2000. Less than 10% of the courses were complicated by infections necessitating admission. Collection of bone marrow and peripheral blood stem cells, storage of cells, and infusion of cells have been reported elsewhere.

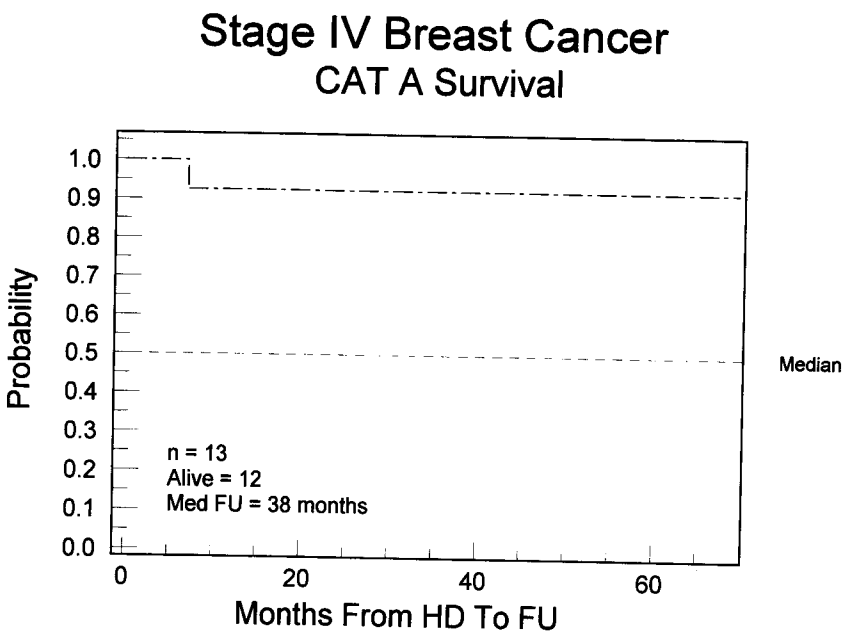


**Figure 2.** *Survival of category A metastatic breast cancer. On the horizontal axis, the time from onset of high dose (HD) to last follow-up (FU) has been plotted. N, number; Med FU, median follow-up of all patients.*

Responses were measured by progression-free survival estimates. Also, overall survival was determined as response to treatment since progression of disease was determined as early as possible by using the cytokeratin assay measuring small numbers of breast cancer cells in the marrow and peripheral blood[5] as well as by change in tumor markers in the serum without radiographic changes.

## RESULTS

Patients were divided into three groups: category A (13 patients), no evidence of disease at time of starting intensive chemotherapy; category B (31 patients), were patients who responded to normal dose but demonstrated evidence of disease at time of intensive chemotherapy, i.e., chemotherapy-sensitive; category C were patients with progression of disease after normal-dose chemotherapy, i.e., chemotherapy-resistant. The best results were obtained in category A patients: median progression-free survival (PFS) of 32 months, and a 90% projected survival of 5 years as documented in Figs. 1 and 2, respectively. The median PFS and overall survival of category B patients were 23 and 36 months, respectively, as documented in Figs. 3 and 4. The median survival of category C patients was 13 months (Fig. 5).

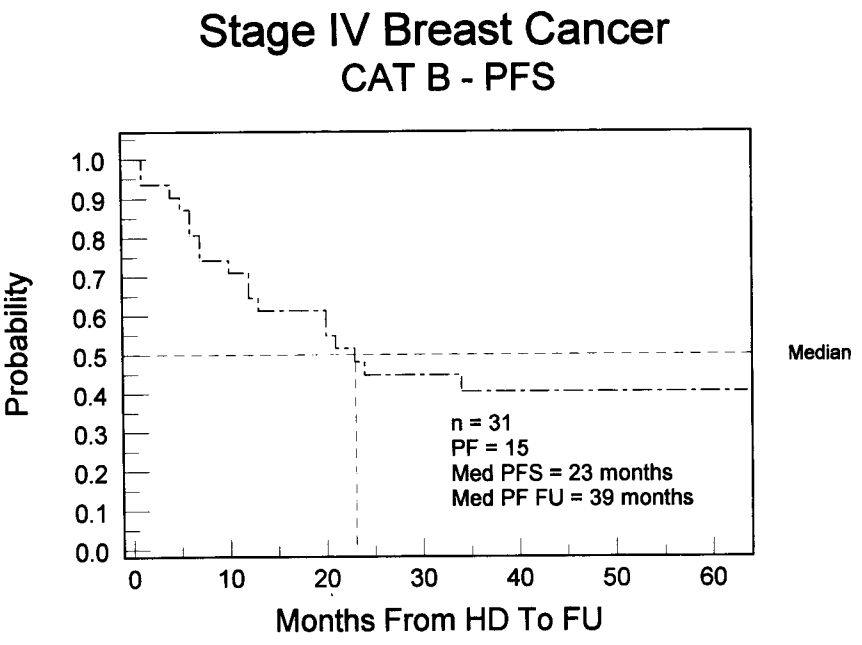


**Figure 3.** *Progression-free survival (PFS) in category B metastatic breast cancer. Kaplan-Meier estimate. On horizontal axis, months from high dose (HD) to last follow-up (FU) has been plotted. N, number; PF, progression-free; Med PF FU, median follow-up of progression-free patients; Med PFS, median progression-free survival.*

## DISCUSSION

It is clear from the results that patients with disease completely responding to normal-dose chemotherapy do better than patients with a partial response and those with progression of disease. This is in line with results and the concept published by Philip et al.[6] The overall survival of the 78 patients is >42 months, which is favorable to the median survival of 19 months of metastatic breast cancer in the CALGB study as published by Mick et al.[2] Patient selection cannot be excluded and may account for the favorable results. Our program is totally outpatient and therefore patient-friendly and cost-effective. The frequency of "infectious episodes" that necessitate hospitalization is <10%. The program with three courses of high dose followed by maintenance (schema B) is more patient-friendly than schema A, in which intensive chemotherapy, CVP and TIP, are alternated for 2 years. The maintenance program does not interfere with day-to-day life, which is not the case in schema A; the CVP and TIP protocols are aggressive, cause severe neutropenia, and tie the patients to Arlington for 3-week periods. The difference in hematopoietic recovery between bone marrow and peripheral blood stem cells is striking: 2.3 times

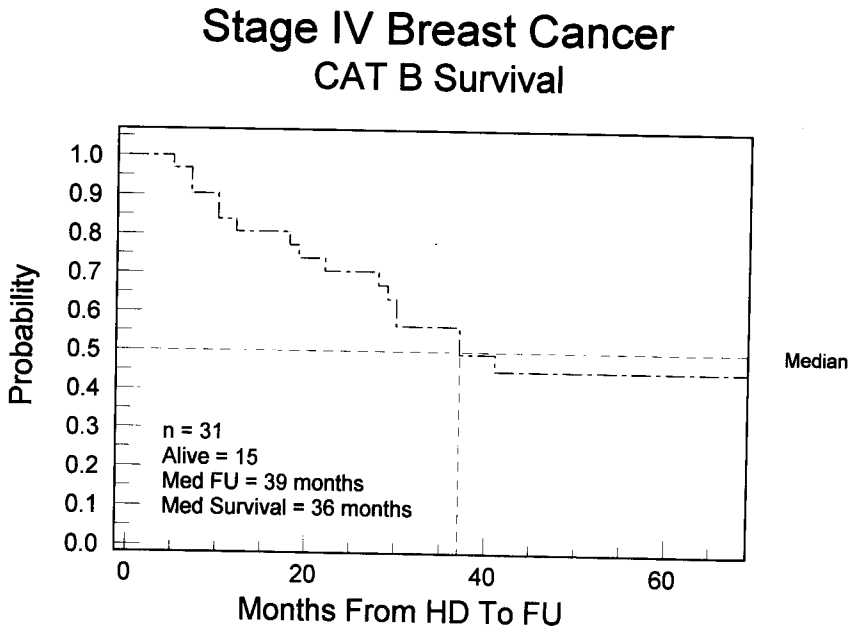


**Figure 4.** *Survival of category B metastatic breast cancer. On the horizontal axis, the time from onset of high dose (HD) to last follow-up has been plotted. N, number of patients under study; Med FU, median follow-up of patients who are alive; Med Survival, median survival.*

more stem cells could be harvested from the peripheral blood and not only hastened hematopoietic recovery, but also the recovery was complete in that white cell as well as platelet counts normalized. These patients sustained maintenance chemotherapy very well without major neutropenia or thrombocytopenia.

Key to preventing major progression in patients is follow-up with sensitive detection methods such as the cytokeratin assay as well as serological tumor markers.[5] With these methods, small numbers of tumor cells can be detected without significant changes on CT scans, MRI or bone scans. The next step is to investigate the role of herceptin in Her-2 neu positive patients in the maintenance part of the program. Early results are promising but longer follow-up is necessary to assess its definite role.

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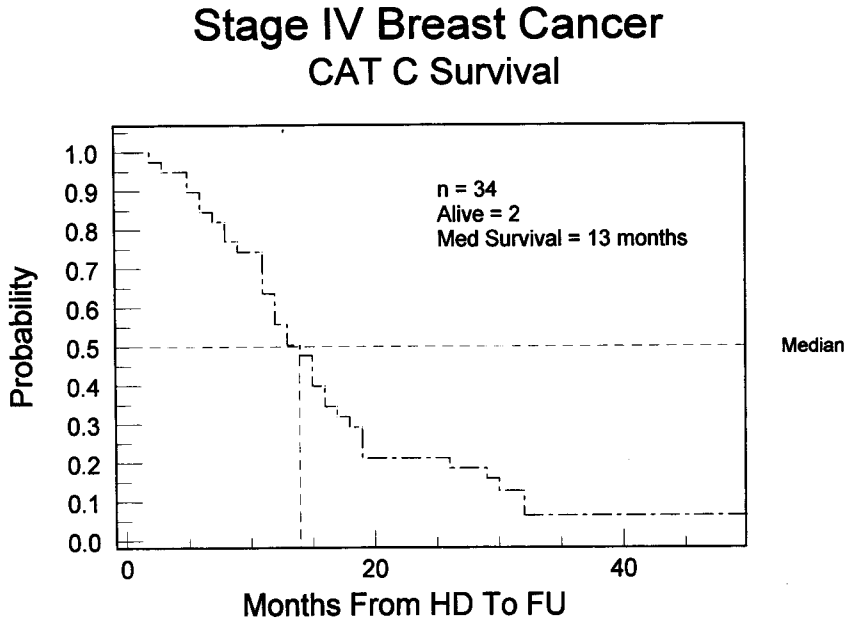


**Figure 5.** *Survival of category C metastatic breast cancer. On the horizontal axis, the time from onset of high dose (HD) to last follow-up has been plotted. N, number of patients under study; Med Survival, median survival.*

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# Sequential High Dose Chemotherapy for Breast Cancer

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While breast cancer is considered a highly curable disease if detected early in its course, the disseminated disease imparts a grave prognosis with a median survival of less than 3 years.[1,2] High-dose chemotherapy increases the number of patients achieving a complete response (CR) to cytotoxic chemotherapy when compared with conventional dose chemotherapy; however, it does not appear that this increased response rate has reliably translated into a similarly increased disease-free survival rate.[3] There are several published series of sequential high-dose chemotherapy regimens in breast cancer.[4–6] Early clinical trials of sequential high-dose chemotherapy were hampered by high morbidity and mortality rates due to the sole use of bone marrow as a stem cell source and lack of hematopoietic growth factors. The use of peripheral blood progenitors and growth factors hastens blood count recovery and has significantly lowered the morbidity and mortality of this approach.

Many strategies have been employed to increase the complete response rate, including the incorporation of new drugs or schedules into established chemotherapy treatment regimens. Other approaches include novel posttransplant consolidation strategies with various types of immune modulation.[7–16] There are several arguments in favor of sequential high-dose chemotherapy as opposed to a single high-dose cycle. Most important among them, according to registry data, is that a CR to therapy is considered the most important prognostic factor associated with a prolonged disease-free survival. Hence, if multiple cycles can increase the CR rate, then perhaps this may translate into prolonged disease-free survival. Several trials of sequential high-dose chemotherapy have been completed over the past 8 years. A recent compilation revealed that the CR rate ranged from 23 to 93% with as many as 35% disease free at 44 months of follow-up.[17]

Paclitaxel is one of the most exciting drugs to emerge in the last 20 years and has been incorporated into transplant regimens by many other investigators. Response rates vary, with some reported as high as 93%.[18] The group at Columbia has piloted a regimen that incorporated paclitaxel at doses ranging from 400 to 825 $mg/m^2$ as the first of three separate high-dose cycles of chemotherapy supported

with peripheral blood progenitors and hematopoietic growth factors. The second cycle was melphalan at 180 mg/m$^2$, administered predominantly as an outpatient, and the third and final cycle was CTCb (cyclophosphamide, thiotepa, and carboplatin). Stem cells were collected before the first intensification. Thirty-one patients completed all three cycles. Overall, the regimen was moderately well tolerated, with transient neurotoxicity observed in virtually all patients. There were no toxic deaths. The complete response rate was 68%, and 52% are progression-free with a median follow-up of 26 months. The phase II dose of paclitaxel is 825 mg/m$^2$. Certainly, it appears that these regimens are uniformly active in controlling disease (even for short periods of time). The impact of paclitaxel for prolonging disease-free survival will ultimately need to be addressed in phase III studies. In addition, the role of posttransplant consolidation strategies will require better delineation.

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# Evaluation of Prognostic Factors for Patients With Breast Cancer Undergoing High-Dose Chemotherapy and Autologous Stem Cell Transplant: A Single Institution Experience

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## ABSTRACT

We treated a total of 442 patients with breast cancer from October 1989 through December 1997 on a variety of phase I and II protocols including ifosfamide, carboplatin, and etoposide; mitoxantrone and thiotepa with or without paclitaxel; ifosfamide, topotecan, and etoposide; busulfan and cyclophosphamide; and cyclophosphamide, thiotepa, and carboplatin. Patients received autologous bone marrow ($n$=127), peripheral blood ($n$=248), or both ($n$=67). Stage of disease included stage II ($n$=49); stage III, noninflammatory ($n$=69); stage III, inflammatory ($n$=17); and stage IV ($n$=307). All patients with stage II and III disease received standard adjuvant chemotherapy before transplant with an anthracycline-based regimen, chest wall irradiation, and, in estrogen receptor–positive patients, hormonal therapy with tamoxifen for up to 5 years. Patients with stage IV disease received induction therapy and were classified based on chemosensitivity before high-dose therapy as complete responders (CR), partial responders (PR), or less-than-partial responders (<PR), which included stable disease and progressive disease. Minimal follow-up for all patients was 6 months at the time of analysis. Median age of all patients was 45 years (range 23–65). Progression-free survival at 5 years for 49 patients with stage II disease was 60%, for stage III noninflammatory disease was 52%, for inflammatory breast cancer was 28%, and for stage IV disease was 31, 9, and 5% for CR, PR, and <PR patients, respectively. By univariate analysis, factors associated with an improved outcome in the adjuvant setting were the absence of minimal bone marrow metastases at the time of transplant and the use of autologous bone marrow rather than peripheral blood stem cells. For patients with metastatic breast cancer, factors associated with an improved outcome included a complete remission at the time of transplant, the absence of minimal bone marrow metastases, and limited metastatic disease. We

conclude that high-dose therapy is associated with durable remissions in patients with high-risk breast cancer and that prognostic factors are useful for identifying candidates for this therapy.

## INTRODUCTION

Since 1992, breast cancer has been the most common indication for high-dose chemotherapy and autologous stem cell transplantation in North America.[1] In 1997, the Autologous Blood and Marrow Transplant Registry reported outcomes for >5800 women who had received such therapy.[1] Despite the large number of women receiving this therapy, the appropriate use of high-dose chemotherapy for the treatment of breast cancer remains controversial. The available data are generally based on small series of phase I and II trials rather than on the results of large, prospective clinical trials. In metastatic breast cancer, for example, there is only one published prospective, randomized trial comparing high-dose chemotherapy to standard chemotherapy.[2] Although this trial demonstrates a superior outcome for patients treated on the high-dose chemotherapy arm, the study has been criticized due to its small size as well as for the treatment regimen and associated clinical outcomes seen in the standard therapy arm. In early stage, high-risk breast cancer, however, two randomized studies have been published comparing standard chemotherapy to high dose chemotherapy, both of which fail to demonstrate an advantage for high-dose chemotherapy.[3,4] Yet, criticisms can be raised again for the relatively small number of patients enrolled in each trial, a fact that limits the statistical power to detect a difference between standard and high-dose therapy. Without the results of large prospective, randomized trials, the application of high-dose chemotherapy in the treatment of breast cancer will continue to be debated.

Despite these limitations, a large amount of data is currently available concerning clinical outcomes following high-dose chemotherapy. The Autologous Blood and Marrow Transplant Registry has performed multivariate analyses of patients with breast cancer treated in the adjuvant setting as well as patients receiving high-dose therapy for the treatment of metastatic disease.[5,6] At our own center, we have treated >500 patients with breast cancer in a series of phase I and II trials of high-dose chemotherapy and autologous stem cell transplantation. Analysis of clinical outcomes following these clinical trials can be useful in defining prognostic factors helpful in identifying patients likely to benefit from high-dose chemotherapy. In this chapter, we present the results of these clinical trials and describe the pre- and posttransplant factors that influence outcome after high-dose chemotherapy.

## PATIENTS AND METHODS

From 1 October 1989 through 30 December 1997, we enrolled patients with high-risk breast cancer on various phase I and II protocols. All patients gave written informed consent for protocols that were approved and reviewed annually by the Institutional Review Board at the University of South Florida. All patients had pathologically documented disease and were required to have adequate cardiac, pulmonary, hepatic, and renal function as determined by standard examinations. Patients also had bone marrow evaluations performed routinely, generally from the posterior superior iliac crests, bilaterally, to exclude the presence of metastatic disease.

Patients with stage II breast cancer with four or more positive nodes and patients with stage III breast cancer were transplanted in the adjuvant setting after four to six cycles of standard-dose therapy, generally with an anthracycline-based regimen. Patients with stage III disease were defined as having noninflammatory or inflammatory breast cancer. Patients transplanted in the adjuvant setting also received chest wall irradiation, either before or after the completion of high-dose chemotherapy. Patients that were estrogen receptor–positive received tamoxifen after the completion of high-dose chemotherapy, for a minimum of 2 years and for up to 5 years in most cases.

Patients with metastatic breast cancer were transplanted after induction chemotherapy or, in the case of patients with a solitary metastatic lesion, after complete surgical resection with or without the addition of radiation therapy. Patients with metastatic breast cancer were categorized as having achieved either a complete response (defined as complete disappearance of all measurable lesions) or a partial response (defined as ≥50% decrease in the size of all measurable lesions) to induction therapy or as having refractory disease (defined as <50% decrease in the size of all measurable lesions or progression at any site). Induction chemotherapy for patients with metastatic breast cancer consisted of an anthracycline-based regimen for those patients never exposed to anthracyclines in the adjuvant setting. For those patients with prior anthracycline exposure or failing anthracyclines for the treatment of metastatic breast cancer, a paclitaxel-based regimen or a combination of ifosfamide, carboplatin, and etoposide in standard doses (miniICE[7]) was used as salvage therapy before high-dose therapy to evaluate chemoresponsiveness. Patients with bone-only disease were defined as chemosensitive if they had evidence of normalization of abnormalities on a nuclear medicine bone scan or plain radiographs or normalization of associated laboratory abnormalities and resolution of associated symptoms with no progression on nuclear medicine bone scans. From 1989 through 1992, patients with brain metastases were considered eligible for high-dose chemotherapy if their disease was clinically stable for a minimum of 3 months after radiation therapy; however,

after 1992, these patients were no longer treated on high-dose protocols.

The source of stem cells included autologous bone marrow, peripheral blood, or a combination of both. Patients undergoing autologous bone marrow harvesting were required to have no evidence of bone marrow metastases after histologic and immunohistochemical analysis of the bone marrow before harvesting. Bone marrow were also evaluated retrospectively for the presence of micrometastases using a polymerase chain reaction (PCR) method to detect the presence of cytokeratin 19 (K19).[8] Peripheral blood stem cells were harvested after a variety of mobilization techniques using hematopoietic growth factors, alone or in combination with chemotherapy. These regimens have been discussed elsewhere.[9,10]

High-dose chemotherapy regimens have been described elsewhere[11] and included ifosfamide, carboplatin, and etoposide (ICE), thiotepa and mitoxantrone (MITT), thiotepa, mitoxantrone, and paclitaxel (TNT), topotecan, ifosfamide, and etoposide (TIME), busulfan and cyclophosphamide and busulfan (BUCY), and cyclophosphamide, thiotepa, and carboplatin (CTC). Evaluations of ICE, MITT, TNT, and TIME were conducted as part of phase I/II dose-escalation trials. Evaluations of BUCY and CTC were conducted as part of phase II trials. Table 1 describes the maximum tolerated doses for each chemotherapy regimen used in these trials.

Patients were followed with routine physical examinations, radiographic examinations, and laboratory studies for evidence of disease progression at 3, 6, and 12 months after the completion of high-dose therapy and yearly thereafter for a minimum of 5 years. Evaluation of progression-free survival was performed for all patients who were a minimum of 6 months posttransplant and was calculated using the Kaplan-Meier method. Differences in survival were evaluated using the log-rank method.

## RESULTS

We treated a total of 442 patients from October 1989 through December 1997. Patient characteristics are shown in Table 2. All patients were female. As previously noted, the minimum follow-up at the time of this analysis was 6 months.

Figure 1 illustrates progression-free survival for all patients with stage II and stage III disease based on number of lymph nodes in patients with stage II disease or, in patients with stage III breast cancer, the presence or absence of inflammatory breast cancer. There was no difference in progression-free survival based on the high-dose regimen employed ($P$=0.96), estrogen receptor status ($P$=0.28), or age (<55 vs. ≥55 years) ($P$=0.4). Factors that adversely affected progression-free survival included the presence of minimal bone marrow metastases as detected by reverse transcription (RT)-PCR for K19 ($P$=0.059) and the use of peripheral blood

**Table 1.** Chemotherapy regimens.

| *Chemotherapy regimen* | *Maximum tolerated dose* |
|---|---|
| ICE | |
| Ifosfamide | 20.1 g/m$^2$ |
| Carboplatin | 1800 g/m$^2$ |
| Etoposide | 3000 g/m$^2$ |
| MITT | |
| Mitoxantrone | 90 mg/m$^2$ |
| Thiotepa | 1200 mg/m$^2$ |
| TNT | |
| Paclitaxel | 360 mg/m$^2$ |
| Mitoxantrone | 48 mg/m$^2$ |
| Thiotepa | 750 mg/m$^2$ |
| TIME* | |
| Topotecan | 36 mg/m$^2$ |
| Ifosfamide | 10 g/m$^2$ |
| Etoposide | 1500 mg/m$^2$ |
| BuCy | |
| Busulfan | 16 mg/kg |
| Cyclophosphamide | 120 mg/kg |
| CTC | |
| Carboplatin | 800 mg/m$^2$ |
| Thiotepa | 500 mg/m$^2$ |
| Cyclophosphamide | 6000 mg/m$^2$ |

**Dose escalation ongoing at the time of this publication.*

stem cells ($P$=0.04) (Fig. 2A and B). The number of positive lymph nodes did influence progression-free survival in patients with stage II breast cancer. The 5-year progression-free survival for patients with four to nine positive nodes was 80% compared with 51% for patients with more than nine positive nodes ($P$=0.04). Of patients with stage III breast cancer, the presence of inflammatory breast cancer was associated with significantly worse progression-free survival ($P$=0.005).

Figure 3 illustrates progression-free survival for all patients with metastatic breast cancer based on chemosensitivity at the time of transplant. Patients transplanted in complete remission did significantly better than patients transplanted with less than a complete response ($P$=0.00002). Of note, the type of treatment necessary to achieve a complete remission (chemotherapy vs. surgery and/or radiation) did not influence progression-free survival in this subgroup of patients ($P$=0.56). As can be seen from the figures, there was a significant difference in outcome for patients who achieved only a partial response to therapy compared with patients who achieved less than a partial response ($P$=0.02). Of

**Table 2.** Patient characteristics

| *Characteristic* | *n* |
|---|---|
| Number of patients | 442 |
| Age | |
| Median | 45 years |
| Range | 23–65 years |
| Stage at transplant | |
| Stage II | 49 |
| 4 to 9 positive nodes | 17 |
| >9 positive nodes | 32 |
| Stage III | 86 |
| Noninflammatory | 69 |
| Inflammatory | 17 |
| Stage IV | 307 |
| CR | 53 |
| PR | 73 |
| <PR | 155 |
| Stem cell source | |
| Bone marrow | 127 |
| Peripheral blood | 248 |
| Both | 67 |

note, all patients ($n$=6) with central nervous system metastases receiving high-dose therapy relapsed. The treatment regimen used did not influence outcome in patients with chemosensitive disease ($P$=0.19) or in patients with chemorefractory disease ($P$=0.12). The presence of more than one site of metastases was associated with an inferior progression-free survival (Fig. 4). A site is defined as a site of organ involvement rather than a solitary metastatic lesion. The presence of minimal bone marrow metastases detected by K19 was associated with an inferior outcome (Fig. 5A); however, the presence of K19 in the peripheral blood stem cell product did not influence outcome ($P$=0.07). Factors that did not influence outcome included the estrogen receptor status ($P$=0.38), the site of disease (bone only vs. other sites) ($P$=0.23), the source of stem cells ($P$=0.97), or the age of the patient ($P$=0.24).

## DISCUSSION

Selection of appropriate candidates for high-dose chemotherapy remains an area of active research and controversy. We describe the results of a large series of patients receiving high-dose chemotherapy and autologous stem cell transplant for the treatment of breast cancer. These studies evaluate outcomes following different high-dose treatment regimens as well as in multiple clinical settings in an attempt

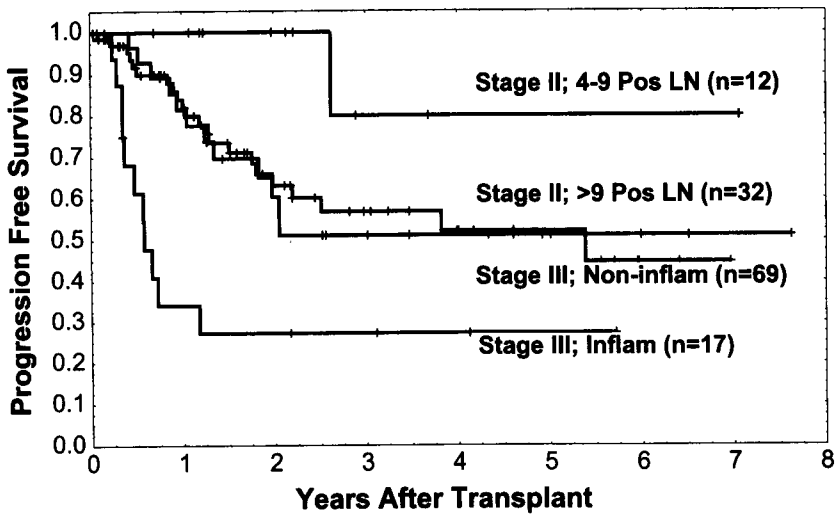


**Figure 1.** *Progression-free survival for all patients with early-stage breast cancer treated in the adjuvant setting.*

to define prognostic factors predictive of long-term, progression-free survival. It is apparent from these data that there are subgroups of patients likely to derive benefit from this type of therapy. Evaluation of standard and novel prognostic indicators can provide further insight into the appropriate role of high-dose therapy in the management of patients with high-risk breast cancer.

In the adjuvant setting, the presence of inflammatory breast cancer remains an important negative prognostic indicator. We and others have consistently described poor outcomes for this group of patients,[5] and future studies should be directed at improving clinical outcomes for this disease. However, novel prognostic indicators may be of more use to identify high-risk populations in patients with noninflammatory breast cancer treated in the adjuvant setting. The current study confirms that the presence of minimal bone marrow metastases as detected using PCR for cytokeratin 19 is associated with a poor prognosis, not only in patients with known metastatic disease as previously described,[8] but also in patients treated in the adjuvant setting. These findings have been confirmed by several authors, in both early and advanced-stage disease.[11–13] In addition, the presence of contaminating tumor cells in peripheral blood stem cell collections may influence outcome. Whether this phenomenon represents residual chemoresistant disease in the patient at the time of high-dose therapy or clonogenic cells within the graft with metastatic potential remains unclear. Future studies to target residual tumor cells in both the patient and the graft may lead to improved outcomes for this group of patients.

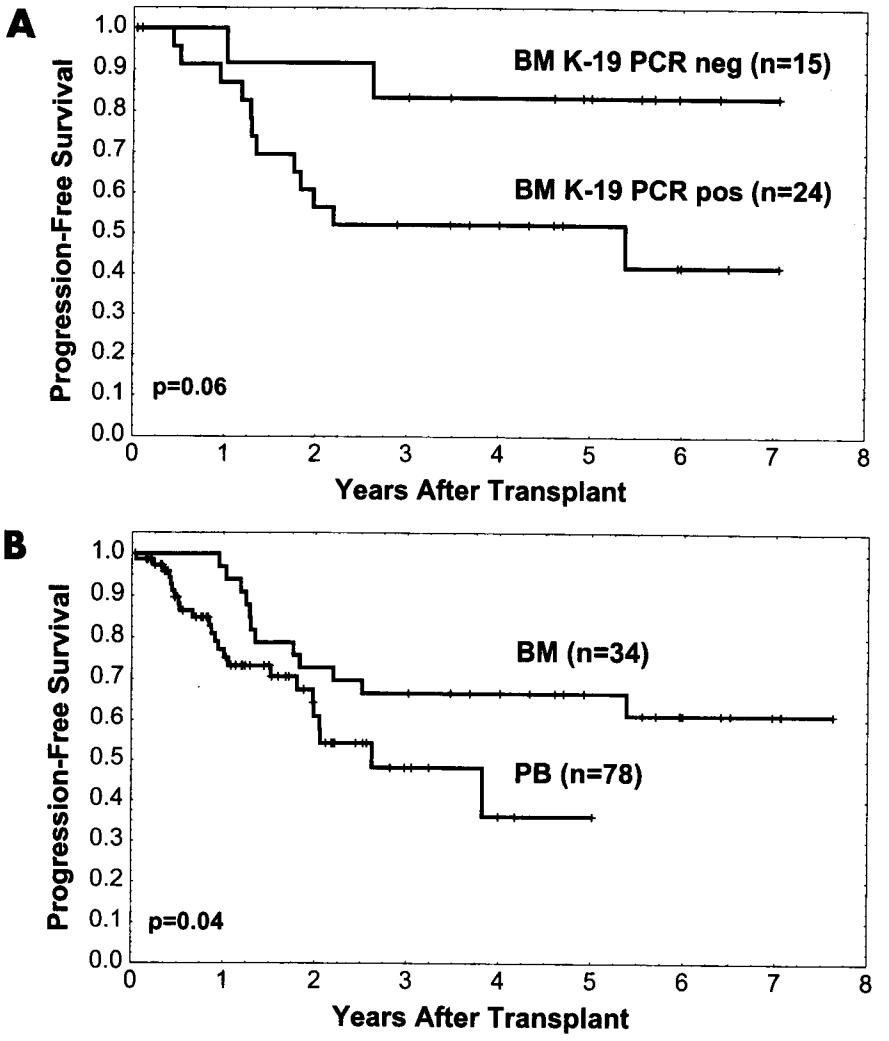


**Figure 2.** *Negative prognostic factors for patients with stage II and III noninflammatory breast cancer undergoing high-dose therapy and autologous stem cell transplant in the adjuvant setting. A: presence or absence of K19 in the bone marrow; B: source of stem cells, bone marrow vs. peripheral blood.*

Although not previously described as a potential negative prognostic indicator, it is of concern that patients receiving peripheral blood stem cells in the adjuvant setting had a worse outcome than patients receiving autologous bone marrow. This finding was not demonstrated in patients with metastatic breast cancer, suggesting that the source of stem cells may not have been the only factor involved. Other

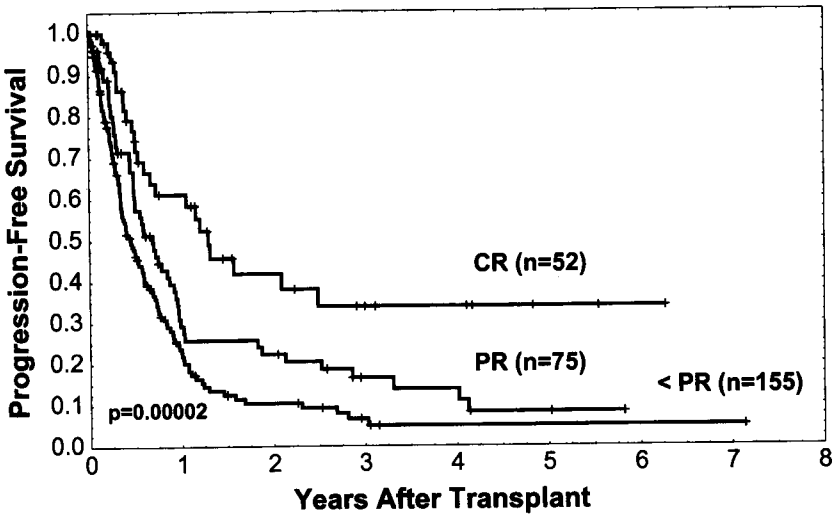


**Figure 3.** *Progression-free survival for all patients with metastatic breast cancer based on chemoresponsiveness at the time of transplant. (CR, complete remission; PR, partial remission; <PR, stable disease or progressive disease.)*

factors such as changes in the treatment regimen over the study period, patient selection, or other patient variables may account for these differences. Multivariate analysis and further follow-up will be necessary to confirm these findings.

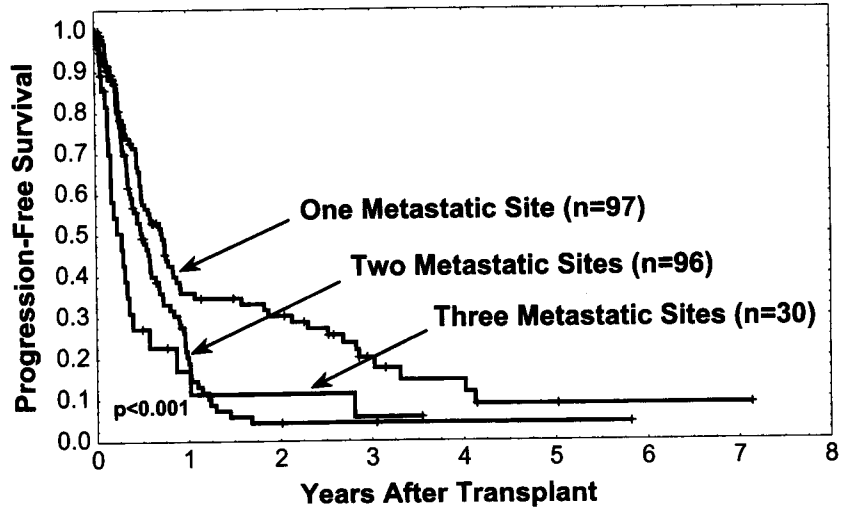


**Figure 4.** *The influence of number of metastatic sites on outcome for patients with metastatic breast cancer undergoing high-dose therapy and autologous stem cell transplant.*

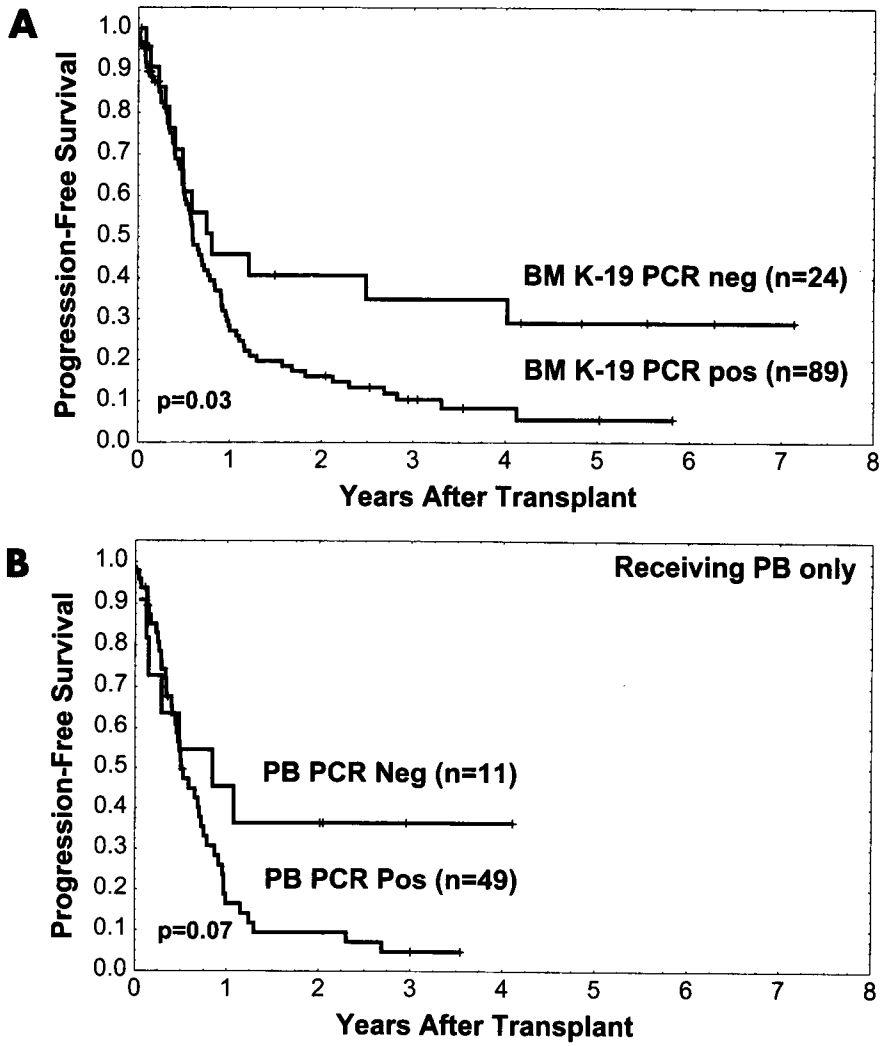


**Figure 5.** *The influence of minimal metastases to the bone marrow (A) and peripheral blood stem cells (B) in patients with metastatic breast cancer undergoing high-dose therapy and autologous stem cell transplant.*

The Autologous Blood and Marrow Transplant Registry reported that age >45 years, less than a complete response to induction chemotherapy, absence of estrogen receptors, and the presence of visceral and central nervous system metastases were associated with a poor outcome after transplant in patients with metastatic breast cancer.[6] Also influencing outcome was the interval from diagnosis to the development of metastases, with patients developing metastases

between 1 and 2 years having the most favorable prognosis. In our study, chemosensitivity before high-dose therapy remains one of the most important predictors of outcome, with patients transplanted in a complete remission having the best outcome. The only other significant predictors of outcome included a limited number of metastatic sites, although the site of metastases did not influence outcome except in the case of central nervous system metastases, and the absence of minimal bone marrow metastases. These variables all suggest that tumor burden at the time of transplant plays an important role in the risk of relapse after high-dose therapy. Thus, future strategies to reduce tumor burden before high-dose therapy may improve outcome.

In conclusion, high-dose chemotherapy followed by autologous stem cell transplantation results in durable remissions for patients with high-risk, early-stage breast cancer and for patients with metastatic breast cancer, most notably for patients with chemosensitive disease. We have demonstrated that one of the most important predictors of outcome is the presence or absence of minimal bone marrow metastases at the time of transplant. These observations likely extend to the presence or absence of minimal tumor cell contamination of the peripheral blood stem cells. It appears that using prognostic factors such as minimal metastases to the bone marrow or peripheral blood, the clinician can select a high-risk group of patients for which novel transplant strategies may be indicated. The results of prospective randomized clinical trials may well define other prognostic factors useful for selecting patients likely to benefit from high-dose therapy.

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# European Trends and the EBMT Database

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## ABSTRACT

High-dose chemotherapy for breast cancer patients is becoming a widely accepted treatment policy for metastatic disease as well as in the adjuvant setting, despite the fact that no data exist from randomized clinical trials. In the last few years, with the introduction of peripheral blood progenitor cell autografts and hematopoietic growth factors, the toxic death rate has been lowered to 2%, even if better patient selection is taken into account as a possible explanation. In this chapter, we outline the European activity in the field as well as the main results from the European Group for Blood and Marrow Transplantation (EBMT) Registry. For metastatic patients, the best results are obtained when high-dose consolidation is performed at the time of complete remission, while with adjuvant patients so far the results seem the same regardless of the number of positive nodes involved. However, a longer follow-up is needed. To control, but of course not to limit, the proliferation of this treatment modality, EBMT and International Society of Hematotherapy and Graft Engineering (ISHAGE) are preparing accreditation policy procedures. At present in Europe, high-dose chemotherapy remains an experimental treatment option.

## THE MAGNITUDE OF THE PROBLEM

In the last five years, high-dose chemotherapy has enormously expanded throughout the continent as a possible treatment modality for patients with breast carcinoma. In 1993, only 400 patients received this type of treatment whereas in 1997, according to a recent unpublished survey, more than 2600 patients underwent high-dose programs. Non-Hodgkin's lymphoma remains the first indication in Europe, exceeding breast carcinoma by about 200 cases per year, while in the United States, breast carcinoma has been the most common indication for the past few years. The vast majority (95%) of autotransplants are conducted using peripheral blood progenitor cells (PBPC) as hematopoietic rescue, and the policy of combining autologous bone marrow transplantation (autoBMT) and PBPC is very seldom used, in the range of nearly 3% (Fig. 1). If we compare these data with the most recently published reports by the Autologous Blood and Marrow Transplant Registry (ABMTR),[1] the shift from marrow to blood autografts has

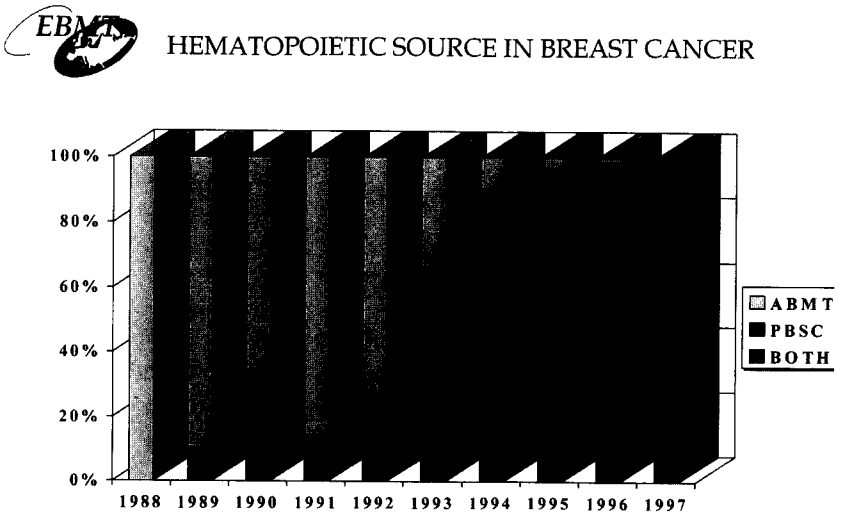


**Figure 1.** *Sources of hematopoietic rescue in patients with breast carcinoma through the years. EBMT Database.*

been more rapid in Europe compared with North America. This may be explained by the fact that U.S. trials started earlier during the ABMT era when PBPCs were not routinely available. Hematopoietic growth factors are used in 70–80% of cases after PBPC reinfusion.

The toxic death rate (defined as death in the first 100 days after transplantation) has dramatically lowered from 15–18% a decade ago to 2% today (Fig. 2). While this may be due to the use of PBPC and hematopoietic growth factors, better patient selection is an important likely factor. In fact, since 1995 high-dose chemotherapy for refractory breast carcinoma has become a very rare treatment option in Europe (no more than 3% of all patients receiving PBPC).[2]

One of the major reasons for concern lies in the fact that while more than 300 centers are performing high-dose chemotherapy, fewer than 20% treat at least 30 patients per year. Accreditation policies for transplant centers are expected in the near future.

## CHEMOTHERAPY FOR METASTATIC DISEASE

High-dose chemotherapy for metastatic disease still represents nearly one half of the European activity in this field. From the EBMT database, significantly better outcome is observed among patients grafted in complete remission compared with patients treated in a less favorable phase of their disease (partial remission, stable disease, or even progression) (Fig. 3).

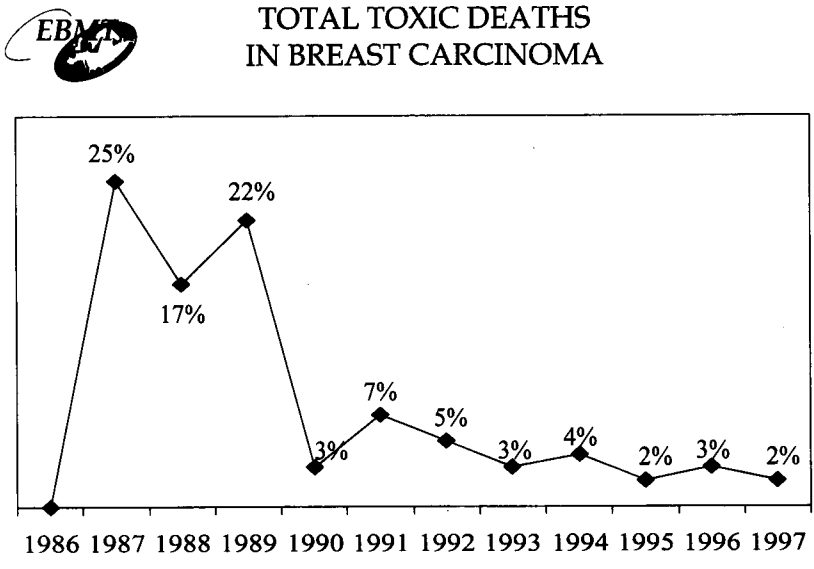


**Figure 2.** *Toxic death rates (100 days from transplantation) for breast cancer patients. EBMT Database.*

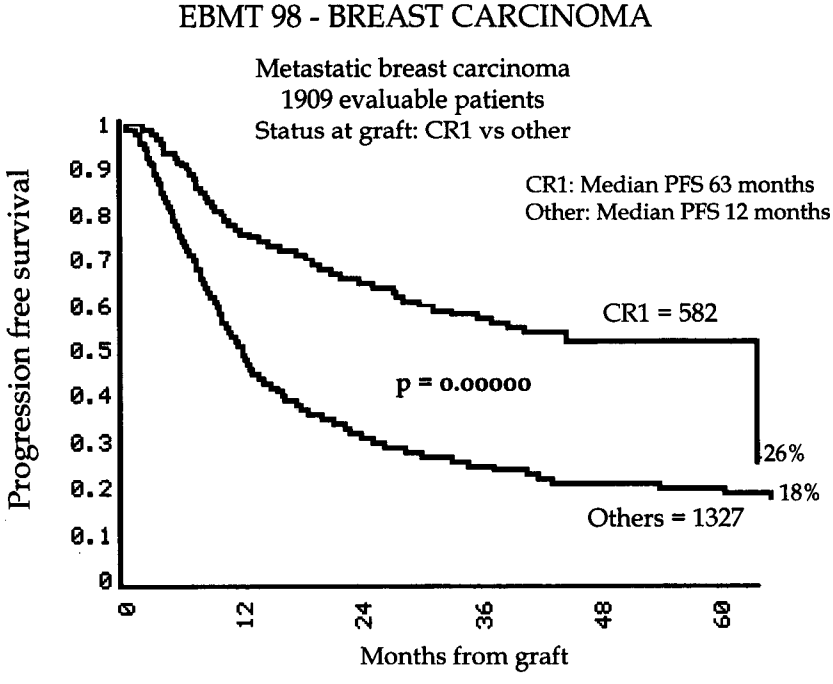


**Figure 3.** *Metastatic breast carcinoma: event-free survival according to status at transplantation. EBMT Database.*

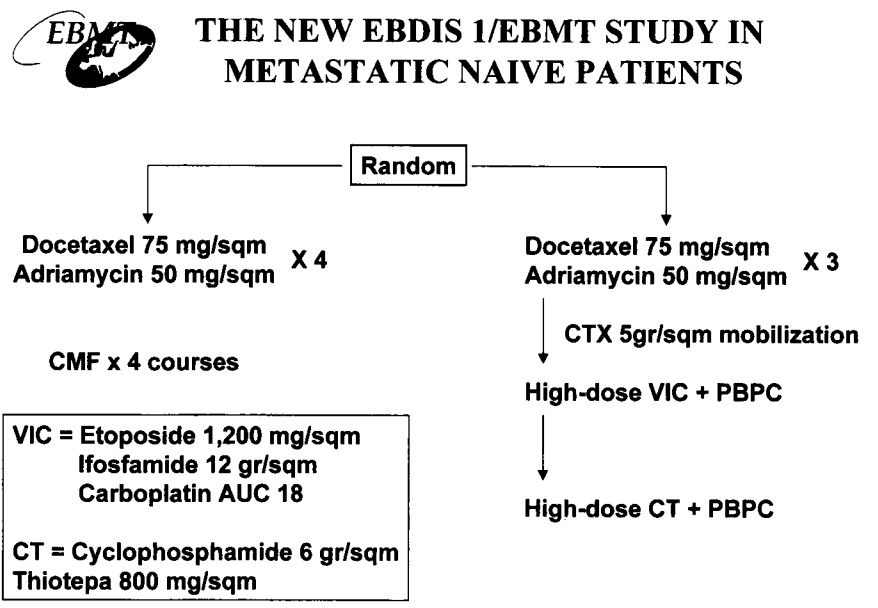


**Figure 4.** *The new EBDIS 1/EBMT randomized trial for metastatic breast cancer.*

Similar outcomes have also recently been reported by the ABMTR.[1] After a period of early phase II studies, in the last few years some randomized phase III trials have been launched. The first, PEGASE 03 from the French National Group compares four courses of 5-fluorouracil, epirubicin, cyclophosphamide (FEC) with epirubicin delivered at the dose of 100 mg/m$^2$ vs. the same FEC schedule followed by an intensification of high-dose cyclophosphamide (6 g/m$^2$) and thiotepa 800 mg/m$^2$. This study, chaired by Dr. Pierre Biron in Lyon, stopped accrual at the end of 1998 (240 patients).

In 1996, a Belgian study set up by Dr. Martine Piccart employed docetaxel upfront at the dose of 100 mg/m$^2$ and then randomization to another course of docetaxel followed by four courses of adriamycin 60 mg/m$^2$ and cyclophosphamide 600 mg/m$^2$ or to docetaxel with lenograstim with the intent to mobilize PBPC, followed by two cycles of melphalan, 140 mg/m$^2$, and mitoxantrone, 35 mg/m$^2$. The most recent proposal has come from a joint venture of the EBMT Solid Tumors Working Party and the European Breast Dose Intensity Study (EBDIS) and the result is the EBDIS 1 trial chaired by Dr. John Crown in Dublin. Figure 4 shows the diagram of the trial. Two hundred sixty-four patients are expected to enter by the year 2000. After successful treatment with high-dose sequential chemotherapy of very high-risk operable breast cancer by the Milan group[3] in patients with 10 or more positive axillary nodes, an Italian Cooperative Group recently started a similar program in naive patients with metastatic disease and good performance

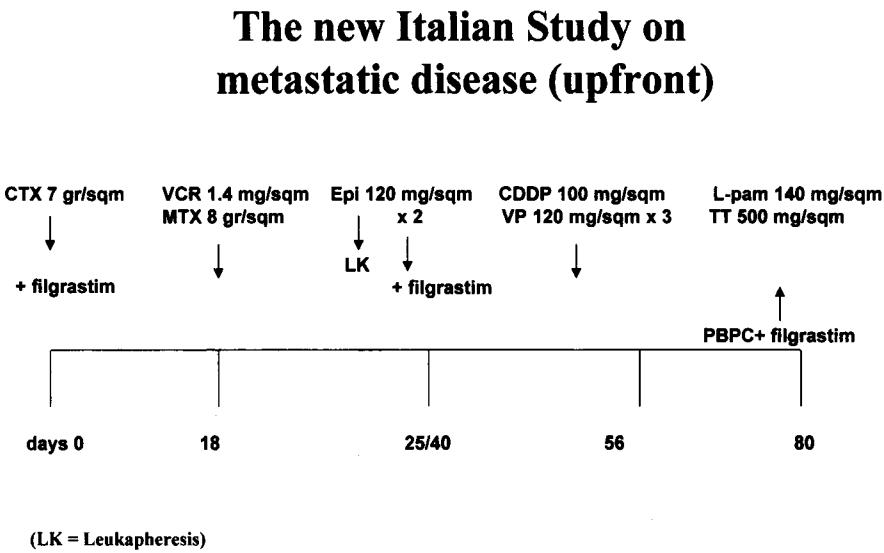


**Figure 5.** *The new high-dose sequential Italian study for naive metastatic patients.*

status (Fig. 5). Patients are initially treated with high-dose cyclophosphamide 7 g/m$^2$, followed by hematopoietic growth factors, and at the time of full hematological recovery, vincristine followed by high-dose methotrexate rescued by folinic acid is delivered. This is followed by two courses of high-dose epirubicin to mobilize PBPCs. The choice of this mobilizing schedule and sequence lies in the theoretical possibility of an in vivo purging effect of cyclophosphamide and methotrexate as well as employing a safe and effective mobilization regimen that has been extensively tested in Italy.[4] After a course of cisplatin and etoposide, patients undergo a conditioning regimen with high-dose thiotepa and melphalan. Total inpatient length of stay for the entire program is in the range of 20 days, and all mobilizations, so far, have been conducted as outpatient procedures. As of July 1998, 40 patients have been treated with a toxic death rate of 0%, while stomatitis grade III or IV (World Health Organization [WHO] criteria) occurs in 75% of the high-dose bialkylator courses.

## CHEMOTHERAPY FOR HIGH-RISK OPERABLE BREAST CANCER (ADJUVANT)

High-dose chemotherapy in the adjuvant setting was introduced in Europe slightly later than in North America, but generally with a lower number of positive axillary nodes as an entry criterion compared to U.S. trials. From the phase II studies that have been published so far, interesting results have been produced by the National Cancer Institute in Milan[3] with 57% disease-free survival at a follow-

**Table 1.** Major European ongoing adjuvant studies in breast cancer (July 1998)

| *Group* | *Nodes (n)* | *Standard* | *High-dose* | *Patients (n)* | *Mobilizing* |
|---|---|---|---|---|---|
| Italian | ≥4 | EPI CMF | HDS | 384 | EPI 120mg/m$^2$ |
| Anglo-Celtic | ≥4 | ADM CMF | ADM+CT | 446 | CTX 4g/m$^2$ |
| Scandinavian | ≥5 or >8* | FEC | FEC + STAMP V | 560 (closed) | FEC |
| Netherlands | ≥4 | FEC | FEC + STAMP V | 750 | FEC |
| EBMT/PEGASE | ≥8 | FEC | FEC + CMA | 286 | FEC |
| German | ≥10 | EC CMF | EC CMT | 197 | FEC |
| IBCSG 15–95 | ≥10 or >5* | EC CMF | HD-EC | 199 | Filgrastim |

*HDS, high-dose sequential; STAMP V, cyclophosphamide, thiotepa, carboplatin; CMA, cyclophosphamide, mitoxantrone, alkeran; CMT, cyclophosphamide, mitoxantrone, thiotepa; HD-EC, high-dose epirubicin/cyclophosphamide. *Depending on estrogen receptor status, tumor size, or biological features.*

up of 5 years; from a pilot experience in Spain,[5] 70% at 4 years; and in Germany as well.[6]

Several phase III randomized studies are underway in Europe, all employing peripheral blood progenitor cells as hematopoietic support. Table 1 shows the major strides with the updated accrual for July 1998. Except for the Inter-Scandinavian trial, which closed accrual in June 1998, all are expected to be concluded by the end of 1998 or by December 1999. Therefore, like the major North American Studies, final results with an adequate follow-up will not be available until the year 2001. As outlined in Table 1, STAMP V (carboplatin, cyclophosphamide, and thiotepa) is one of the most often employed regimens, but with a double carboplatin dose compared with the one.

As previously published by the EBMT Solid Tumors Working Party survey,[7] the vast majority of the centers mobilize with the combination of chemotherapy and hematopoietic growth factors, while only one study (IBCSG 15-95) uses filgrastim alone at the dose of 10 μg/kg.

From the EBMT Database (Fig. 6), a difference among patients treated with less or more than nine positive nodes is not observed (at a relatively short follow-up of 1.5 years, $P$=0.08), but of course more mature data are warranted.

## INFLAMMATORY BREAST CANCER

The large French National Study (Pegase 02) on inflammatory breast carcinoma is presented in the article by Dr. Viens (p. 279–292) and will not be reported here.

Due to the very low frequency of this disease (2–4% of all breast carcinomas) few data are available in the literature.

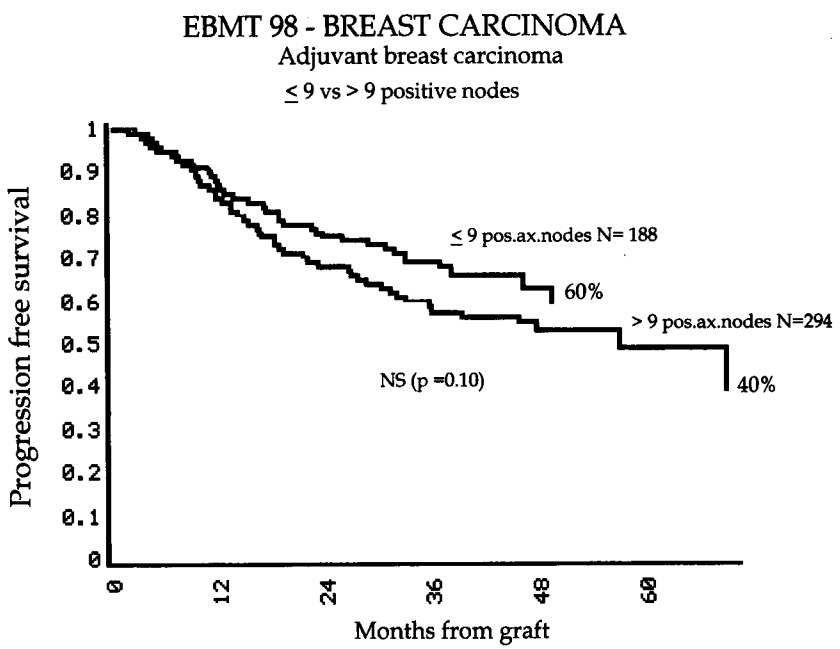


**Figure 6.** *Event-free survival for patients with breast carcinoma by number of positive axillary nodes. EBMT Database.*

In 1994, an Italian Cooperative Group started a phase II pilot study. As of December 1997, 20 patients have been enrolled. Inclusion criteria: age ≤55 years, absence of distant metastases, good performance status (Eastern Cooperative Oncology Group [ECOG] score 0–1), left ventricular ejection fraction (LVEF) ≥50%. The median age is 45 years (29–55). Patients were to receive four courses of epirubicin 150 mg/m$^2$, PBPC mobilization after third or fourth cycle, followed by two high-dose courses of mitoxantrone (40 mg/m$^2$), cyclophosphamide (200 mg/kg), and thiotepa (500 mg/m$^2$), when feasible. Surgery was scheduled at the end, and radiation therapy was administered in case of positive resection margins. Thirty-eight leukaphereses were performed (median day 11, range 8–13) after epirubicin. Seven patients received a double graft, and the reasons for not performing the double treatment were inadequate number of CD34$^+$ cells (five patients), mucositis grade IV (three patients), toxic death due to cardiac failure (one patient), and other reasons (including two patients with transient cardiac failure). At a median follow-up of 24 months, 15 patients are alive with no disease (75%), confirming the good results previously published by our group,[8] with a shorter follow-up.

The results are promising, and we are moving toward a less potentially cardiotoxic schedule (reduction of total anthracycline dose) and a possible tandem transplant with partially non–cross-resistant drugs.

## DIFFERENCES BETWEEN EUROPE AND NORTH AMERICA

Apart from the difference in the choice of the number of positive lymph nodes for high-risk operable breast cancer and the use of the combination of peripheral blood progenitor cells and autologous bone marrow, other differences exist between Europe and North America. First, the policy of treating patients outside a clinical trial seems less accepted in Europe,[7] partly due to the fact that insurance coverage in Europe generally is provided by the treating center and not by the patients themselves.

In many countries, insurance is still not required and this allows an easier accrual of patients in trials as in the case of other solid tumors.

A common accreditation policy is not yet well established in Europe, and the EBMT together with ISHAGE published some guidelines (minimum number of patients treated per year, laboratory and blood bank facilities, etc.) which, it is hoped, will become mandatory for each European center in the near future. So far in Europe, high-dose chemotherapy and hematopoietic rescue has not been considered standard therapy for breast carcinoma of any stage,[9] and the policy of treating patients outside clinical trials should be discouraged.

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# Expression of C-ErbB-2/Her-2 in Patients With Metastatic Breast Cancer Undergoing High-Dose Chemotherapy and Autologous Blood Stem Cell Support

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## ABSTRACT

C-erbB-2/HER-2 (designated HER-2) is overexpressed in both primary and metastatic breast cancer and has been shown to predict for poor prognosis. We investigated the expression of HER-2 in patients with metastatic breast cancer undergoing high-dose chemotherapy (HDCT) with autologous blood stem cell (ABSC) support and correlated the presence (positive) or absence (negative) of HER-2 overexpression in these patients to response to treatment, progression free survival (PFS) and overall survival (OS). The level of HER-2 expression was analyzed in 57 patients with metastatic breast cancer undergoing HDCT with ABSC support. Plasma from peripheral blood was taken at three different time points during the course of treatment and was analyzed using an enzyme immunoassay (EIA) to detect circulating levels of the extracellular portion of HER-2. HER-2 levels were found to be elevated (>0.2 U/mg protein) in 27 of 57 (47.4%) patients at one or more time points during treatment. The level of HER-2 varied during course of treatment. Following induction chemotherapy (ICT), five patients who were negative at the initial measurement showed overexpression of HER-2. Three patients overexpressed HER-2 only at the post-HDCT/ABSC measurement. Response rates to treatment was similar in patients independent of plasma HER-2 levels. Overexpression of HER-2 was associated with a significantly shorter PFS ($P$=0.004) and OS ($P$=0.003) following HDCT/ABSC. We conclude that overexpression of HER-2, measured by EIA in patient plasma, predicts a shorter PFS and OS in patients with metastatic breast cancer treated with HDCT and ABSC support. These data also suggest that optimal timing or several measurements of circulating HER-2 levels using EIA are necessary before HDCT to determine overexpression, since levels may vary during the course of treatment.

## INTRODUCTION

The proto-oncogene c-erbB-2/neu HER-2 resides on chromosome 17 and encodes a 185-kDa transmembrane glycoprotein (designated HER-2) with intracellular tyrosine kinase activity.[1] HER-2 is a member of the EGF growth factor receptor family which consists of four closely related family members: the EGF receptor (EGFR, ERBB1), HER-2 (ERBB2/NEU), HER-3 (ERBB3) and HER-4 (ERBB4).[2] Both homodimers and heterodimers are formed between the various family members mediating a complex system of signal transduction affecting both cell proliferation and growth. [2–6]

The importance of these growth factor receptors on the growth and regulation of breast cancer has been clearly demonstrated. Overexpression and/or amplification of HER-2 has been found in 20–30% of primary breast tumors and to varying degrees in other cancers (ovarian, gastric, colorectal, lung, salivary, bladder, pancreas, endometrial, cervical, oral, and prostate).[7–12] Both HER-2 overexpression and gene amplification have been often found to correlate with decreased survival, particularly in patients with node-positive breast cancer.[7–14,47]

Although HER-2 oncoprotein overexpression correlates with a reduction in patient survival, only a few studies have evaluated the incidence of HER-2 overexpression in patients with metastatic breast cancer (MBC) and examined whether increased levels of this protein indicates a poorer prognosis and/or reduced responsiveness to systemic treatment.[14–24] In these studies, 30–50% of patients with MBC have been shown to have elevated levels of circulating (plasma, serum) extracellular domain of HER-2.[14–19] Overexpression has been shown to correlate with an increased tumor burden and reduced survival.[14–16,18,22–25]

Since EIA is a relatively simple assay commonly used in clinical medicine, the possibility that elevated levels of extracellular HER-2 could indicate poor prognosis in patients with MBC is of particular interest.

HDCT followed by ABSC support is rapidly becoming the preferred treatment for patients with MBC, high-risk stage II and III breast cancer in the U.S.[26] Little is known about the incidence of HER-2 gene overexpression in patients with MBC undergoing this treatment and whether increased levels of this protein indicates a poor prognosis and/or a reduced responsiveness to this treatment.

In this retrospective study, we have examined the incidence of overexpression and changes in the levels of HER-2 protein in the plasma of 57 patients with advanced breast cancer treated with HDCT and ABSC support. Results were analyzed and correlated to response to treatment, progression-free survival (PFS) and overall survival (OS).

## MATERIALS AND METHODS

### Patients

Peripheral blood and ABSC specimens were collected during the course of treatment of 57 patients with metastatic breast cancer enrolled into clinical trials using HDCT and ABSC transplantation. Patients were between 18 and 55 years of age and had either hormone receptor–negative tumours or had failed at least one hormone treatment regimen for MBC (patients with CNS involvement were excluded).

All patients received treatment initially consisting of two cycles induction chemotherapy (ICT). This consisted of cyclophosphamide, 500 mg/m$^2$ body surface area (BSA) intravenously; doxorubicin, 50 mg/m$^2$ BSA intravenously, or epirubicin, 60 mg/m$^2$ BSA intravenously; 5-fluorouracil (5-FU), 500 mg/m$^2$ BSA intravenously. Patients without progressive disease after these two cycles continued on the study which included further ICT using dose-escalation of cyclophosphamide to 2000 mg/m$^2$ BSA intravenously with the same doses of doxorubicin or epirubicin and 5-FU. CD34$^+$ cells were mobilized with either rhGM-CSF or rhG-CSF at 5 or 10 μg/kg body weight starting 1 day after ICT. Apheresis (AP) was performed on average for 4 consecutive days (when WBC values increased to >2.5/nL) using a Fenwal CS 3000 Plus (Baxter Healthcare, Deerfield, IL) or COBE (COBE Laboratories, Lakewood, CO) blood cell separator. A total blood volume of 10 L per AP was processed at a flow rate of 60–70 mL/min. AP collections were continued until a minimum of $2\times10^6$ CD34$^+$ cells/kg was obtained.

High-dose chemotherapy consisted of cyclophosphamide (6 g/m$^2$ BSA intravenously), mitoxantrone (70 mg/m$^2$ BSA intravenously) and either carboplatin (800 mg/m$^2$ BSA intravenously) or vinblastine (12 mg/m$^2$ BSA intravenously), administered over 4 days. Three patients did not receive HDCT due to disease progression and were excluded in the analysis after ICT. Four patients received a second regimen of HDCT with cyclophosphamide and mitoxantrone and carboplatin. One patient received a second HDCT with thiotepa (500 mg/m$^2$ BSA intravenously) and cyclophosphamide (6 g/m$^2$ BSA intravenously). CD34$^+$ cells were reinfused from cryopreserved AP product containing $>2\times10^6$ CD34$^+$/kg that were pooled and washed according to the method established by Glück et al.[27]

### HER-2 plasma levels

HER-2 plasma levels were determined using the HER-2 serum EIA kit (Chiron Diagnostics, formerly Triton Diagnostics, Alameda, CA) according to the manufacturer's instructions. This kit uses a monoclonal antibody–based immunoenzymatic assay to quantitate the shed HER-2 fragment in plasma.

Blood samples obtained during the course of treatment were collected in ethylene-diamine tetraacetic acid (EDTA) vacutainer tubes. Plasma was collected by centrifugation at 400*g* for 10 minutes. The plasma was removed, frozen in aliquots, and stored at –70°C until required for analysis. Plasma HER-2 levels were examined at three different time points: *1*) at initial enrollment to the clinical program; *2*) at the time of first or second apheresis (following ICT with rhGM-CSF or rhG-CSF); and *3*) ~1 month after HDCT and ABSC reinfusion. Before assay, frozen plasma samples were thawed gradually and centrifuged at 8000 rpm for 5 minutes in Eppendorf tubes. Protein concentration was measured by the Bradford protein dye method,[28] using bovine serum albumin as the standard.

Measurements of HER-2 levels in plasma of seven healthy women resulted in a mean concentration of 13.8 U/mL ± 2.3 SD. To further offset any variation due to patient plasma differences during treatment, data was expressed as U/mg plasma protein or 0.134 U/mg protein ± 0.02. The cut-point for positivity (i.e., overexpression) was defined as 21 U/mL or 0.2 U/mg protein (using the mean plus three times the standard deviation calculation from Isola et al.[24]). Plasma samples expressing HER-2 levels below this cut-point were defined as "HER-2 negative" and those samples equal to or above this cut-point were defined as "HER-2 positive." More significant differences between the HER-2 positive and negative patient populations were found when the data were calculated and analyzed using the 0.2 U/mg protein cut-point and all data was subsequently evaluated using this cut-point.

## PATIENT EVALUATION AND STATISTICAL ANALYSIS

Progression free survival was defined as the time from study entry to documented signs of recurrence or progression of disease. Overall survival was defined as the time from study entry to death due to MBC. Survival curves were calculated using the Kaplan-Meier method. Differences between survival times were analyzed by the log-rank, Wilcoxon, and Cox tests for survivorship functions and hazard ratios were calculated by Cox's proportional hazards method for univariate analysis.

All patients had a physical and radiological evaluation based on World Health Organization criteria at various times during the course of treatment and subsequently every 2–3 months for 2 years and then every 6 months until evidence of relapse.

Patients were considered to have achieved a complete response (CR) if no evidence of disease was found for at least 4 weeks. Partial response (PR) was defined as a reduction in measurable disease volume to <50% of prestudy size for at least 4 weeks. Stable disease (SD) was defined as no significant change (no more than 25% above or 50% below prestudy size) in evaluable disease for at least 4

weeks. Progressive disease (PD) was defined as a ≥25% increase in the minimum size of one or more measurable lesions or the presence of new metastatic lesions.

Only patients with CR or PR were considered to have responded to treatment. Fisher's exact test was used to test for response difference according to HER-2 expression levels.

## RESULTS

### HER-2 levels during treatment

The data shown are derived from plasma samples of 57 patients with MBC treated with HDCT and ABSC support. HER-2 plasma levels in these samples were determined using the c-erbB-2 serum EIA kit (Chiron Diagnostics). Plasma HER-2 levels were examined at three time points: *1*) At initial enrolment to the clinical trial; *2*) after ICT, at the time of apheresis; and *3*) at a 4- to 6-week restaging after HDCT and ABSC support.

Table 1 shows the number of samples overexpressing HER-2 at each serial measurement. The overall percentage of patients that overexpress HER-2 is within the range of that found in the few studies that have examined overexpression in MBC; i.e., 20–57% of patients with MBC have elevated HER-2 plasma concentrations.[11–17] Of the 57 patients examined, 27 (47.4%) overexpressed HER-2 on at least one of the examined time points. The overall median concentration of positive values during the course of treatment was 0.61 U/mg protein. Patients were included if they overexpressed HER-2 at any one or more of the three time points. A further examination of all HER-2 overexpressing patients (at any one or more of the time points) showed that 16 of 25 (64%) evaluated patients were positive at the first measurement (at initial enrolment to the clinical trial). At the second measurement (following ICT, at time of apheresis), 21 of 27 (77.8%) evaluated patients overexpressed HER-2. This included five patients (five of 27) that were negative at the initial measurement. At the third serial measurement, following

**Table 1.** Plasma HER-2 overexpression in peripheral blood

| | *Initial* | *At apheresis* | *Posttransplant* | *Overall positive* |
|---|---|---|---|---|
| *n* (%) | 16 (32.7) | 21 (37.5) | 14 (29.2) | 27 (47.4) |
| Mean | 0.89 | 0.59 | 0.33 | 0.61 |
| Range | 0.22–4.65 | 0.20–3.18 | 0.21–0.55 | 0.20–4.65 |
| Total | 49 | 56 | 47 | 57 |

*No., number of HER-2 positive; %, percent HER-2 positive. The lower limit for the amount of overexpressed protein is 0.2 U/mg total protein. Overall positive plasma HER-2 refers to patients with HER-2 levels >0.2 U/mg protein at any one or more of the three time points.*

HDCT with ABSC support, 13 of 24 (54.2%) evaluated patients overexpressed HER-2. This included three patients (three of 24) who were negative at the two prior measurements. Eight of 24 (33.3%) evaluated patients overexpressed HER-2 at all three time points.

The mean expression values of secreted HER-2 decreased during the course of treatment in 17 of 22 evaluated patients who were HER-2 positive initially and/or at time of apheresis. Eleven of these 17 values decreased to below the cut-point of 0.2 U/mg protein after HDCT and ABSC reinfusion.

## Survival and clinical course analysis

Patients that overexpressed levels of HER-2 (>0.2 U/mg) on at least one measurement were considered HER-2 positive. Twenty-four HER-2 negative patients responded to chemotherapy (17 PR and seven CR) and 21 HER-2 positive patients responded to treatment (17 PR and four CR). The total number of nonresponders (PD + SD) was five for both HER-2 positive and HER-2 negative patients. There was no significant difference in the response to chemotherapy between HER-2 positive and HER-2 negative patients (Fisher's exact test, data not shown).

OS and PFS analysis using the Kaplan-Meier method on the basis of patient HER-2 expression levels are shown in Table 2. The median OS for all patients on this study was found to be 25.1 months, and the median PFS was 10.4 months.

The difference in OS was highly significant between patients with HER-2 negative and HER-2 positive plasma using the log-rank test ($P$=0.003), Wilcoxon

**Table 2.** Overall survival and progression-free survival: results according to HER-2 overexpression

| *Plasma HER-2 expression* | *Median overall survival* | *Median progression-free survival* |
|---|---|---|
| (+)HER-2 ($n$=27) (CI) | 17.2 months (8.2, 19.9) | 7.5 months (5.5, 10.4) |
| (-)HER-2 ($n$=30) (CI) | 28.9 months (25.1, indeterminate) | 14.3 months (8.3, 24.6) |
| Hazard ratio (relative risk) | 2.5 (1.3, 4.7) | 2.3 (1.3, 4.1) |
| $P$* | 0.0030 | 0.0040 |
| $P$† | 0.0018 | 0.0070 |
| $P$‡ | 0.0040 | 0.0051 |

*(+)HER-2 and (–)HER-2 designates those patients with >0.2 U/mg protein or <0.2 U/mg protein, respectively, at one or more time point. CI, 95% confidence interval. *Log-rank test of survivorship. P values are derived from a comparison of survival functions for (+)HER-2 and (–)HER-2 patients; †Wilcoxon estimate of survivorship; ‡Cox estimate of survivorship. The hazard ratio designates a relative risk of death or relapse between HER-2 positive and HER-2 negative patients.*

($P$=0.0018), and Cox ($P$=0.0040) tests. The overall median survival for HER-2 positive patients was 17.2 months and for HER-2 negative patients, 28.9 months. The relative risk or hazard ratio was 2.5. This means that on average that HER-2 positive patients die at 2.5 times the rate of HER-2 negative patients.

Differences in the duration of PFS was also highly significant using the log-rank (0.004), Wilcoxon (0.007), and Cox (0.0051) tests (see Table 2) between HER-2 plasma positive and negative patients. The median PFS of HER-2 plasma positive patients was 7.5 months compared with 14.3 months for HER-2 negative patients. The relative risk or hazard ratio was 2.3. Disease progression in HER-2 positive patients occurs at 2.3 times the rate of HER-2 negative patients.

Figure 1A and B shows the OS and PFS curves, respectively, of patients with plasma positive and negative HER-2 expression.

There were no significant differences in OS and PFS between the group of patients who initially overexpressed HER-2 (at the first measurement) and that continued to overexpress HER-2 (six patients) following HDCT/ABSC support and those patients that initially overexpressed HER-2 and then became HER-2 negative following HDCT/ABSC support (11 patients) (data not shown).

An independent analysis at each of the three serial measurements for HER-2 expression was performed. Analysis of the PFS showed that only the hazard ratio at the second measurement (at the time of apheresis) was significant for those patients who overexpressed HER-2 and those who did not (hazard ratio 2.04, 95% CI 1.14–3.65, $P$=0.017).

Analysis of OS at each time point showed significant differences in hazard ratio between the HER-2 positive and HER-2 negative group at this second measurement (hazard ratio 2.66, 95% CI 1.40–5.05, $P$=0.003) and also at the initial measurement (hazard ratio 2.25, 95% CI 1.15–4.43, $P$=0.018).

## DISCUSSION

A simple, quantitative EIA procedure was used to examine HER-2 overexpression in patient plasma samples. No clear cutoff value has been established in a variety of studies which have examined overexpression using this procedure and previous studies have individually determined the cut-point for HER-2 overexpression in patient serum and plasma in various ways. Isola et al.[24] examined HER-2 levels in preoperative and follow-up serum samples in patients with breast cancer using the EIA assay manufactured by Triton Diagnostics. Serum levels in 113 patients with benign breast disease had a mean of 5.7 ± 4.8 U/mL and the cut-point for positivity was defined as 20 U/mL (using the mean plus three times the standard deviation calculation). Revillion et al.[17], using the HER-2 plasma assay manufactured by Triton, determined a cutoff value of 27 U/mL from healthy women ($n$=30). Volas et al.[20] selected a cut-point of 30 U/mL, also based on serum

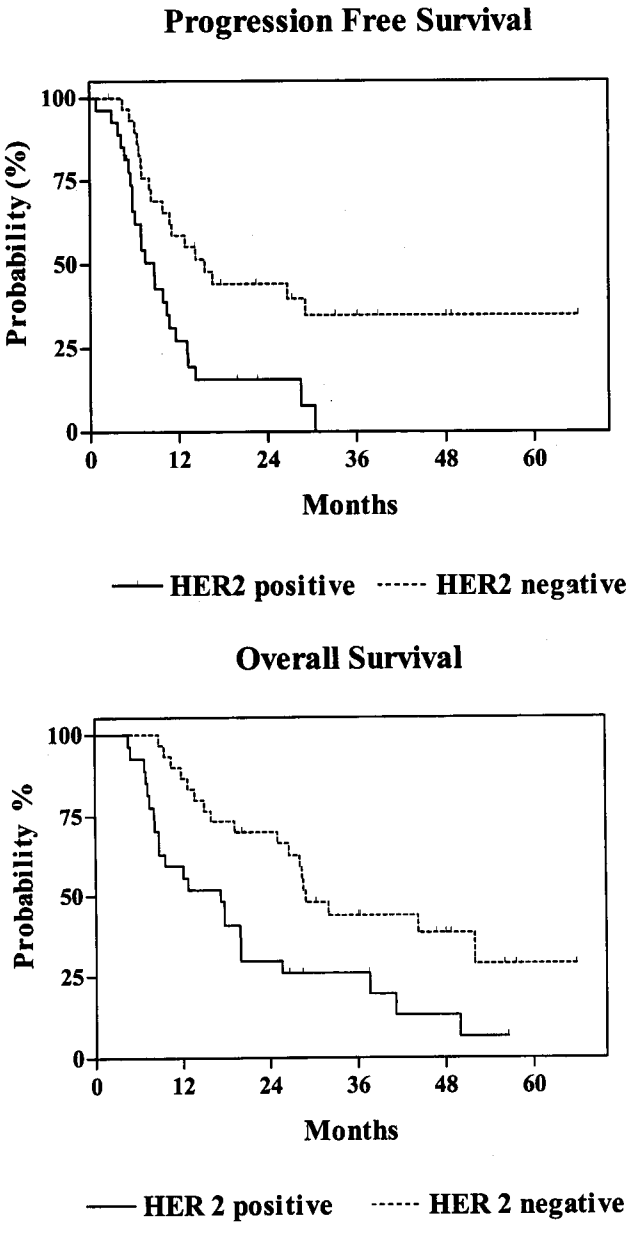


**Figure 1.** *Kaplan-Meir survival plots according to the levels of circulating HER-2 fragment in plasma of 57 patients with MBC undergoing HDCT with ABSC support. Levels of HER-2 were determined using an enzyme immunoassay. HER-2 pos represents patients with overexpression of plasma HER-2 (≥0.2 U/mg protein), and HER-2 neg represents patients with control levels of plasma HER-2. Tick marks represent censored patients. A: Kaplan-Meir plot of progression free survival. B: Kaplan-Meir plot of overall survival.*

results of healthy controls also using the EIA kit by Triton Diagnostics. Kandl et al.[14] measured the HER-2 antigen fragment in the serum of 79 women with advanced breast cancer. They found that a serum concentration of >10 U/mL had a significant impact on OS from the time of diagnosis of metastatic disease.

We determined the cut-point for HER-2 overexpression using plasma from normal blood samples to be 21 U/mL. After normalizing for plasma protein, a cut-point of 0.2 U/mg plasma protein was obtained. Any variations due to patient differences during treatment were therefore offset. Protein in plasma samples was found to vary considerably particularly during ICT (45.6–125.7 mg/mL) using the Bradford protein determination method.[28] More significant discrimination between various HER-2 expressing populations was found using data analyzed in relation to total plasma protein.

Serial changes in HER-2 overexpression were measured in this study. These results show that changes in HER-2 levels can occur during treatment and that patients can have both negative and positive HER-2 levels during the course of treatment. In three of the 27 HER-2 overexpressing patients, HER-2 was only detected at the post-HDCT and ABSC transplantation measurement. Five patients that were negative at the initial measurement were found to overexpress HER-2 at the second measurement after ICT at apheresis. Revillion et al.,[17] using the same EIA method, also observed that five of 23 initially HER-2 negative advanced breast cancer patients became HER-2 positive (overexpressing) during treatment with chemotherapy. Serial changes in HER-2 plasma levels were also observed by Isola et al.[24] In patients who overexpressed HER-2 prior to HDCT, there were no significant differences in OS and PFS between those patients which lost HER-2 overexpression (11 of 17) or maintained overexpression (six of 17) post-HDCT (data not shown). This again is similar to studies which have shown that changes in HER-2 overexpression do not correlate with clinical outcome.[17–20]

It has been shown that chemotherapy and cytokines used to mobilize hematopoietic stem cells into blood can also mobilize tumor cells and the observed increase in the number of patients with circulating HER-2 after ICT may be related to this effect.[29,30] In this study, if only an initial measurement of HER-2 had been determined, eight patients who were actually positive for HER-2 overexpression would have been excluded. Therefore, during treatment more than one measurement (or a determination of the optimal timing of measurement) with this method may be necessary in order to evaluate HER-2 expression. An analysis of PFS and OS at each time-point in relation to HER-2 overexpression showed that for both PFS and OS, the hazard ratio at the second measurement (at apheresis, following ICT with rhG-CSF or rhGM-CSF growth factor) showed significant survival differences and therefore, this may be the optimum time to evaluate HER-2 overexpression prior to HDCT.

In this study, we found that 47.4% of patients with MBC overexpress HER-2. These results are consistent with the few studies that have examined overex-

pression in MBC, i.e., 20–57% of patients with MBC have elevated HER-2 plasma/serum concentration.[14–20,22–25] These HER-2 positive patients had a significantly decreased OS and PFS following HDCT and ABSC transplantation (Table 2 and Fig. 1A and B). Therefore, the presence of elevated plasma HER-2 may be an important prognostic factor that could be used to select more effective and additional therapies for these patients (e.g., directly targeted treatments using immunologic therapy).

It is still not certain whether patients with elevated levels of HER-2 would benefit more from specific and/or increased dosages of chemotherapeutic agents as suggested previously by some clinical studies.[31–34] Results from a study by Muss et al.[31] suggest that patients with node positive early breast cancer who overexpress HER-2 may benefit from higher doses of adjuvant chemotherapy particularly when using doxorubicin. Results of this retrospective study indicate that increased dosages of drugs used in HDCT do not significantly improve response and survival in patients with MBC who overexpress HER-2. (Four of the five patients which received an additional HDCT treatment were HER-2 positive.)

HER-2 overexpression has been observed to result in resistance to a wide variety of drugs both in vitro and clinically. Correlations have been found between HER-2 overexpression and resistance of breast tumor cells in vitro to a wide variety of agents, including alkylating agents such as *cis*-platinum and cyclophosphamide,[35] tumor necrosis factor,[36] and natural killer cells.[37] Several clinical studies have also shown an association between HER-2 overexpression and drug resistance to alkylating agents, anthracyclines, and CMF-like regimens.[31,38–40] Additional studies have shown a correlation of HER-2 overexpression with tamoxifen[41–44] and hormone therapy[25,45] resistance in breast cancer. Alternatively, the poor prognosis associated with HER-2 overexpression may occur as a result of increased proliferation and metastasis as indicated by other studies.[13,24,46] In another recent study, HER-2 overexpression was described as having both prognostic and predictive value with regard to adjuvant therapy in patients with lymph node positive breast cancer.[47] Other biomarkers, e.g., estrogen receptor, high S-phase fraction, p53 mutation, may also contribute estimating survivals. This together with our finding could be of potential clinical relevance by making choices to alternative or more aggressive treatments available to selected patients.

In conclusion, in our retrospective study, we are the first to identify a potential prognostic factor relating to the overexpression of HER-2 in plasma using an EIA method, in women with MBC undergoing HDCT and ABSC transplantation. These data need confirmation in a larger prospective study including a multivariate analysis to discriminate whether or not this biomarker is an independent prognostic factor. Evaluation of PFS and OS will also require possible correlation with other biomarkers, e.g., MDR1, MRP, Topo II(, p53, BAG-1, which are all included in a prospective study currently underway.

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# CHAPTER 7
# OTHER SOLID TUMORS

# High-Dose Chemotherapy and Autologous Stem Cell Transplantation for Ovarian Carcinoma: Comparisons to Conventional Therapy and Future Directions

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## ABSTRACT

High-dose chemotherapy and peripheral blood stem cell (PBSC) rescue is increasingly used to treat patients with advanced epithelial ovarian cancer. Its greatest benefit appears to be in patients responding to initial therapy or relapsed patients with platinum-sensitive disease and minimal tumor <1 cm. We report on our updated series of 164 patients treated at our center between September 1989 and June 1998 with this disease. While initially all patients received high-dose carboplatin, mitoxantrone, and cyclophosphamide ($n$=79), more recent patients with platinum-sensitive disease received 24-hour infusional paclitaxel (700 mg/m$^2$), 120-hour infusional carboplatin (area under the curve [AUC] dose of 28), and bolus mitoxantrone (90 mg/m$^2$) ($n$=38) to capitalize on the antitumor benefits of paclitaxel in this disease. Platinum-resistant patients with minimal bulk disease receive 96-hour infusional paclitaxel (650 mg/m$^2$), and bolus melphalan (180 mg/m$^2$) and mitoxantrone (90 mg/m$^2$; $n$=40). Of the 164 patients transplanted, 53% had platinum-resistant disease, and in 46% the tumor bulk was >1 cm. The median progression-free survival (PFS) and overall survival (OS) for the entire group is 7.7 and 17.3 months. Those with platinum-refractory disease appear not to benefit, with a 5.6-month PFS and 10.6-month OS. In contrast, patients with relapsed platinum-sensitive, low tumor bulk have a 16.0-month PFS and a 43.3-month OS, and approximately 20% remain progression-free up to 5 years. When treated with the paclitaxel regimen as consolidation therapy ($n$=10), the PFS was 65% at 26 months from diagnosis. High-dose therapy is associated with an improvement in PFS and OS compared with conventional options for relapsed, responding, low tumor burden patients. Its role as consolidation therapy of an initial remission should be verified in a phase III trial.

## INTRODUCTION

Ovarian cancer, like the leukemias and lymphomas, is initially chemosensitive but frequently incurable using conventional therapies.[1] New paclitaxel/platinum chemotherapy regimens have improved the median survival for patients with advanced disease; however, they do not increase the proportion of patients with a surgical complete remissions (CR) and thus will likely cure few additional patients.[2] Because of its dismal prognosis and the fact that it shares some of the features of other initially chemosensitive tumors that respond favorably to intensive high-dose chemotherapy regimens, much interest in high-dose therapy has been given to this tumor over the past 10 years. There are considerable in vitro and in vivo data on the use of dose intensity in ovarian cancer, including regional high-dose therapy via the intraperitoneal route using cisplatin. In fact, ovarian cancer shares many of the features of hematologic malignancies, lymphomas, and pediatric round cell tumors that benefit from such high-dose therapy, including chemosensitivity, and occasionally can be cured with conventional therapy despite large bulk at diagnosis. There is a favorable dose-response curve for a variety of agents and synergy of active agents in vitro.

While conventional salvage therapy produces responses in the range of 15–25%,[3–7] early transplant trials reported response rates for drug-resistant patients in the range of 75%.[8–10] Shea et al.[8] treated a large number of patients with refractory tumors with high-dose carboplatin and autologous bone marrow transplantation in a phase I trial. Of the 11 patients treated with ovarian cancer, seven (77%) responded, one of whom had a CR. Shpall et al.[9] treated a group of 12 patients who had failed a median of three prior regimens, all of whom were also platinum-resistant with trialkylator therapy consisting of thiotepa, cyclophosphamide, and cisplatin. All were debulked surgically before transplant, and all received their cisplatin via the intraperitoneal route. Of eight evaluable patients, six (75%) had a pathologic partial remission that lasted 6 months. At the same time, our group treated seven patients with refractory ovarian cancer with a combination of high-dose carboplatin, cyclophosphamide, and mitoxantrone as part of a phase I trial.[10] Mitoxantrone was chosen based on its in vitro activity for platinum-resistant disease at high doses using the tumor cloning assay. This trial demonstrated that the mitoxantrone doses (75 mg/m$^2$) gave serum levels in the steepest part of the in vitro dose-response curve. Of six evaluable patients (one early death), all responded, with a median response duration of 7.5 months. One patient who had failed platinum-based induction therapy was progression-free at >2 years, and 29% were alive at 2 years.

A phase II trial of this regimen was conducted at the maximum tolerated dose (MTD): carboplatin 1500 mg/m$^2$, cyclophosphamide 120 mg/kg, mitoxantrone

75 mg/m$^2$.[11] Of the 30 patients, only one-third were platinum-sensitive and 73% had bulky disease as defined by maximum residual disease >1 cm at the time of transplant. Of the 27 patients with measurable or evaluable disease, 89% responded, with seven of platinum-sensitive vs. nine of 19 platinum-resistant disease patients obtaining a clinical CR ($P$=0.06). The median survival for the entire group was 29 months, and the median PFS was 7 months. The median survival for the 10 platinum-sensitive patients was not reached, with an 80% survival at 1 year.[12] Given the short overall survival for platinum-resistant patients of 10.4 months, one of the conclusions of the study was that high-dose therapy may not be of value to patients with platinum-resistant disease. We subsequently verified these findings in a multivariate analysis of the first 100 patients treated at our center. As of May 1998, our group has treated a total of 164 patients, who are described in this report. Many of our initial impressions remain for these patients who have now been followed for a median of 36 months.

## PATIENTS AND METHODS

### Patient selection

Patients were eligible if they either failed conventional chemotherapy or relapsed after a remission or had poor-risk disease in first remission. As of May 1996, only patients with platinum-resistant disease patients and tumor burdens <1 cm were eligible for transplant based on the poor survival outcome for those with bulky disease. All were required to have either surgery or chemotherapy to minimize tumor bulk at transplant. Starting in May 1996, patients were also eligible if they had either bulky stage III or IV disease for transplants as part of initial therapy.

### Patient eligibility

Patients were eligible to age 65, with a SWOG performance status of 0–2. Prior to transplant, a determination of platinum sensitivity and maximal tumor diameter was made. Other eligibility criteria included a cardiac left ventricular ejection fraction of >45%, bilirubin <2.0 mg/dL, and a creatinine clearance >60 mL/min, before the adoption of area under the curve (AUC) dosing. Pretreatment white blood cell (WBC) and platelet count had to be >2500 and > 100,000/μL, respectively. Initially, histologically negative bilateral bone marrow aspirates and biopsies were required. However, after the first 70 examinations were negative, these were dropped. Audiograms documenting normal hearing in the voice tones were required.

### Preparative regimens

The initial regimen tested was 120-hour infusion carboplatin (1500 mg/m$^2$) and bolus mitoxantrone (75 mg/m$^2$) and cyclophosphamide (120 mg/kg). Because nonhematologic toxicity appeared greater in a retrospective analysis of patients with an AUC equivalent dose >30, and the median AUC dose was calculated to be 27.5, as of May 1996, all subsequent patients received carboplatin dosing at an AUC of 28. For platinum-resistant disease, a small cohort of patients (14) were treated with the same chemotherapy combined with escalating doses of cyclosporine in an attempt to inhibit multidrug and platinum resistance. However, the nonhematologic toxicity was too great to move this into a phase II trial. Subsequent platinum-resistant patients have received treatment on a non-platinum-containing regimen: split dose bolus melphalan (180 mg/m$^2$) and mitoxantrone (90 mg/m$^2$) and paclitaxel as a continuous infusion over 4 days (400–650 mg/m$^2$). Finally, starting in May 1996, all platinum-sensitive patients were treated with paclitaxel (700 mg/m$^2$) given over 24 hours, mitoxantrone similar to above (90 mg/m$^2$), and carboplatin at an AUC of 28 over 5 days. The inclusion of paclitaxel was based on its favorable effect in the initial management of this disease and its in vitro favorable dose-response curve.

All patients treated after 1994 received blood stem cell grafts rather than bone marrow. Stem cells were universally mobilized by cytokines alone, with a target dose of $4\times10^6$ CD34/kg, which provides rapid neutrophil and platelet engraftment.

## RESULTS

### Patient characteristics

The patient characteristics of the 164 patients are shown in Table 1. The differences of this group from an unselected group with this disease: only 12% had stage I or II at diagnosis, 70% had optimal cytoreduction at the time of initial surgery, and only 40% had either a clinical or pathologic CR to initial therapy. The median time from diagnosis to transplant was 16 months, and nearly one-half had bulky disease (>1 cm) at the time of transplant. The median number of chemotherapy regimens before transplant was two, and at the time of transplant 53% of the patients were platinum resistant. At the time of this analysis the median follow-up time posttransplant was 36 months. The number of patients treated with each regimen were 79 for carboplatin, mitoxantrone, and cyclophosphamide; 40 for melphalan, mitoxantrone and paclitaxel; and 38 for paclitaxel, carboplatin and mitoxantrone. Seven received a variety of alternative regimens including thiotepa, cisplatin, and cyclophosphamide on a Southwest Oncology Group (SWOG) multicenter phase II trial.

**Table 1.** Clinical characteristics

| | |
|---|---|
| Number treated | 164 |
| Age (years) | 49 (23–65) |
| Stage at diagnosis | |
| I | 9 |
| II | 12 |
| III | 108 |
| IV | 34 |
| Histology | |
| Serous | 120 |
| Endometrioid | 21 |
| Clear cell | 6 |
| Other | 17 |
| Relapsed/refractory disease | 152 |
| Consolidation therapy | 12 |
| Platinum sensitive | 47% |
| Bulk at transplant | |
| NED | 11% |
| Microscopic | 12% |
| <1 cm | 31% |
| >1 cm | 46% |
| Median time from diagnosis to transplant | 16 months |
| Median follow-up as of 7/98 | 36 months |

## Responses

The median PFS for all 164 patients was 7.7 months, and their OS was 17.3 months, of whom 53% had platinum-resistant disease at the time of transplant. In keeping with our prior reports, those with platinum-resistant disease did poorly, with PFS and OS of only 5.6 and 10.5 months (Fig. 1). Unlike the survival reported in the analysis of our first 100 patients, the OS for those reported now with platinum-resistant, low tumor bulk (<1 cm) was only 12.0 months vs. 28 months.

The PFS and OS for those with platinum-sensitive disease was 11.7 and 30.8 months. For those with minimal bulk disease (<1 cm), the PFS was 16.0 months and the OS was 43.3 months (Fig. 2), both significantly higher than for those with platinum-sensitive bulky disease at 7.8 and 23.3 months, respectively. Approximately 40% of this group appear to be disease-free at a median follow up of 2 years, and approximately 20% are projected to be disease-free at 4–5 years. Similar to our initial report, the OS for those below the median age of 48 was 22.8 months vs. 13.5 months for those >48 ($P$=0.018).

Ten patients received high-dose chemotherapy with the paclitaxel, carboplatin, and mitoxantrone regimen as part of initial therapy. Five had stage IV disease, and

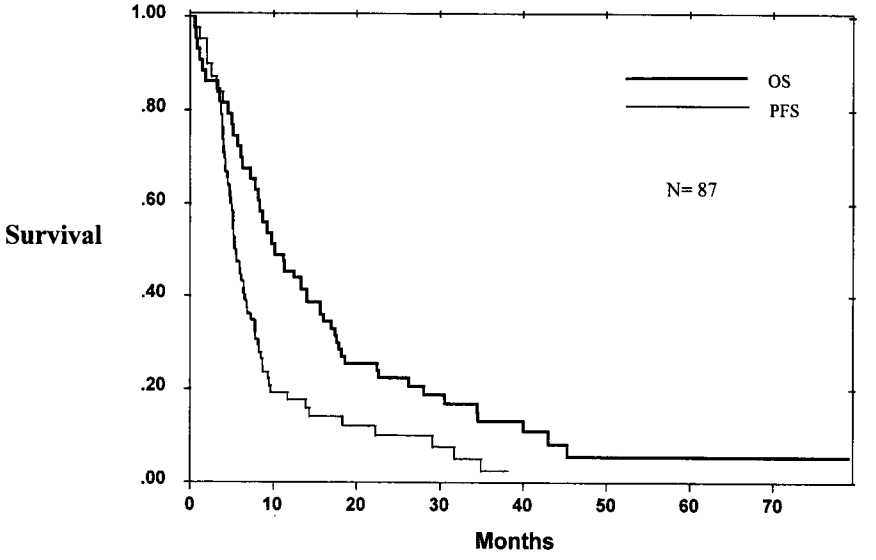


**Figure 1.** *Progression-free and overall survival of patients with platinum resistant ovarian cancer following an autologous transplant.*

four had suboptimal III disease at diagnosis. At a median time from diagnosis of 26 months, 65% are disease-free.

## Comparisons to conventional therapy

To date there have been no randomized trials comparing transplant to conventional therapy for patients relapsing after an initial remission. This is largely due to the fact that there is no curative conventional therapy available for this group. However, we have performed several comparisons using matched patients from our patient cohort to defined trials of conventional salvage therapy.[13,14] These comparisons support our conclusions of the benefit of transplant in patients with relapsed, chemosensitive, low-tumor-burden ovarian cancer. For patients in first relapse not previously exposed to paclitaxel, this agent administered in conventional doses for platinum-resistant disease gives a 4-month PFS[13], which is identical to a matched platinum-resistant group that received transplant. In contrast, those with platinum-sensitive disease have a 4.5-month PFS with paclitaxel[13] vs. 8 months with transplant. In addition, while all conventionally treated platinum-sensitive patients had relapsed by 20 months, 30% of the transplants were progression-free and at 3 years 18% remain progression-free.

A similar analysis was performed for those with sensitive disease whose first remission lasted more than a year before first salvage therapy was administered.

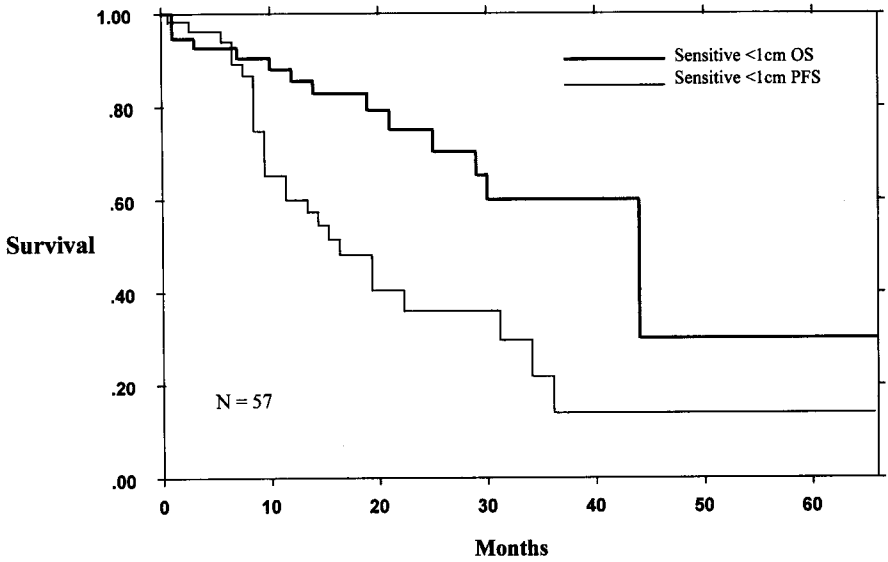


**Figure 2.** *Progression-free and overall survival of patients with platinum sensitive, minimal tumor burden ovarian cancer following an autologous transplant.*

Treated with either cyclophosphamide, doxorubicin and cisplatin or single agent paclitaxel, the median survival for these groups were 20 and 24 months, respectively. In contrast, a similar group undergoing transplant for platinum-sensitive disease whose initial remission lasted >12 months had a 93% survival at 22 months.

Finally, a comparison was made between transplant and conventional dose topotecan and paclitaxel for patients failing a single chemotherapy regimen.[7] These data for the two agents were taken from a study used to support the licensing of topotecan in the United States by the Food and Drug Administration (FDA) as second-line therapy for patients with advanced ovarian cancer.[7] Compared with either option, the PFS is longer for the 90 patients transplanted who met the same eligibility criteria in this 164-patient series (50.1 vs. 14.7 and 18.9 weeks for those treated with paclitaxel and topotecan, respectively). The OS was also higher for those transplanted: 97.4 vs. 63.0 and 53.0 weeks for the topotecan and paclitaxel groups.

## DISCUSSION

To determine the value of high-dose therapy, it is appropriate to compare the results to conventional options. As patients with relapsed disease have no chance for cure with conventional therapy, it is unlikely that a randomized trial will ever be performed for this group. The standard approach to relapsed disease is based on prior responsiveness to platinum, with sensitivity defined as a remission lasting >6

months off therapy. For this platinum-resistant group, a variety of agents may produce remissions, including paclitaxel,[7,13] topotecan,[7,8] liposomal doxorubicin,[15] oral low-dose etoposide,[16] and hexamethymelamine.[17] Remissions occur in approximately 20% and last 3–4 months, and survival from the onset of first relapse is 5–12 months. In contrast, those whose remissions lasted more than 6 months can expect to respond to platinum in the range of 35–40%. Remissions for these patients usually last 6–9 months, and survival averages 20–24 months. No conventional single agent or combination chemotherapy is superior to the reintroduction of platinum for this patient group.[13]

Our results indicate that for patients with recurrent platinum-sensitive, low-tumor-burden disease, transplant offers a superior overall survival to conventional options, with approximately 20% having long-term PFS. However, for those with platinum-resistant and, especially, bulky disease, transplant appears to offer similar PFS and OS to conventional therapies and, given its toxicity and costs, should not be generally recommended. Patients meeting the criteria of platinum-sensitive, low-tumor-burden disease then should be offered the option of transplant at the time of first relapse.

Regarding the comparisons reported here, the only significant difference between the transplant patients and those treated with conventional-dose chemotherapy is that the median age for the transplant patients was approximately 10 years younger. While we found that age was an important prognostic factor for survival of the transplant patients, it was not so for PFS. In addition, Duska et al.[18] recently reported that controlling for stage and tumor grade, the median survival for women in the reproductive age group with advanced ovarian cancer was not different from that for older patients. None of the patients described here were treated for low malignant potential tumors.

With the suggestion that platinum sensitivity and low tumor burden are important prognostic factors for patients undergoing transplants for this disease, it is possible that transplanting patients at an earlier point in their disease will yield superior results. The results with 10 patients described here treated with taxene-based therapy suggest that transplants are of value in this setting, with the best PFS reported for suboptimal III/IV disease with conventional platinum/taxene therapy at 18 months compared with the >26 months reported here. Several larger pilot studies have recently been reported that also describe transplants at the time of second-look surgery. The largest report to date is that by Legros et al.[19] Patients received chemotherapy with a platinum-based combination after debulking surgery, and after demonstrating platinum sensitivity, all were treated with high-dose chemotherapy with either melphalan at 140 mg/m$^2$ (23 patients) or carboplatin 1600 mg/m$^2$ and cyclophosphamide 6.4 g/m$^2$ (30 patients). At a median follow-up of >6.5 years, 23% are in continuous CR, and 45% are alive. Of 31 patients with no or only microscopic disease at second look, the disease-free survival at 5 years was 26.9%. For the 19

patients in this group with a negative second look, the 5-year disease-free survival (DFS) of 32.8% implies that the 12 patients with microscopic residual disease at second look have a similar DFS after the single transplant procedure. For those patients with bulky disease at the initiation of second-look surgery, the 5-year DFS was 19.2%, and the 5-year survival was 33.8%.

These data appear superior for patients with a positive second look laparotomy to that described for those treated with only conventional therapy after second-look surgery. Considering recent reports of survival after second-look surgery for optimal or suboptimal disease, and the fact that 42% of this group had suboptimal disease at diagnosis, we would expect the median survival for this group to be ~45 months treated with conventional therapy rather than the 66 months reported.

Given the improvements in the conventional therapy for this tumor and the patient selection biases inherent in these pilot studies of transplants as consolidation therapy, the true value of transplantation in the initial management of patients with advanced ovarian cancer will come only from randomized trials. Certainly, the preliminary results of our ongoing paclitaxel, mitoxantrone, and carboplatin trials would indicate that this therapy may be useful for this patient group, and in the absence of a comparative trial, should be considered. However, all eligible patients should be enrolled in any available comparative phase III trial. Under the auspices of the National Cancer Institute (NCI), the GOG as well as SWOG, CALGB, and ECOG recently began a randomized phase III trial. Patients with stage III or IV disease who respond to four to six cycles of a platinum-based regimen after debulking surgery and have a clinical CR (suboptimal III and IV) or a PR documented by a second-look laparotomy will be randomized between six cycles of paclitaxel (175 $mg/m^3$ over 3 hours) combined with carboplatin at an AUC of 7.5 and a single transplant using the carboplatin, mitoxantrone, cyclophosphamide regimen described by our program. The trial, which is expected to take 5 years, will include those initially debulked suboptimally including those with a pathologic CR in view of their high expected relapse rate. Accrual has been slow primarily because of the slow acceptance of this form of therapy by patients and their physicians. Also, some transplant physicians feel that no conventional therapy is acceptable for patients with a disease that has such a poor prognosis. Efforts to improve the understanding of this therapy by patients and physicians and a clear explanation of its rationale should help to increase accrual. Unlike the breast cancer trials, the goal was to initiate this trial before the publication of extensive pilot data indicating its effectiveness, which would result in patients demanding this therapy at all costs. Fortunately, the insurance industry has in large measure supported this trial, as it has the breast cancer transplant trials.

Alternative approaches such as that reported by Fennelly et al.[20] that explore double-dose chemotherapy in rapid succession at the time of initial diagnosis with stem cell rescue (dose-dense), followed by a single cycle of high-dose melphalan,

have been reported. However, in lieu of standard-dose chemotherapy followed by a myeloablative regimen, this form of therapy has not been successful in increasing the number of patients with a pathologic CR at the completion of the treatment. Whether alternative regimens given shortly after diagnosis will increase the pathologic CR rate is yet to be determined but is an attractive possibility.

In summary, it appears that high-dose chemotherapy may improve the survival of patients both when used after relapse or as part of initial therapy. This type of therapy is the only treatment available to date that provides a long-term PFS of periods up to 4–5 years when used for patients with relapsed platinum-sensitive low-tumor-burden disease. In addition, pilot data are suggestive of an improvement in the PFS or OS of patients transplanted with responding first-remission disease. The benefit for this group of patients should be verified in an ongoing randomized trial and not be arbitrarily employed in subjects.

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# High-Dose Chemotherapy in the Management of Germ Cell Tumors

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## ABSTRACT

Although the majority of patients with disseminated germ cell tumors (GCT) are cured with first-line therapy containing cisplatin, etoposide, and bleomycin (BEP), 20–30% of patients are not cured with this treatment. The use of vinblastine, ifosfamide, and cisplatin (VeIP) as a second-line therapy cures about 30% of patients. High-dose chemotherapy (HDCT) is used early in the management of germ cell tumors in two settings: first, as initial salvage therapy, and second, as first-line therapy in patients with newly diagnosed poor-prognosis disease. The latter is given in an intergroup trial that compares the efficacy of two cycles of BEP followed by two cycles of high-dose carboplatin, etoposide and cyclophosphamide with autologous bone marrow (BM) or peripheral blood progenitor cells (PBPC) to four cycles of BEP. This trial is accruing well, with >90 patients enrolled to date. Earlier observations from a single institution demonstrated the feasibility of this approach. When the outcome of HDCT was compared with that of a historical group treated with conventional therapy, there was a significant advantage to using HDCT. With regard to rescue of HDCT as initial salvage therapy, we recently reported our results on 49 patients. After patients received zero to two cycles of cytoreductive therapy consisting of VeIP or a similar regimen, autologous BM ($n$=10) or PBPC were collected. All patients were treated with two cycles of etoposide (750 mg/m$^2$) and carboplatin (700 mg/m$^2$) a day for 3 consecutive days. There was no treatment-related mortality. At a minimum follow-up of 12 months, 51% of patients are continuously disease-free. Eleven of the 17 patients (65%) who achieved complete remission with initial chemotherapy are continuously disease-free, compared with nine of 20 patients (45%) with partial remission (PR) and negative markers and four of 11 patients (36%) with PR and positive markers. In contrast to the 69% of patients with good prognostic factors, only 18% of those with intermediate prognostic factors are continuously disease-free. These data indicate that high-dose chemotherapy is effective as initial salvage chemotherapy and has acceptable toxicity.

## INTRODUCTION

With >7600 new cases expected to be diagnosed in 1998, germ cell tumors are the most common cancer among young people between the ages of 15 and35 years.[1] Fortunately, the introduction of cisplatin-based combination therapy 20 years ago led to a high rate of cure; those who present with minimal to moderate dissemination can expect a cure rate of >95%.[2,3] However, of the patients presenting with more advanced disease, only 70% are cured with first-line therapy.[4] Second-line salvage therapy, which includes vinblastine, ifosfamide, cisplatin with or without cyclophosphamide, and dactinomycin (VIP or VAB-6), is associated with only a 25–30% cure rate.[5,6]

Recently, third-line salvage therapy, high-dose chemotherapy, has shown a cure rate of 20–25%.[7–9] Although HDCT in these heavily pretreated patients is associated with a high mortality rate (15–20%), the introduction of cytokines and the use of PBPCs in the treatment of high-risk germ cell tumors at an earlier stage have resulted in reduced treatment-related mortality.

HDCT is being evaluated in two settings. First, several groups are involved in a study using HDCT as first-line therapy in the management of patients who historically have had low rates of cure. Second, we are testing HDCT as an initial salvage therapy for those with relapsed or refractory disease after first-line cisplatin-based regimens.

## HIGH-DOSE CHEMOTHERAPY AS INITIAL THERAPY FOR POOR-RISK PATIENTS

Since germ cell tumors are highly responsive to chemotherapy, and a dose-response relationship has been suggested for etoposide, cisplatin, and more recently, carboplatin, it is logical to use such agents at higher doses for patients with poor-prognosis disease. The early use of HDCT in this setting is intended to bring the cure rate in line with that achieved for good-prognosis patients. Employing this strategy in this group of patients is possible because of their young age and ability to tolerate HDCT.

A recently published study from the Medical Research Council and the European Organization for Research and Treatment of Cancer tested the hypothesis that more intensive chemotherapy early in the management of patients with poor-prognosis nonseminomatous germ cell tumor may increase survival.[10] This was a randomized trial that included 380 patients and accrued patients between May 1990 and June 1994. The first arm of the treatment consisted of three cycles of bleomycin, vincristine, and cisplatin at 10-day intervals (BOP) followed by three cycles of etoposide, ifosfamide, and cisplatin (VIP) with additional bleomycin after BOP and twice in each cycle of VIP. In the second arm, four cycles of BEP were

followed by two cycles of EP. More complicated and toxic intensive regimens did not improve cure rate, failure-free survival, or overall survival ($P$=0.687, $P$=0.101, and $P$=0.190). In addition, the 5-year progression-free survival for those in the poor-prognosis group based on the international criteria was only 41%, with a 1-year failure-free survival of 49%. Many researchers speculated that the dose intensity in this study was insufficient to improve the cure rate, and that higher doses might yield greater success.

Investigators at Institute Gustave-Roussy (IGR) have conducted a phase III trial testing the addition of HDCT to conventional-dose induction therapy for patients with untreated poor-risk germ cell cancer.[11] 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 × two cycles followed by high-dose intensification with PEC.[12] Preliminary results suggest no benefit to patients receiving high-dose intensification. Of 49 patients randomized to receive PVeBV × four, there were two early deaths and one refusal. Complete response was obtained in 30 of the 49 (61%), and 82% of patients were 2-year survivors. Of 53 patients randomized to receive two cycles of modified PVeBV plus consolidation, there were eight early deaths and two refusals. Complete response was obtained in 21 of the 53 (41%), and 61% of patients were 2-year survivors. A 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 intensive dose 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, the role of more intensive therapy in poor-prognosis patients remained to be defined.

The Memorial Sloan Kettering Cancer Center group investigated initial high-dose chemotherapy in two consecutive trials. Patients were given conventional chemotherapy (VAB-6), and those who showed a suboptimal decline in serum human chorionic gonadotropin (HCG) or alpha-fetoprotein (AFP) after two to three cycles of treatment were given high-dose carboplatin and etoposide with autologous marrow support.[13] The majority of patients entered in the protocol had required transplantation. This trial presented evidence of improved outcome compared with a similar group from earlier trials. In the first trial, 16 patients were treated with high-dose carboplatin and etoposide after suboptimal response to VAB-6. Fifty-six percent obtained complete remission, and 50% remained free of disease, with a median event-free survival of 68 months. These reports were more promising than those of a similar prognostic group treated with VAB-6 alone, in which only 17% had a durable response.

A more recent extension of this trial at Memorial Sloan Kettering enrolled a similar population of poor-risk patients with sluggish declines in serum markers.[14] Patients with poor-risk features were begun on VIP; if markers failed to decline by the 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 16 received VIP alone. Fourteen had conventional therapy truncated and moved to HDCT due to poor marker decline. Overall, 15 of the 30 (50%) remain progression-free, a significantly better response rate than that of a historical group of poor-prognosis patients from the same institution. Again, whether this represents a therapeutic advance is being assessed in a randomized clinical trial in poor-risk patients. A large intergroup trial has been started in the United States, enrolling patients with poor-prognosis disease defined by the new International Prognostic System and randomizing them to receive either standard therapy (BEP $\times$ four) or high-dose therapy (BEP $\times$ two followed by carboplatin, etoposide, and cyclophosphamide $\times$ two). This trial is ongoing; whether it supports or refutes the role of HDCT in patients presenting with poor-risk disease remains to be seen.

## INITIAL SALVAGE THERAPY

Since the overall cure rate for recurrent germ cell tumor with ifosfamide-cisplatin based therapy is 20–25%, the proper next investigative step is incorporation of HDCT as a component of initial salvage therapy.[15] 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. Only six of the 25 patients enrolled did not enter the transplantation portion of the protocol. Overall, eight patients obtained a CR, 14 PR, two stable disease, and one progressive disease. There was only one therapy-related death. With a median follow-up of 18 months, 14 of the 25 (56%) remain progression-free. Three additional patients who progressed after protocol treatment are disease-free after subsequent surgeries or additional chemotherapy. Although it was unclear whether these results were superior to conventional salvage approaches since these patients were highly selected, the excellent tolerance of therapy and the high response rate were encouraging and set the stage for subsequent trials.

A recent study from our institution yielded more insight into the role of HDCT as an initial salvage therapy.[16] Forty-nine patients with relapsed or refractory testicular cancer were treated with salvage therapy that included HDCT between August 1992 and November 1996. Patients with extragonadal primaries were excluded because of our prior work showing no benefit with this therapy for this

**Table 1.** Patient characteristics

| | |
|---|---|
| No. patients | 49 |
| Median age | 30 (16–47) |
| Initial therapy | |
| BEP | 42 |
| PVB | 3 |
| VIP | 2 |
| others | 2 |
| Response to initial therapy | |
| CR | 17 |
| PR/Marker negative | 19 |
| PR/Marker positive | 9 |
| Refractory to cisplatin | 4 |
| Cytoreductive regimen | |
| VeIP | 36 |
| PVB | 3 |
| VIP | 2 |
| BEP | 2 |
| None | 6 |

group. Forty-three of these patients received cytoreductive therapy before HDCT (Table 1). HDCT consisted of carboplatin 700 mg/m$^2$ and etoposide 750 mg/m$^2$ daily for 3 consecutive days on days –6, –5, and –4. Hematopoietic cells were infused on day 0; their source was bone marrow (BM) in 10 patients, BM and PBPCs in nine patients, and PBPC in the remaining patients. A second course of HDCT was given 7–10 days after hematopoietic recovery. The median patient age was 30 years (range 16–47), and the majority of the patients (31) had mixed nonseminomatous disease or pure choriocarcinoma. All patients received a prior cisplatin-based regimen, with 42 patients receiving BEP.

The response to prior therapy was complete in 17 patients, partial with normalization of the marker in 19 patients, and partial with a positive marker in nine patients. Four patients were refractory to cisplatin and progressed on treatment. The response to HDCT was complete in 24 patients (49%). Eight patients showed no evidence of active disease and on posttherapy surgery had only teratoma. Ten patients had partial resolution of their radiographic abnormality with normalization of HCG or AFP. The last seven patients had either stable disease or progressed during HDCT. Of those who achieved complete response, 75% remained free of recurrence, and of the six patients who recurred, two were cured with subsequent surgery. On the other hand, of those who achieved a partial response and a negative marker, only 20% remained free of recurrence. Moreover, none of the patients whose HCG or AFP remained positive or progressed during HDCT were cured. It

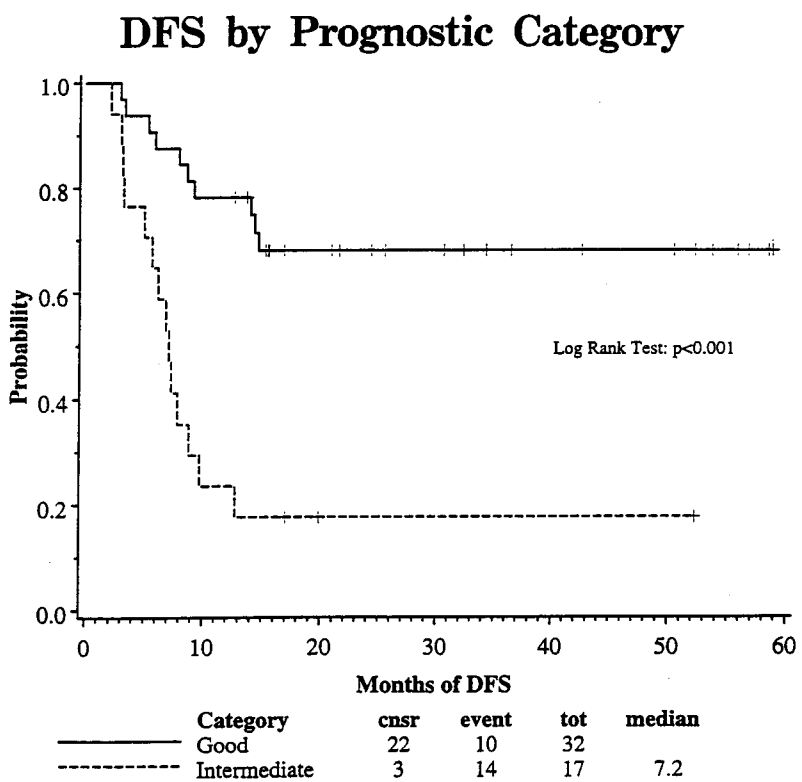


**Figure 1.** *Disease-free survival based on prognostic category. Scores were calculated by giving 1 point each for progressive or refractory disease before HDCT and 2 points for absolute refractory disease or HCG >1000 before HDCT.* [17] *Thirty-two patients had a score of 0 (good risk category), and 17 patients scored 1–2 ( intermediate risk group). Median survival was not reached for the good category group.*

is of interest that six of the nine patients who had a partial response to first-line therapy and persistent elevation of their tumor marker were cured after HDCT, while one of the four patients who were cisplatin refractory was cured. Based on the multivariate analysis of prognostic factors, 32 patients were in the good category group and 17 patients were in the intermediate group (Fig. 1) According to this prognostic model, 1 point is given for progressive or refractory disease before high-dose chemotherapy and 2 points for absolute refractory disease or HCG >1000 before HDCT. Of the 32 patients in the low-risk category, 72% are currently free of disease compared with only 29% of those in the intermediate category. Ninety-one of the 98 planned cycles of HDCT were given with limited toxicity. Febrile neutropenia was seen in 58% of the cycles and documented infection in 18% of the cycles. Grade 3–4 gastrointestinal toxicity rate was 39%

(mucositis 39%, vomiting 31%, and diarrhea 29%). Nine patients developed renal toxicity, and one patient required short-term dialysis. Twelve patients developed significant ototoxicity and peripheral neuropathy.

In conclusion, we report that including HDCT as a salvage treatment for relapsed germ cell tumors yields a high cure rate, with 51% of patients remaining free of disease and 57% currently free of disease with a minimal follow-up of 14 months. However, those with refractory disease had a cure rate of only 18%. Thus, it appears that high-dose chemotherapy can be employed safely and effectively as initial salvage therapy for relapsed disease.

## SUMMARY

Whether HDCT will play a major role in the management of untreated poor-prognosis patients or simply remain a minor option for rare patients failing multiple chemotherapy is currently being defined by a large multicenter study. However, high-dose chemotherapy has been shown to have a major role in the management of relapsed patients who do not have extragonadal primary GCT. Because treatment of patients presenting with cisplatin-refractory disease remains problematic, further research is needed to understand the basis for such resistance and to explore new treatment options.

## ACKNOWLEDGMENTS

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# Three-Fold Intensification by Sequential High-Dose Chemotherapy for Small Cell Lung Cancer: A Multicenter Phase II Study

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## ABSTRACT

In a phase II multicenter study supported by the European Group for Blood and Marrow Transplantation (EBMT), we were able to demonstrate the feasibility of administering sequential high-dose ifosfamide, carboplatin, etoposide (ICE) chemotherapy with circulating progenitor cell rescue to patients with small cell lung carcinoma. Sixty-nine patients were included and the majority (71%) received the three courses of intensive chemotherapy. Toxicity was tolerable, with six toxic deaths. The complete remission rate was 49% for the whole group and 68% in limited-disease patients. Median overall survival was 19 months in limited-disease patients compared with 11 months in patients with extensive disease ($P$=0.001). Based on these results, a randomized trial is now ongoing in various European centers comparing standard chemotherapy to sequential high-dose ICE.

## INTRODUCTION

More than 25 years ago, small cell lung carcinoma (SCLC) was shown to be a distinct histopathologic entity from other lung carcinoma cell types, based on a high-dose fraction, a short doubling time and a high metastatic potential.[1] This finding provided the basis for testing the use of chemotherapeutic agents. Even if different regimens appeared to yield similar therapeutic results, the combination of cyclophosphamide, adriamycin, vincristine (CAV) became standard, as has the current combination of cisplatin and etoposide (PE).[2,3] These regimens appeared equivalent when properly compared and led to a 5-year long-term survival of 3–4%.[4,5] Any new treatment strategy should focused on improving long-term

**Table 1.** Results of four randomized trials (2-year survival data)

| *Author (year)* | *Regimen* | *DI (%)* | *No. patients* | *No. LD* | *No. ED* | *CR (%)* | *RR (%)* | *OS (months)* | *2-year survival (%)* |
|---|---|---|---|---|---|---|---|---|---|
| Arriagada | CAEP | — | 50 | 50 | — | 54 | | 13 | 26 |
| (1993) | CAEP | 25–33* | 50 | 50 | — | 67 | | 17 | 43 |
| Woll | V-ICE | — | 31 | 28 | 3 | 58 | 93 | 16 | 15 |
| (1995) | V-ICE | 7 | 34 | 32 | 2 | 56 | 94 | 17 | 32 |
| Steward | V-ICE | — | 153 | 85 | 68 | 51 | 77 | 12.5 | 18 |
| (1998) | V-ICE | 26 | 147 | 93 | 54 | 51 | 90 | 16 | 33 |
| Thatcher | ACE | — | 202 | | | | 86 | 11 | 39† |
| (1998) | ACE | 33 | 201 | | | | 89 | 12.5 | 47† |

**First cycle only. †At 1 year. DI, increase in dose intensity; OS, median overall survival.*

survival rate. It is not known yet if high-dose chemotherapy will have a significant long-term impact, but the 2-year survival data of four randomized trials testing the early intensification strategy all showed an improvement compared with the standard regimen[6–9] (Table 1). Interestingly enough, neither the overall response rate, the complete remission rate, nor the median survival were predictive of the 2-year survival improvement. Furthermore, these results were obtained by only a small increase in dose intensity, from 7 to 33%. Within the EBMT, it was proposed that a higher increase in dose intensity might lead to a higher increase in long-term survival and that only a randomized trial could prove it. To prepare for such a trial, a phase II study was designed to test a threefold intensification of dose and was conducted from March 1994 until October 1997. It aimed to test the feasibility of an intensive approach within a multicenter setting.[10]

## RESULTS

Sixty-nine patients were included in the study (Table 2). Median age was 53 years (range 35–65), and median performance status was 0 (range 0–1). There were 58 men and 11 women. Thirty patients (43.5%) were diagnosed as having limited disease and 39 patients (56.5%) had extensive disease, of whom 27 had more than two metastatic sites (69%). Abnormal lactate dehydrogenase (LDH) level was observed in 30 patients. Twenty-five percent of patients with extensive disease had metastases in liver, 15% in bone, 14% in distant lymph nodes, 13% in bone marrow, 11% in adrenal glands, and 3% in brain.

### Mobilization/collection phase

Mobilization of progenitor cells (PBPCs) was performed with high-dose epirubicin (Farmorubicin) 75 mg/m$^2$/d intravenous bolus on days 1 and 2, followed

**Table 2.** Patient characteristics

| | |
|---|---|
| *n* | 69 |
| Sex (M/F) | 58/11 |
| Median age (years) | 53 (35–65) |
| PS (0/1) | 35/34 |
| LDH (normal/abnormal) | 36/30 |
| Stage (limited/extensive) | 30/39 |
| Number of metastatic sites | |
| 1 | 12 (31%) |
| 2–3 | 19 (49%) |
| 4–5 | 8 (20%) |

by filgrastim 5 μg/kg/d subcutaneously. Grade IV infection occurred in two patients, resulting in toxic death in one of them, due to septic shock. Another patient died during the mobilization phase due to cardiac failure. Leukaphereses were performed after a medium of 11 days (range 8–14). After a median of three leukaphereses (range 1–5), $3.6\times10^8$ mononuclear cells/kg (range 2.2–21.3) and $16.6\times10^6$ CD34 cells/kg (range 1–96) were collected.

## ICE chemotherapy

The intensification program consisted of the high-dose ICE regimen (ifosfamide 10 g/m$^2$/d as a 17-hour continuous infusion, carboplatin 300 mg/m$^2$/d as a 3-hour infusion, and etoposide 300 mg/m$^2$/d as a 3-hour infusion were given on days 1 to 4). The first intensive course was given starting within 21 days from the beginning of mobilization and repeated every 28 days three times. PBPCs were reinfused 48 hours after the end of high-dose chemotherapy, and the cycles were followed by filgrastim 5 μg/kg/d subcutaneously until leukocyte recovery. Chest radiotherapy and prophylactic cranial irradiation were left to center policy but were recommended in complete responders on chemotherapy completion.

All three cycles of high-dose ICE could be administrated in 49 patients (71%). Toxicity was the main cause of program discontinuation. ICE toxicity was analyzed in 172 cycles (Table 3). Severe mucosal toxicity was observed in 10.5% and gastrointestinal toxicity with abdominal pain and diarrhea in 14.4% of the cycles. Moderate to severe nausea and vomiting occurred in 20% of the courses. Severe infection complicated 13% of the cycles, with four patients dying of septic shock. The six toxic deaths (during mobilization and high-dose ICE) occurred most predominantly during the early phase of the trial, before prophylactic use of antibiotics during neutropenia was recommended.

Hematologic recovery time (calculated from day 1 of PBPC reinfusion) was 9 days (4–12) to reach $\geq1\times10^9$/L leukocytes and 9 days (5–27) to reach $\geq20\times10^9$/L

**Table 3.** ICE toxicity (172 cycles)

| | *WHO grade* | | | | | |
|---|---|---|---|---|---|---|
| | *0* | *I* | *II* | *III* | *IV* | *% III+IV* |
| Mucositis | 94 | 33 | 27 | 16 | 2 | 10.5 |
| Diarrhea | 80 | 37 | 30 | 21 | 4 | 14.5 |
| Nausea/vomiting | 38 | 47 | 51 | 34 | 2 | 21 |
| Neurologic | 150 | 14 | 3 | 4 | 1 | 3 |
| Otological | 162 | 4 | 5 | — | 1 | 0.5 |
| Cardiac | 162 | 4 | 2 | 4 | — | 2 |
| Infection | 119 | 7 | 24 | 17 | 5 | 13 |

platelets with no difference between cycles 1 and 3. The platelet or red cell transfusion rate was similar when comparing cycle 1 and 3. Febrile neutropenia occurred in 117 of the 172 cycles (68%). Overall, intravenous antibiotics were prescribed during 9 days (range 0–62); the median duration of hospital stay was 20 days (5–75).

### Response rate and survival

Sixty-seven patients were evaluable for response (two were too early). After high-dose chemotherapy, 33 patients (49%; 95% confidence interval [CI] 37–62%), achieved a CR or near CR according to Dana Farber Cancer Institute criteria.[11] Twenty-four patients achieved a PR, resulting in overall response rate of 85% (95% CI 74–93%). Response rate was also analyzed according to established prognostic factors such as disease extension, performance status, sex, liver metastases, and elevated LDH (Table 4). Disease extension was the only significant predictive factor for response, with 68% of the patients with limited disease achieving complete response compared with only 36% of the those with extensive disease ($P$=0.01).

Median OS was analyzed according to disease extension. In limited-disease patients, the OS was 19 months compared with 11 months in patients with extensive disease ($P$=0.001) (Fig. 1). Survival was also analyzed according to established prognostic factors. Patients with LDH below normal values had a significantly better overall survival compared with the patients with abnormal values (median OS: 17 months vs. 11 months; $P$=0.001).

## CONCLUSIONS

This phase II multicenter study supported by the EBMT demonstrated the feasibility of administering sequential high-dose ICE chemotherapy with PBPC

**Table 4.** Complete response

| | *No. patients* | *CR* | *%* | *P* |
|---|---|---|---|---|
| Stage | | | | 0.01 |
| Limited | 28 | 19 | 68 | |
| Extensive | 39 | 14 | 36 | |
| PS | | | | 0.18 |
| 0 | 33 | 19 | 58 | |
| 1 | 34 | 14 | 41 | |
| Sex | | | | 0.35 |
| Female | 11 | 4 | 36 | |
| Male | 56 | 29 | 52 | |
| Metastases | | | | 0.86 |
| Liver | 16 | 6 | 37 | |
| Other | 23 | 8 | 25 | |
| LDH | | | | 0.13 |
| Normal | 36 | 21 | 58 | |
| Abnormal | 28 | 11 | 39 | |

rescue in patients with small cell lung cancer. The majority of the patients (71%) could receive the three courses of intensive chemotherapy. Toxicity was tolerable, with six toxic deaths (9%) observed mostly at the beginning of the study. The complete remission rate was 49% for the entire group and 68% in limited-disease patients. With a median survival of 19 months in limited-disease patients, the high-dose ICE regimen compares favorably with the best results obtained using other

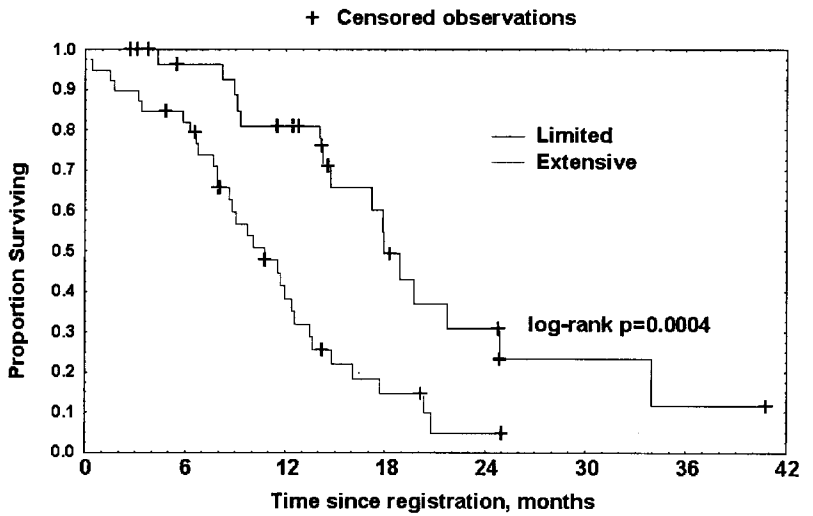


**Figure 1.** Overall survival according to disease extension.

intensive treatments. These results justified a phase III EBMT study, comparing six cycles of standard ICE to intensive sequential high-dose chemotherapy with PBPC support. It will test a doubling in 3-year survival. This randomized trial is now ongoing in various European centers.

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# High-Dose Therapy for Small Cell Lung Cancer With Stem Cell Support

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## INTRODUCTION: RATIONALE FOR DOSE-INTENSIVE THERAPY IN SCLC

Small cell lung cancer (SCLC) histology constitutes approximately 15–25% of all bronchogenic carcinomas and represents the fourth leading cause of death from cancer in both men and women in the United States.[1] Patients invariably present with systemic metastatic disease, overt in two-thirds (extensive stage [ED]) and subclinical in a third (limited stage [LD]). Combination chemotherapy achieves excellent immediate palliation when using the many chemotherapeutic agents which have major activity against SCLC. However, combination regimens constructed from these established agents (etoposide or teniposide, cisplatin or carboplatin, ifosfamide, cyclophosphamide, vincristine, and doxorubicin) produce similar short- and long-term results. Consensus conventional-dose treatment consists of four to six cycles of etoposide and cisplatin or carboplatin with concurrent chest radiation therapy for the third of patients with limited-stage disease[2] and combination chemotherapy alone for extensive-stage disease. Complete response rates range from 50 to 70% for LD and 15 to 30% for ED patients. By 2 years, however, 20–40% of LD and <5% of ED patients remain alive.[3,4] Five-year survival is about half that at 2 years. New agents with promising activity include paclitaxel, gemcitabine, and the topoisomerase I inhibitors (topotecan, irinotecan). The role of these new agents are being evaluated in ongoing first-line therapy trials. Even though ifosfamide improved survival in ED patients in conjunction with platinum and etoposide, this lead has not been tested in LD patients.

## DOSE INTENSITY: WITHOUT CELLULAR SUPPORT

Near log-linear dose-response relationships are consistently demonstrated for the alkylating agents and radiation in preclinical in vitro and in vivo experiments.[5–8] The impact of dose or dose intensity of chemotherapy on clinical outcome remains controversial in the clinic. Analysis of dose intensity (expressed in drug dose administered per $m^2$ per week) of individual agents or regimens delivered in numerous SCLC trials using the methodology of Hryniuk and Bush[9] indicated that higher dose intensities of cyclophosphamide and doxorubicin with vincristine

(CAV) and with etoposide (CAE), but not etoposide and cisplatin (EP), were associated with a longer median survival in extensive-disease patients. The relative range of doses administered and response and survival advantages were modest.[10]

Seven randomized trials have tested the role of dose intensity in SCLC, almost exclusively in the extensive-stage setting.[11–17] The actual delivered doses when reported were significantly less different between the arms than the planned dose intensity differences (1.2- to 2-fold). Three of these trials showed a modest survival advantage for the higher-dose therapy. Arriagada et al. treated LD patients with six cycles of conventional dose chemotherapy wherein the first cycle only was randomly assigned conventional dose vs. modest intensification.[17] A complete response and survival advantage for the patients receiving the intensified chemotherapy was remarkable, since the relative difference in the two groups was so small. While this result could reflect chance, it is possible that dose intensity, particularly if given early in the course of treatment, may be more effective in the limited- rather than the extensive-stage setting. Early intensification and treatment of earlier stage disease are two themes to consider when designing new trials.

Multidrug cyclic weekly therapy was designed to increase the dose intensity of treatment by taking advantage of the differing toxicities of the weekly agents. Although patient selection effects were evident, early phase II results were quite promising.[18,19] No survival benefits were documented in the randomized trials,[20–23] perhaps related to the greater dose reductions and delays required for the weekly schedules compared with every-3-weeks conventional therapy, thus the actual delivered dose intensities were not that different. Moreover, not only were doses and schedules varied, but so were the regimens, leading to interpretation obstacles. Follow-up is still too limited to evaluate late disease-free survival plateau differences.

Currently established cytokines (e.g., granulocyte and granulocyte-macrophage colony-stimulating factors [G-CSF and GM-CSF]) were able to maintain dose intensity across multiple cycles[24] without demonstrable survival advantage. With cytokine use, a modest increment in dose intensity, limited by cumulative thrombocytopenia, can be achieved (1.5- to 2-fold). The effectiveness of various thrombopoietins or other cytokines to increase achievable dose intensity remains uncertain. The underlying cardiovascular and pulmonary comorbidity, median age of 60–65 years, and enhanced risk of secondary smoking-related malignancies inherent in lung cancer patients contribute to an increased risk when applying dose-intensive therapy.

## DOSE INTENSITY: WITH CELLULAR SUPPORT

This summary includes older trials of patients with SCLC undergoing autologous bone marrow transplantation (autoBMT) if specifics about their response status (relapsed or refractory; untreated; or responding to first-line

chemotherapy [partial or complete response]) and their extent of disease (limited or extensive stage) were provided in the published reports. While not part of a formal meta-analysis, patients in these various categories were pooled for aggregated relapse-free and overall survival characteristics.

Fifty-two patients with relapsed or refractory disease achieved complete and partial responses in 19 and 37%, respectively, in 14 small studies.[25–38] The median response durations and survivals were about 2–4 months. Combination chemotherapy regimens, especially those containing multiple alkylating agents, were slightly more effective (response rate 58%, CR 26%), but more toxic (18 vs. 6% deaths). The high complete response rate substantiates a dose-response relationship, but was insufficient for cure.

One hundred three patients with untreated SCLC (71% limited disease) received single- or double-cycle high-dose therapy as initial treatment. Overall and complete response rates of 84 and 42% were achieved.[39–46] Relapse free, 2-year, and overall survivals were comparable to treatment with conventional multicycle regimens. Seven percent achieved durable remissions. Transplantation in the newly diagnosed SCLC setting is potentially hazardous because of the frequency of life-threatening complications from uncontrolled disease and the likelihood of tumor cell contamination in untreated autografts. On the other hand, early intensification may have greater impact on the disease.

Approximately 344 patients in response to first-line chemotherapy received high-dose chemotherapy with autologous marrow support as consolidation.[47–63] Conversion from partial to complete response occurred in 40–50%, but without durable effect. In patients with limited disease in complete response at the time of high dose therapy, 35% remained progression-free at a median follow-up >3 years at the time of publication.

One randomized trial has been reported.[60] Of 101 patients with SCLC who received five cycles of conventional chemotherapy with prophylactic cranial irradiation (PCI), 45 (45%) were eligible for randomization to one further cycle of either high- or conventional-dose therapy using cyclophosphamide, etoposide, and carmustine.[60] Dose-response was proven. Conversion from partial to complete response occurred in 77% of evaluable patients after high-dose therapy, and in none after conventional-dose treatment. Disease-free survival was significantly enhanced, and a trend toward improved survival was observed with high-dose therapy. However, overall outcomes were poor, and an 18% toxic death rate in the autoBMT arm led the investigators to conclude that dose-intensive therapy should not be considered a standard therapy in SCLC. Almost all patients recurred in the chest, reflecting the fact that chest radiotherapy was not given in this trial.

High rates of relapse in sites of prior tumor involvement may be expected, due to the greater tumor burden and/or drug-resistant clones in the chest by poorer drug delivery and/or intratumoral resistance factors such as hypoxia in areas of bulk

tumor, or in the case of autograft contamination, the possibility of homing with microenvironmental support for the tumor in local-regional sites.[41,56] After conventional-dose therapy, chest relapse is reduced from 90 to 60% with 50 Gy radiotherapy. Thus, high-dose curative treatment approaches should include radiotherapy to sites of bulk disease.

Much of the high dose SCLC experience just reviewed occurred during the initial developmental phase of high-dose therapy for solid tumors. The majority of trials employed either single high-dose chemotherapeutic agents (with or without low dose agents in addition) (six trials; two with chest radiotherapy),[41,42,48–53] or single alkylating agents (six trials; four with chest radiotherapy).[42,44,47,54–57] Combination alkylating agents were employed in a minority of patients (10 trials; six with chest radiotherapy).[29,32,43,58–64] Dosing was suboptimal compared with modern standards. Treatment-related morbidity and mortality were higher than currently expected.

## DOSE INTENSITY: NEWER REPORTS USING CELLULAR SUPPORT

A number of new and updated experiences have been published since high-dose therapy for SCLC was last reviewed and are summarized below.[46] In one study, six of 10 partial responders with ED were transplanted with high-dose methotrexate and etoposide after high-dose cyclophosphamide for mobilization. All achieved near complete response but relapsed a median of 4 months later. Half had tumor contamination of their peripheral blood progenitor cells (PBPC) documented while in response.[53]

In the updated Polish experience, six LD and 20 ED patients received two cycles of high-dose cyclophosphamide and etoposide as induction followed by the same drugs in six or with BCNU in 20.[63] Seven patients were already in complete response and an additional seven of 18 achieved CR. Five patients remain progression free 3 to 89 months later. Twenty-nine percent of complete responders remained disease free >2 years.[63]

Thirteen of 18 LD patients (72%) received high-dose ifosfamide, carboplatin, and etoposide (ICE) with epirubicin as consolidation after two cycles of mobilization chemotherapy.[64,65] Event-free survival was 69% (median follow-up was 14 months). Nine (50%) remained progression-free. About 25% had stage I or II SCLC, and surgical resection was performed in seven patients. None of the PBPCs collected after the second cycle of mobilization chemotherapy contained microscopic tumor cells as measured by immunocytochemistry using keratin and ENM-125 antibodies. Further follow-up at 44 months had an event-free and overall survival of 56%.[65]

At the Dana Farber Cancer Institute and Beth Israel Deaconess Medical Center, more than 55 patients with limited-stage and more than 30 with extensive-stage

SCLC have been treated with high-dose combination alkylating agents after response to conventional-dose induction therapy. Of the original cohort of 36 limited-stage SCLC (all had stages IIIA or B disease), 29 were in or near complete response before treatment with high-dose cyclophosphamide, carmustine, and cisplatin (CBP) with marrow ± PBPC support followed by chest and PCI.[62,66] For this group, the 5-year event-free survival is 53% (minimum follow-up 40 months, range to 11 years). Multivariate analysis suggests that response to induction is most important (CR or near CR vs. PR), but that short induction (four cycles or fewer) and the use of ifosfamide during induction also impart better prognosis. Of the extensive-stage patients, 17% remain progression free >2 years after high-dose therapy. Local regional relapse represents about 50% of all relapses.

## FUTURE DIRECTIONS

### Intensify involved field radiotherapy

Meta-analyses of randomized trials indicate that for LD SCLC, thoracic radiotherapy (TRT) provides a 25–30% improvement in local-regional control and a 5% increase in long-term progression-free survival for limited-stage SCLC.[67,68] Local-regional relapse remains unacceptably high (about a 60% actuarial risk of local relapse by 3 years) with the routinely given 45–50 Gy TRT,[69–71] and may be underestimated due to the competing risk of systemic relapse.[72] Further enhancement of local-regional control might increase the proportion of long-term survivors.

Dose intensity of chest radiotherapy has not been sufficiently studied. The Eastern Cooperative Oncology Group (ECOG) and the Radiation Therapy Oncology Group (RTOG) compared 45 Gy TRT given either daily over 5 weeks or twice a day over 3 weeks concurrent with cisplatin and etoposide chemotherapy.[73] Two-year local-regional failure was reduced from 61 to 48% with the more intense TRT. With extended follow-up, a survival advantage for the more intensive radiotherapy is observed.[74] In a prolonged phase I CALGB trial, Choi et al. escalated the dose of TRT in cohorts of five to six patients with limited-stage SCLC.[75] Using a shrinking-field technique, TRT was given concurrently with cisplatin and etoposide either as daily 180 cGy fractions or as twice-a-day 150 cGy fractions. The maximal tolerated doses defined by acute esophagitis was 45 Gy for twice-a-day administration and 70 Gy when given once a day. Intensification of TRT dose is feasible and should be evaluated in a randomized setting.

### Intensify induction

The benefits of induction therapy include the reduction of tumor burden, stabilization of rapidly progressive systemic and local symptoms from SCLC,

selection of patients possessing chemosensitive tumors for subsequent intensification, and diminution of micrometastases in the marrow and/or PBPCs as discussed below. On the other hand, chemoresistant tumor cells might proliferate or even be induced across treatment and may outweigh tumor burden reduction. Indeed, the Arriagada trial suggests that initial intensification of induction may improve disease-free and overall survival.[17] Administration of multicycle dose-intensive combination therapies supported by cytokines and PBPCs using either repeated cycles of the same regimen[76–79] or a sequence of different agents[80–83] is a logical extension of this concept. Thatcher and colleagues explored different methods to collect hematopoietic stem cells to achieve greater dose intensity with the ICE regimen for the treatment of good performance status SCLC patients. Conventional-dose ICE was supported by autologous whole blood cells given on day 3 of chemotherapy for six cycles.[78] Cycle length was 3 weeks using cryopreserved apheresis products or 2 weeks using either apheresis products or 750 cc whole blood stored at 4°C, thus increasing dose intensity. Cycles were repeated on platelet recovery to 30,000/μL. In this phase I trial of 25 patients, the full planned dose intensity for each of the arms was reached across the first three cycles, although only 56% completed all six. Mortality was 12% and complete response rate was 64%, but the longer-term outcomes are unknown as the median follow-up was 10 months. The authors note that the collection of whole blood without cryopreservation reduced the cost and complexity of cellular support for nonablative therapy substantially.[78] In a subsequent randomized phase II study, 50 "good prognosis" patients were given ICE every 2 or 4 weeks.[77] The median dose intensity delivered over the first three cycles was 1.8 (0.99–1.97) vs. 0.99 (0.33–1.02) on the 2-week vs. 4-week cycles, respectively. More hematopoietic and infectious events occurred on the standard-dose 4-week arm.

In the EBMT study, 47 patients underwent mobilization with epirubicin and G-CSF followed by three cycles of moderately intensive ICE.[79] Radiation to chest and head was recommended. Of 35 evaluable, the complete and near complete response rate was 69%. Mortality was 14%.

Humblet et al. treated 37 limited-stage patients with four intensive alternating cycles of etoposide with either ifosfamide or carboplatin with stem cell support.[83] TRT (10 Gy) in five fractions was given concurrently with each chemotherapy administration. Mortality was 3%. The median event-free survival was 18 months, and 80% remain alive at 30 months. Perhaps due to the fact that no PCI was given, eight of 13 relapses occurred in the brain.

## Minimal residual tumor/autograft involvement

Autograft contamination by tumor cells may cause relapse. Gene marking studies have definitively proven that residual tumor cells do directly contribute to

relapse in certain hematologic malignancies and neuroblastoma.[84–86] Similar experiments in solid tumors have not yet demonstrated gene-marked tumor cells in relapse sites.[87] It remains that these cells may also serve as a marker to indicate the patient has increased systemic chemotherapy-resistant tumor burden.

In SCLC, the marrow is one of the most common metastatic sites. By immunohistochemical techniques (sensitivity of 1 in $10^4$ cells), subclinical micrometastatic disease is detected in marrow in 13–54% of newly diagnosed limited-stage and 44–77% of newly diagnosed extensive-stage SCLC patients.[88–92] Of patients in complete response, two tiny series suggest two-thirds have subclinical disease in marrow.[93,94] In one paper, residual tumor appeared to predict relapse.[94] Brugger and colleagues have reported circulating tumor cells in patients with metastatic SCLC or breast cancer mobilized with G-CSF and IPE chemotherapy.[95] Circulating tumor cells were not observed after the second cycle of chemotherapy, supporting the contention that in the short term, in vivo chemotherapy induction may "purge" the patient and the autologous stem cell source.[65] In our unpublished data, up to 77% of limited disease patients in or near complete response before high-dose therapy have detectable tumor cells in their marrow by keratin staining.

Although many chemotherapeutic agents have major clinical activity against overt SCLC, the uniform clinical outcomes suggest that these different systemic drugs fail to eradicate a coincident population of tumor cells, presumably enriched for in vivo resistance mechanisms. Molecular and antigenic characterization of these residual cancer cells may guide strategies for further treatment. We are using a fluorescence microscope with automated computerized scanning with one set of fluorescent probes for detection and a second set with different fluorophores for biologic characterization to analyze patterns of coexpression of various markers in these cells.[96] Prospective trials to determine the clinical significance of marrow or peripheral blood tumor contamination and the impact of novel stem cell sources to support high-dose therapy are underway.

## CONCLUSION

Two major strategies to administer high-dose therapy for SCLC include dose-intensive multicycle approach as initial treatment and the "later" intensification in responders. Advantages for each approach are evident. The multicycle approach can achieve early dose intensity and maintain it for about three to four cycles. Disadvantages to this approach include subtransplant doses, high mortality rates, late administration of chest radiotherapy (except for the relatively low-dose radiotherapy in the recent Humblet trial), and the collection of stem cells early in treatment when they are more likely to be contaminated with tumor cells. Advantages to later intensification include a patient with decreased tumor burden and tumor-related symptoms with consequent improved performance status, a

partial purge of the autograft, and the ability to deliver early dose-intensive thoracic radiotherapy. The drawback of later administration of the dose-intense cycle can be surmounted in part by intensification and shortening of induction chemoradiotherapy. The optimum may be to merge the two strategies into one: a brief dose intensive induction followed by a single or double cycle of stem cell supported therapy followed by TRT and PCI. Ultimately, a randomized trial in patients with limited comorbid disease will be necessary to determine whether the increased toxicity is worthwhile and for which subsets of patients this approach is curative.

High-dose therapy has a strong scientific basis: it kills more tumor cells and achieves minimal tumor burden in most. In clinical situations in which toxicity has been acceptable, it typically results in prolonged progression-free survival in a subset of patients. An additional group of patients may be near-cure. High-dose therapy may have increased value if additional targets of residual tumor cells can be identified for novel treatment strategies and modalities. Most biologic strategies such as active or adoptive immunotherapy, gene function replacement (retinoblastoma gene and/or *p53*), or interruption of autocrine or paracrine growth loops work best against minimal tumor burden.

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# Transplantation With PBSC, Manipulated or Unmanipulated, for the Treatment of Childhood Cancer

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## AUTOLOGOUS PBSCT FOR THE TREATMENT OF CHILDREN >1 YEAR OLD WITH STAGE IV NEUROBLASTOMA

Previously, we reported long-term results in 22 children >1 year old with stage IV neuroblastoma treated with multidrug therapy using the "high-MEC" regimen consisting of carboplatinum (400 mg/m$^2$) and VP-16 (200 mg/m$^2$) on days $-7$ through $-4$ and melphalan (90 mg/m$^2$) on days $-3$ and $-2$ and who received autologous peripheral blood stem cell transplantation (PBSCT).[1] After PBSCT, three patients died of regimen-related toxicities and one patient transplanted in the refractory stage of the disease died of disease progression. Hematologic recovery was evaluated in 21 patients, excluding one early death. The median number of days required to achieve an ANC of $>0.5\times10^9$/L and platelet count of $>50\times10^9$/L were, respectively, 11 and 46. Eleven patients relapsed 3 to 50 months after PBSCT, and currently seven patients (five of 13 who were transplanted in CR and two of seven in PR) are disease-free at 52 to 84 months.

Based on the data obtained in this pilot study, a prospective study was started and by March 1998, 45 patients have been registered. Five patients died of complications before PBSCT and seven are in the pretransplant phase of chemotherapy. Thirty-three patients had already undergone PBSCT, and regimen-related mortality was observed in two (6%). Currently, 15 have relapsed after PBSCT (Fig. 1). It appears that a new modality is required to decrease posttransplant relapse.

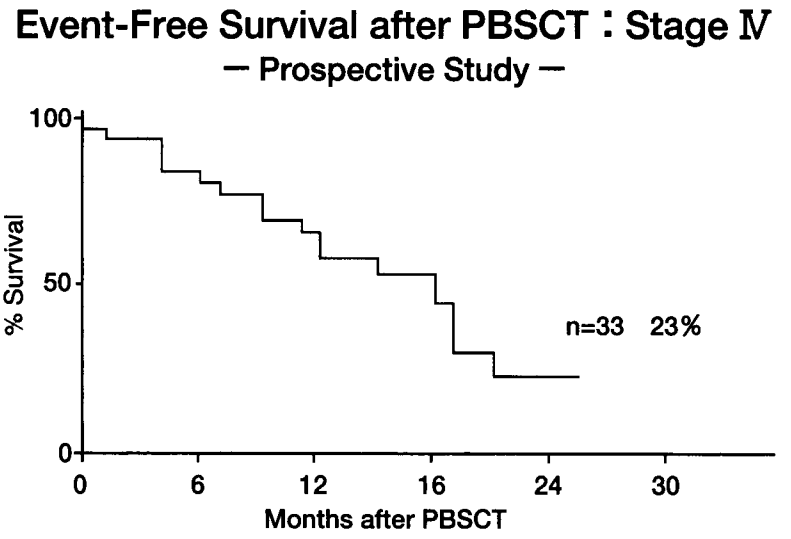


**Figure 1.** *The Kaplan-Meier estimates of event-free survival for children >1 year old with stage IV neuroblastoma who underwent PBSCT.*

## G-CSF TREATMENT AFTER AUTOLOGOUS PBSCT: A PROSPECTIVE RANDOMIZED TRIAL

There is a possibility that additional use of granulocyte colony-stimulating factor (G-CSF) may further enhance the already fast recovery rate of hematopoiesis after PBSCT. However, in our study we found that growth factor activities, such as G-CSF and thrombopoietin, can be identified in the serum of transplant recipients.[2,3] Thus, it is very probable that these endogenously secreted CSFs provide sufficient stimulation for hematopoietic engraftment without the need for exogenous cytokines, as previously observed.[4]

To clarify this issue, we performed a clinical trial, in which a total of 74 children scheduled to undergo PBSCT were prospectively randomized at diagnosis to evaluate the effectiveness of exogenous G-CSF treatment in accelerating hematopoietic recovery after PBSCT.[5] A total of 63 patients finally underwent PBSCT; 32 patients in the treatment group (300 $\mu g/m^2$ of G-CSF intravenously over 1 hour from day 1 of PBSCT) and 31 in the control group without treatment. There was no significant difference in the numbers of transfused MNC, $CD34^+$ cells, or colony-forming units granulocyte/macrophage (CFU-GM) between the treatment and control groups. After PBSCT, the time to achieve an ANC of $>0.5\times10^9$/L in the treatment group was 1 day less than that in the control group (median; 11 vs. 12 days, $P<0.05$), although the last day of red blood cells (RBC)

transfusion (day 11 vs. day 10) and the duration of febrile days (>38°C) after PBSCT (4 vs. 4 ) were identical in both groups. However, there was a significant difference in the time to achieve a platelet count of $>20\times10^9$/L (26 vs. 16 days; $P$=0.01) and platelet transfusion-dependent days between treatment and control groups (27 vs. 13 days, $P$<0.05). We suggest that the marginal clinical benefit of 1 day earlier recovery of granulocytes could be offset by the delayed recovery of platelets in this setting.

## AUTOLOGOUS TRANSPLANTS WITH CD34+ CELLS

Although the cells responsible for both short- and long-term hematopoietic recovery are not precisely defined, the effective depletion of cancer cells or T cells may be possible through the use of purified CD34+ cells. Moreover, the cell isolation procedure may eliminate toxicities during graft infusion. In a previous study with 54 infusions of unmanipulated PBSC graft in 52 children,[6] we assessed toxicity and found the volume of PBSC infused varied from 46 to 500 mL (mean 219.6 ± 118.4 mL). We found that transient toxicity was rather common during graft infusion.

In contrast, in our pilot study of autologous transplantation with purified CD34+ blood cells in 23 children, the mean volume of the grafts the patients received was 8 mL (range 3–58), and no adverse effects directly related to graft infusion were observed. After the infusion of cells, the median number of days to achieve an ANC of $>0.5\times10^9$/L and a platelet count of $>50\times10^9$/L was, respectively, 11 and 26. Comparison of days to engraftment between different modes of transplantation with purified or unmanipulated blood cells is shown in Table 1.

Although the potential disadvantage of CD34+ cell selection is that the reconstitution of immunohematologic functions may be delayed, these engraftment data were identical to our historical data of 74 transplants that were performed with unmanipulated PBSC containing an equivalent number of CD34+ cells. We also showed that, once recovered, hematopoietic function is stable over the long term. However, the recovery of the T4/T8 ratio as a marker of immune reconstitution after ex vivo manipulation tended to be slow compared with regular autologous PBSCT with unmanipulated cells.

**Table 1.** Comparison of engraftment days between different modes of transplantation with purified or unmanipulated blood cells

| | *AutoPBSCT* | *Auto CD34+* | *AlloPBSCT* | *Allo CD34+* |
|---|---|---|---|---|
| *n* | 72 | 23 | 12 | 13 |
| ANC> $0.5\times10^9$/L | 12 (16–25) | 11 (9–18) | 10 (8–19) | 15 (9–20) |
| Platelets $>50\times10^9$/L | 16 (10–195) | 26 (13–55) | 16 (12–39) | 18 (12–23) |

## TANDEM TRANSPLANTS WITH CD34-POSITIVE AND -NEGATIVE CELLS

Dose intensification and sequential use of agents to overcome drug resistance may benefit some patients, and interest in the concept of tandem transplants has been growing. To improve the therapeutic efficacy under these conditions by depleting contaminating tumor cells in the grafts, positively selected $CD34^+$ cells have been used. To maximize the intensity of therapy at an acceptable cost in toxicity, we developed a new tandem transplant strategy, in which cells recovered in the $CD34^-$ fraction, which still contains a large amount of clonogenic progenitor cells identified as CFU-GM, are used in the initial transplant.[7] This was followed by two to three courses of regular-dose chemotherapy without rescue for further in vivo purging before the final transplant with purified $CD34^+$ cells, which enables the ultimate eradication of reinfused cancer cells. Identical carboplatinum-based regimens consisting of cyclophosphamide (1.2 $g/m^2 \times 2$ days) and melphalan (90 $mg/m^2 \times 2$ days) were used for both courses of high-dose therapy. Currently, 13 patients have been treated. The number of CFU-GM infused and the subsequent engraftment speed after each transplant procedure were identical.

Based on the results, we conclude that this approach makes multiple-course combination high-dose therapy more feasible by ameliorating cytopenia with an improved cost/benefit ratio. Although tumor cells contaminating the $CD34^-$ fraction might be of clinical concern, the presence of these cells in the graft is not necessarily capable of producing a relapse of the disease on reinfusion. This risk associated with our procedure must be weighed against the likelihood of a significant benefit. We believe that the number of cancer cells in the first graft is far smaller than the tumor burden remaining in the entire body at the first transplant and that infused cancer cells can be purged by the subsequent transplant. Nevertheless, recognizing that cancer cells might be enriched in the $CD34^-$ fraction, the use of an additional purification of this fraction for negative depletion of cancer cells will be evaluated.

## FACILITATED ENGRAFTMENT BY INTRAMEDULLARY ADMINISTERED ENRICHED $CD34^+$ CELLS?

Stem cell products are commonly infused into the venous system following myeloablative chemoradiotherapy. Hence, it is likely that only a few cells enter the marrow space, since many cells are "trapped" in the microcirculation, including the reticuloendothelial system, pulmonary vessels, and other capillary beds during the first pass through the systemic circulation. When the harvested graft is manipulated, there is a significant loss of cells and graft failure becomes a major concern. Therefore, we hypothesized that direct puncture of the marrow cavity to implant the graft might provide a more stable engraftment. In clinical transplan-

tation, this procedure is feasible only when the cells are purified to reduce the total volume of the graft. Our experience with this approach will be presented.

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# Multiple Courses of Cyclophosphamide, Thiotepa, and Carboplatin: Managing Toxicity by Dose Reduction and Pharmacokinetic Monitoring

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## ABSTRACT

Multiple courses of high-dose alkylating chemotherapy have been made possible by the technique of peripheral blood progenitor cell transplantation, but end-organ toxicities such as hemorrhagic cystitis, veno-occlusive disease of the liver, or hemolytic uremic syndrome are now dose-limiting. Because of this, three subsequent administrations of CTC—cyclophosphamide (6 g/m$^2$), thiotepa (480 mg/m$^2$), and carboplatin (1600 mg/m$^2$)—have been shown to be not feasible. To overcome these problems, we have developed a regimen called "tiny CTC" (tCTC) that contains two-thirds of the CTC dose combination. Three subsequent courses of tCTC, administered every 4–5 weeks, are well tolerated and, in contrast to CTC, allow discharge of the patients after stem cell transplantation. Similar end-organ toxicities as after the full-dose CTC regimen were observed in the first 20 patients to undergo three subsequent courses, but were not life-threatening and were readily reversible. To identify patients at risk for developing severe organ toxicity, we have developed and validated methods for determining plasma concentration vs. time curves of cyclophosphamide, 2-dechloroethyl cyclophosphamide, thiotepa, and carboplatin after tCTC. The pharmacokinetic/pharmacodynamic studies are in progress. These data should serve to individualize doses of alkylating agents so as to improve both the safety and the efficacy of this regimen.

## INTRODUCTION

High-dose chemotherapy in breast cancer continues to represent an experimental approach of uncertain value.[1] Several large, randomized studies are underway that will answer the question of whether a single course of high-dose therapy with peripheral blood progenitor cell (PBPC) transplantation is beneficial in the adjuvant setting. Survival data from a single, small but randomized study done in the Netherlands Cancer Institute suggest that the benefit of high-dose chemotherapy—if it exists at all—is smaller than uncontrolled studies had suggested.[2] In advanced disease, only a single randomized study has been reported thus far in a peer-reviewed journal.[3] The large majority of published articles deal with phase I and feasibility studies. This is reasonable since the techniques of supportive care are rapidly evolving and novel strategies made possible by these advances need to be developed.

Novel treatment strategies include the addition of new agents (such as taxanes) to high-dose regimens, or the administration of multiple courses of high-dose therapy. While some groups are exploring the concept of "high-dose sequential" chemotherapy,[4,5] we have been working on the development of a regimen that would allow the repeated administration of a very high dose of the alkylating agents cyclophosphamide, thiotepa, and carboplatin.[6] We have attempted to deliver three full-dose courses of this combination, but many patients were not able to tolerate this degree of dose intensity, and severe toxicity occurred in the form of veno-occlusive disease of the liver or hemolytic uremic syndrome.[7] Because of this, we lowered the dose of each CTC course by one-third and initiated a phase II study of this combination in patients with advanced breast cancer. This report contains preliminary data from this study and focuses on feasibility, toxicity, and strategies to prevent severe end-organ toxicity.

## MATERIALS AND METHODS

### Patient selection

All patients had biopsy-proven stage IV breast cancer, were <56 years of age, and had excellent performance status (World Health Organization [WHO] 0 or 1). Previous chemotherapy was not allowed, unless it had been limited to non–anthracycline-based adjuvant therapy that had been discontinued at least 1 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 bone marrow 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 anthracycline-based regimen that we previously reported to be highly efficacious in locally advanced breast cancer.[8] 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 (tiny CTC) was begun 3 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.[7] Evaluable patients who did not respond to a $FE_{120}C$ or tCTC course were taken off study.

## PBPC mobilization and harvest

For stem cell mobilization, the $FE_{120}C$ chemotherapy regimen was used, all given as intravenous push on day 1. Filgrastim (5 μg/kg/d subcutaneously) was started on day 2. Leukaphereses began when the white blood cell (WBC) count exceeded $3.0\times10^9$/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.[9]

Based on earlier findings,[10] we considered a graft size of $3.0\times10^6$ CD34$^+$ cells/kg body weight sufficient for sustained bone marrow recovery and $1.0\times10^6$ CD34$^+$ cells/kg sufficient for rapid (but possibly transient) granulocyte recovery after high-dose therapy.

## High-dose chemotherapy regimen: tCTC

The high-dose chemotherapy regimen tCTC was administered as published previously.[7] Briefly, carboplatin was administered intravenously as daily 1-hour infusions on days −6, −5, −4, and −3. The total dose of carboplatin was 1060

$mg/m^2$ in patients with normal renal function, but in those with creatinine clearances of 110 ml/min or less, it was determined by the following formula:

$$\text{dose (mg)} = 13.3 \times (\text{creatinine clearance} + 25)$$

Cyclophosphamide (total dose 4000 $mg/m^2$) was divided over 4 daily 1-hour infusions, and thiotepa (total dose 320 $mg/m^2$) was divided over 8 twice-daily half-hour infusions. Both agents were given on days −6, −5, −4, and −3. Mesnum (500 mg per dose) was given six times a day for a total of 36 doses, beginning 1 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 a day. To prevent gram-positive infections, roxitromycin was given orally from day 0 and was discontinued when the neutrophil count exceeded $0.5\times10^9$ cells/L. Irradiated platelet transfusions were administered to maintain platelet counts of at least $10\times10^9$/L, and leukocyte-free irradiated red blood cells were given to maintain hemoglobin levels ≥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.

G-CSF (filgrastim; a gift from Amgen-Roche, Breda, The Netherlands) was administered as a daily subcutaneous injection of 300 μg, regardless of body weight, from day 1 until the WBC count exceeded $5.0\times10^9$/L.

## Sample collection for pharmacokinetic monitoring

Samples were collected before the infusions on all days of chemotherapy, and on days 1 and 4 at 30 minutes after start of the cyclophosphamide infusion ($t$=0) and at $t$=60, 90, 120, 150, 180, 210, 285, 390, and 660 min. The infusion sequence was cyclophosphamide (1 hour), carboplatin (1 hour), thiotepa (30 minutes). After sample collection, blood samples were placed on ice and plasma was separated immediately by centrifuging the sample for 3 min at 3,000$g$ at 4°C. Plasma ultrafiltrate was prepared without delay using the MPS-1 system (Amicon Division, Danvers, MA) by centrifugation (10 min, 1500$g$) of 1.0 mL plasma.

## Carboplatin assay

Free, ultrafiltrable carboplatin was measured using Zeeman atomic absorption spectrometry as described earlier.[11]

### Thiotepa, TEPA, and 2-dechloroethyl cyclophosphamide assays

ThioTEPA, TEPA, cyclophosphamide and 2-dechloroethyl cyclophosphamide levels in plasma were determined using capillary gas chromatography with nitrogen/phosphorous selective detection after liquid-liquid extraction of the samples with chloroform.[12]

## RESULTS

### Feasibility of scheduled treatment

A total of 41 patients were entered on the protocol. Pertinent patient characteristics are given in Table 1. Not all patients proceeded to high-dose therapy: seven patients had progressive disease or no sign of response at all to $FE_{120}C$ and were taken off protocol since high-dose therapy was not considered to be in their best

**Table 1.** Characteristics of the 41 patients on study

| | |
|---|---|
| Age (years) | 44 (27–55) |
| Hormone responsiveness | |
| ER negative | 28 |
| ER +, failed FLHT1 | 7 |
| ER +, failed SLHT2 | 1 |
| ER unknown, failed FLHT | 5 |
| Number of sites of disease | |
| 1 site | 11 |
| 2 sites | 20 |
| 3 sites | 6 |
| 4 or more sites | 4 |
| Sites of disease | |
| Lymph nodes | 23 |
| Bone | 18 |
| Liver | 11 |
| Lung | 11 |
| Prior therapy | |
| Radiation therapy | |
| Adjuvant | 20 |
| For advanced disease | 2 |
| Chemotherapy | |
| Adjuvant | 8 |
| For advanced disease | 13 |

*FLHT, first-line hormonal therapy; SLHT, second-line hormonal therapy.*

interest. One patient did respond to $FE_{120}C$, but had estrogen receptor–positive lobular carcinoma. The authors believed that high-dose therapy was not the best treatment option, and she was taken off protocol as a protocol violation. The treatment of one further patient was too early for evaluation and will be reported in a later publication.

A total of 80 tCTC courses were administered. Thirty-two patients received a first tCTC course, 28 patients a second, and 20 patients completed the full planned sequence of three tCTC courses. Two patients are still undergoing treatment after tCTC-1 and will be reported elsewhere. The other two patients were taken off protocol after tCTC-1 either because of lack of response (one patient) or because of prolonged abnormalities of liver function tests (one patient). Six of eight patients who did not proceed to a third course of tCTC after tCTC-2 stopped treatment because of excessive toxicity (one unexplained death, three hemorrhagic cystitis, one veno-occlusive disease, one refractory thrombocytopenia). A seventh patient showed no further disease regression after the second tCTC course and was advised to discontinue protocol treatment. The eighth patient was still on protocol at the time of this writing.

The protocol required that second and third courses of tCTC be started on day 22–29 after the previous PBPC transplantation; the earliest time point of 22 days was preferred. Following this policy, the median day after PBPC reinfusion on which tCTC-2 was begun was 28 (range 21–53). This was day 35 (range 20–42) for the third tCTC course. In addition, dose adaptations were required in three of the third courses.

## Major toxicity

The tCTC regimen was well tolerated by most patients, and the large majority were able to leave the hospital on the first day after PBPC transplantation. Details of the outpatient management of these transplantation patients will be published elsewhere (A.M.W., et al., manuscript submitted); this section will focus on major toxicities, such as grade IV gastrointestinal (GI) toxicity, organ toxicity (liver, kidney, lung, heart, or lung), hemorrhagic cystitis, and other complications that are important for the feasibility and acceptability of the triple tCTC regimen.

One patient died on the day 18 after reinfusion while apparently recovering from her second tCTC course. Her death was sudden and in her sleep, at a time when the neutrophil count had returned to normal but platelet transfusion dependence had not yet resolved. Unfortunately, no autopsy permission could be obtained and the cause of death remains unclear.

The major (organ) toxicity of tCTC is presented in Table 2. Although none of this toxicity was life-threatening, it is clear that major organ toxicity such as hemorrhagic cystitis and veno-occlusive disease of the liver do occur with some

**Table 2.** Major toxicities of tCTC courses

| *Toxicity* | *Number of courses* |
|---|---|
| Hemorrhagic cystitis (mild to moderate) | 7 |
| Prolonged GI-toxicity (reversible) | 4 |
| Veno-occlusive disease (nonlethal) | 2 |
| Allergic reaction to tCTC | 2 |
| Radiation pneumonia | 2 |
| Symptomatic ototoxicity | 1 |
| Hemolytic uremic syndrome | 1 |

frequency. An important type of toxicity is prolonged GI toxicity, which consists of prolonged nausea—sometimes with vomiting—that may continue for many weeks. Since nutrition is difficult, patients may require tube feeding; any further planned courses of tCTC chemotherapy would need to be postponed.

The occurrence of possibly irreversible alopecia was unanticipated in two patients. All other patients experienced normal regrowth of hair after discontinuing the protocol.

## Progression-free survival analysis

At the time of writing this report, the median follow-up of the patients in this study was only 21 months, with a lead follow-up of 56 months. As a result, even

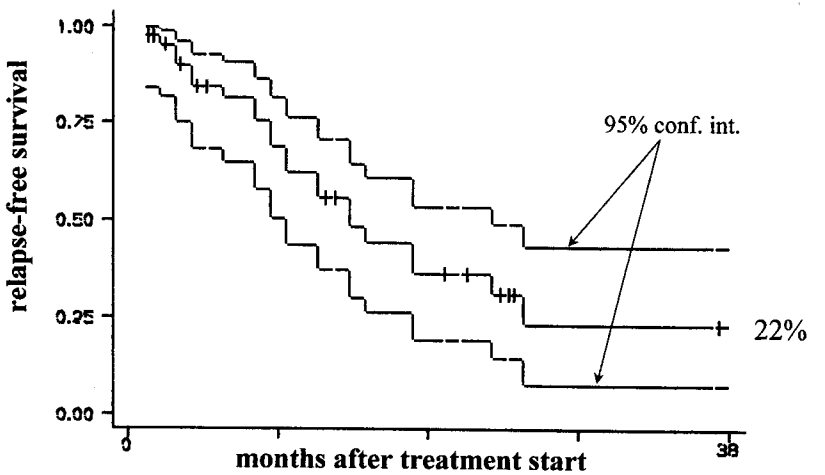


**Figure 1.** *Progression-free survival, all patients. All 41 patients entered on the protocol are represented in the curve, including the nine patients who did not proceed to high-dose therapy (see text).*

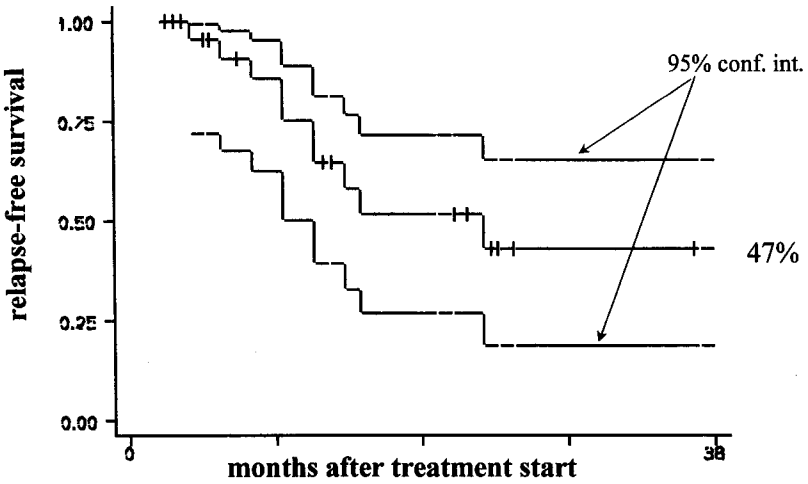


**Figure 2.** *Progression-free survival of patients who responded to $FE_{120}C$. The progression-free survival was calculated from the start of chemotherapy for all patients who had at least 50% regression of measurable or evaluable disease after the two courses of $FE_{120}C$, and who were considered to have "chemosensitive disease."*

the progression-free survival data of this highly selected patient group are immature. Nevertheless, some indication of the level of efficacy at this early time can be obtained from Figs. 1 and 2. Not surprisingly, patients responding to two courses of FEC with at least 50% regression of evaluable tumor appeared to have a better prognosis than those who did not. The 2-year progression-free survival for all patients was 22% (95% confidence interval[CI] 8–44%), and that for FEC-responsive patients was 47% (95% CI 20–66%).

### Pharmacokinetic data

For two patients, the plasma concentration vs. time curves of carboplatin, thiotepa, TEPA, cyclophosphamide, and 2-dechloroethyl cyclophosphamide are shown in Fig. 3A–D. This information establishes the suitability of the methods to obtain adequate curves in this patient group. Detailed analyses of more patients are in progress and will be published elsewhere.

## DISCUSSION

Three subsequent courses of tCTC could be administered to most patients in this trial. As in the study with full-dose CTC,[7] end-organ toxicity such as hemorrhagic cystitis and veno-occlusive disease occurred but were generally mild and did not

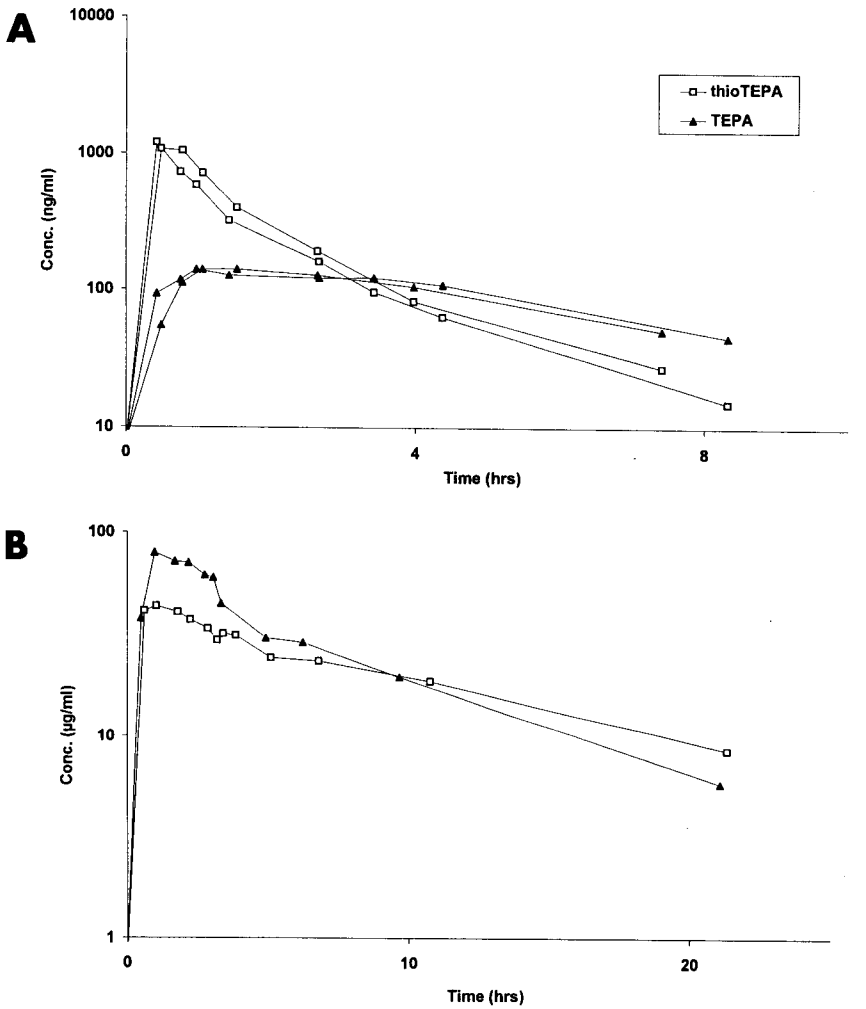


**Figure 3.** *Plasma concentration versus time curves of three alkylating agents on the first day of a tCTC course. A: Plasma concentration of TEPA and thiotepa vs. time curves of two patients. Both had received a thiotepa dose of 40 mg/m². B: Plasma concentration of cyclophosphamide vs. time curves of two patients after their first dose of 1000 mg/m² cyclophosphamide.*

cause mortality. However, this type of toxicity continues to be of major concern. Experience with this treatment strategy is still limited, and severe hemorrhagic cystitis or lethal veno-occlusive disease may occur when larger numbers of patients have been studied. These toxicities are, however, observed in only a minority of patients and may, in part, result from pharmacokinetic variability between patients.

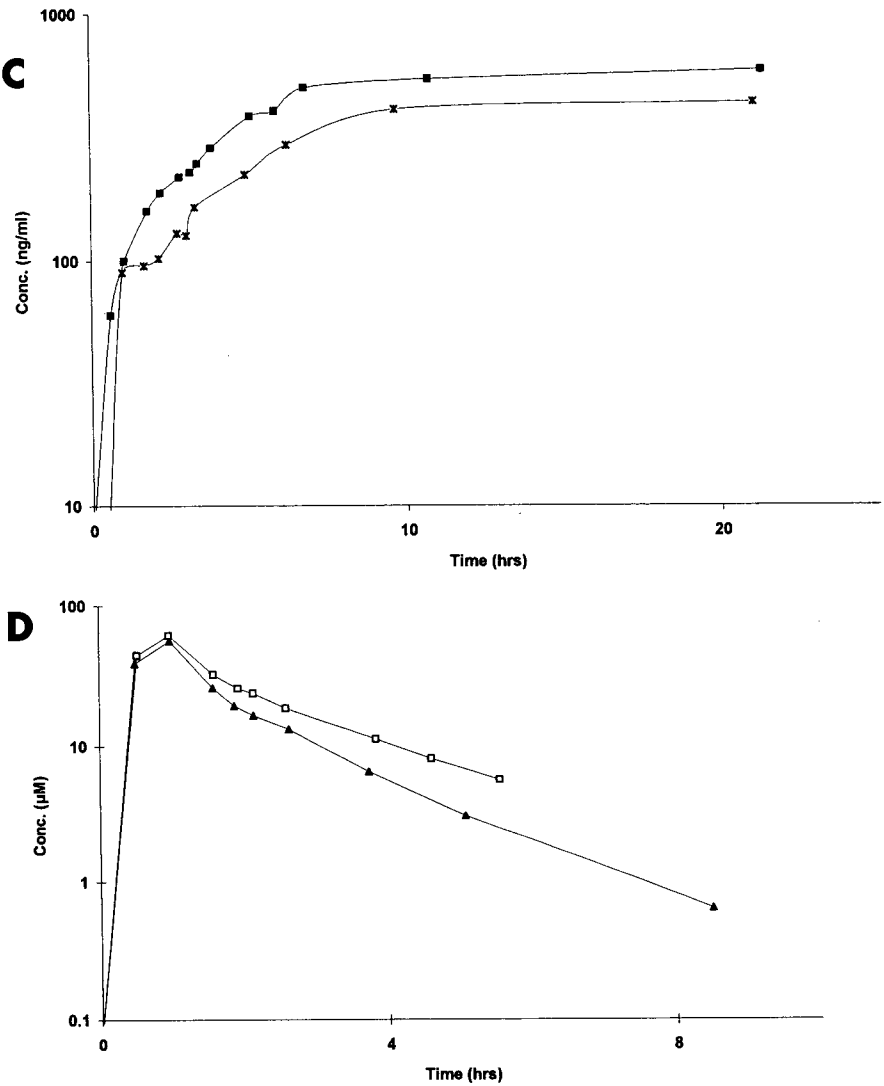


**Figure 3, continued.** *Plasma concentration versus time curves of three alkylating agents on the first day of a tCTC course. C: Plasma concentration of 2-dechloroethyl cyclophosphamide vs. time curves after a dose of 1000 mg/m² cyclophosphamide. D: Plasma concentration of carboplatin vs. time curves of two patients who received carboplatin in a dose targeted to achieve an area under the curve of 3.3 mg · mL$^{21}$ · 1 min$^{21}$.*

It is here that the repeated administration of the same regimen most clearly demonstrates its advantages over other strategies. We have never observed severe toxicity in a first course of tCTC, and pharmacokinetic observations made during this

course could be used to modify dosages or even administration schedules in second and third courses. In theory, it would even be possible to use information gathered on the first day of the first tCTC course to modify the last few doses of tCTC-1.

The first step to achieve such an ambitious goal is to establish analytic methods that conveniently allow the rapid determination of all three alkylating agents: cyclophosphamide and its metabolites; thiotepa and its biologically active metabolic breakdown product, TEPA; and carboplatin. We and others have assayed carboplatin in biological fluids in patients undergoing high-dose therapy with the agent. We have also shown that its kinetics are unaffected by dose and can accurately be predicted using modified Calvert or Chatelut formulas.[13,14] We have recently proceeded to improve the sensitivities of the assays for thiotepa and TEPA[15] and have shown the suitability of our adapted methods for monitoring patients receiving high-dose therapy. A gas-chromatographic assay for cyclophosphamide and 2-dechloroethyl cyclophosphamide that allows the simultaneous determination of thiotepa and TEPA has also been developed recently in our institute.[12] Using this integrated assay, we are attempting to develop limited-sampling strategies that will allow us to determine the relevant pharmacokinetic parameters in the patients receiving CTC from only a small number of timed plasma samples. The suitability of the methods to reliably monitor the pharmacokinetics during the first day of the first tCTC course has been shown and is illustrated in Fig. 3A–D, the plasma concentration versus time curves of carboplatin, thiotepa, TEPA, cyclophosphamide, and 2-dechloroethyl cyclophosphamide in two patients on the first day of their first tCTC course. The pharmacokinetic/pharmacodynamic relationships can be studied when more curves become available.

All these attempts to predict and prevent severe organ toxicity in patients receiving multiple courses of tCTC only make sense if this treatment approach proves to be more effective than standard chemotherapy. At present, this is difficult to predict. The results in patients with advanced (stage IV) breast cancer who have chemosensitive disease (i.e., who achieve objective tumor regression after two courses of $FE_{120}C$) appear to be promising, with a 2-year progression-free survival of 45%, but the confidence interval is large and the follow-up is brief. It is possible, or even likely, that certain individual tumors have "exquisite alkylating agent sensitivity" and that patients bearing an advanced tumor with that characteristic may specifically benefit from this approach. One challenge for the next few years will be the development of methods to recognize exquisite alkylator sensitivity in individual tumors before chemotherapy is initiated.

It is, at present, difficult to predict whether triple tCTC represents an advancement in the treatment of patients with breast cancer. The regimen has certainly shown impressive results in some patients but has failed in others. The number of patients in the phase II study is still small and the follow-up is brief. Nevertheless, the following preliminary conclusions appear to be justified.

First, a response to the conventional-dose induction chemotherapy is required to achieve disease-free survival of 1.5 years or more. All patients in whom all measurable disease was absent after FEC had long progression-free survivals, and the same was true in three of 10 partially responding patients.

Second, the patients in whom the most satisfactory responses were achieved were those with limited disease. Patients in whom all sites of disease could either be resected or irradiated did very well. In addition, most patients with long remissions had only soft-tissue metastases (skin, lymph nodes, local recurrences) and only infrequently had bone or liver involvement. This finding is not surprising, and these features are also prognostically favorable when only conventional therapy is employed.

Eventually, only randomized studies will be able to determine whether this high-dose approach is superior to other high-dose strategies and to conventional dose treatment.

## ACKNOWLEDGMENTS

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# Epirubicin Plus G-CSF–Elicited Peripheral Blood Progenitor Cell Mobilization Is Significantly Enhanced by Amifostine

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## ABSTRACT

Amifostine, a phosphorylated aminothiol, increases the selectivity of anticancer drugs for neoplastic cells, protects normal tissues from radiochemotherapy toxicities, and stimulates in vivo hematopoiesis. The aim of the present study was to investigate whether amifostine in conjunction with epirubicin and granulocyte colony-stimulating factor (G-CSF) might enhance peripheral blood progenitor cell (PBPC) mobilization. Ten patients (three untreated and seven pretreated with seven to 17 chemotherapy cycles) with advanced solid tumors who were given two cycles of epirubicin (120 mg/m$^2$ on day 0) plus G-CSF (5 μg/kg/d, days 1–10) at 3-week intervals were randomized to receive amifostine on the first or second cycle. Amifostine was administered intravenously for 5 days (day 0, 1000 mg; days 1–4, 500 mg/d). Once a day on days 0 to 10, white blood cell and platelet counts, CD34$^+$ cells, and progenitor cell incidence (CFU-Mix, BFU-E, CFU-GM, LTC-IC) were evaluated. White blood cell and platelet counts were not affected by amifostine administration. In contrast, epirubicin + G-CSF + amifostine compared to epirubicin + G-CSF induced a statistically significant ($P$<0.05) increase of peak values for CD34$^+$ cells/mL, CFU-Mix/mL, BFU-E/mL, and CFU-GM/mL. Compared with day 0 values, epirubicin + G-CSF + amifostine vs. epirubicin + G-CSF resulted in significantly higher fold increases for CFU-Mix, BFU-E, and CFU-GM. This randomized study demonstrates that addition of amifostine to epirubicin + G-CSF significantly increases the mobilization of CD34$^+$ cells and primitive and committed progenitors in pretreated cancer patients.

## INTRODUCTION

Autologous stem cell transplantation (autoSCT) permits the administration of high-dose chemoradiotherapy followed by the infusion of the patient's own hematopoietic cells, previously collected and cryopreserved.[1] AutoSCT may be the best treatment option for patients with acute leukemia in first remission and intermediate- or high-grade non-Hodgkin's lymphoma.[2,3] This therapeutic approach may also be the treatment of choice for patients with acute leukemia, Hodgkin's disease and non-Hodgkin's lymphoma at first relapse.[4] Recent reports also indicate that autoSCT may be an effective treatment for patients with a number of different types of solid tumors, including breast cancer, gonadal cancer, and neuroblastoma.[5]

For several years, marrow-derived cells have represented the main source of hematopoietic reconstituting cells.[6] More recently, mobilized peripheral blood progenitor cells (PBPC) have been increasingly used to reconstitute hematopoiesis in patients undergoing high-dose chemoradiotherapy.[7] PBPC collections comprise a heterogeneous population containing both committed progenitors and pluripotent stem cells and can be harvested *1*) in steady state, *2*) after chemotherapeutic conditioning, *3*) after growth factor priming, or *4*) after both chemotherapeutic conditioning and growth factor priming.[8] G-CSF, alone or in combination with chemotherapy, is widely used for stem cell mobilization. The availability of PBPC has opened new therapeutic perspectives to alleviate the severe toxicity related to prolonged myelosuppression[9] and is associated with a number of clinical benefits including a reduction of platelet transfusions and shorter hospital stay.[9] In addition, the availability of large numbers of mobilized hematopoietic stem and progenitor cells that can be easily collected through leukapheresis allows extensive manipulations of the grafts, including positive and negative selection as well as the possibility to exploit these cells as vehicles for gene therapy strategies.[10]

The development of mobilization protocols aimed at improving the quantity and quality of collected PBPC represents an area of active investigation. In fact, *1*) optimal regimens for PBPC mobilization are not yet established; *2*) there is a wide individual variability in mobilization yields (poor yields being achieved in 5–15% of normal donors)[11]; *3*) with currently used mobilization protocols, patients who have received extensive prior chemotherapy or radiotherapy may be poor mobilizers; and *4*) there is an expanding use of high-dose sequential chemotherapy strategies requiring the collection of large numbers of $CD34^+$ cells. Currently, new approaches to improve PBPC mobilization include combinations of G-CSF or granulocyte-macrophage (GM)-CSF with other cytokines such as interleukin (IL)-3, stem cell factor (SCF), Flt3 ligand, and macrophage inflammatory protein 1α (MIP-1α).[12–17]

Amifostine (WR-2721) is a phosphorylated aminothiol compound that increases the selectivity of anticancer drugs for neoplastic cells, protects normal

tissues from radiochemotherapy toxicities, and stimulates both in vitro and in vivo hematopoiesis.[18–25] Amifostine protection on normal tissues has been demonstrated for photoactive agents, alkylating agents, nitrogen mustard, and platinum analogs.[26–33] Amifostine is a prodrug that is dephosphorylated by membrane-bound alkaline phosphatase to the free thiol which is then very rapidly taken up by normal cells, but very slowly, if at all, by tumor cells.[34,35] The mechanisms of amifostine cytoprotection rely on its ability to neutralize the DNA-reactive moieties of cytotoxic agents, its action as a scavenger of oxygen free radicals, and probably its capacity to reduce apoptosis.[36–38] In a recent phase I/II clinical trial conducted in patients with myelodysplastic syndromes, List et al. have demonstrated a stimulation of hematopoiesis in vivo by amifostine with an important response on myeloid progenitors.[39]

We conducted a randomized clinical trial with a crossover design to evaluate the role of amifostine in conjunction with epirubicin and G-CSF in an attempt to improve PBPC mobilization. The effect of amifostine was investigated on the mobilization of $CD34^+$ cells and primitive (LTC-IC) and committed progenitors (CFU-Mix, BFU-E, CFU-GM).

## MATERIALS AND METHODS

### Patients

Ten patients ranging in age from 33 to 54 years were included in this study. Informed consent was obtained from all patients before entry into the study. The main clinical characteristics of the patients at the time of the study are shown in Table 1. Prior to the study, all but three patients had received combination chemotherapy and two had received radiotherapy. The interval between the last chemotherapy cycle and inclusion in the study ranged from 0.5 to 21 months.

### Study design

The effect of amifostine was explored in a randomized trial with a crossover design (Fig. 1). Patients were randomized to receive amifostine on first or second cycle, thus each patient served as their own control. Intravenous (IV) epirubicin (Farmorubicin, 120 mg/m$^2$, day 0) followed by G-CSF (Neupogen, 5 μg/kg/d subcutaneously, days 1–10) was administered at 3-week intervals. Amifostine (Ethyol) was administered as a 15-minute IV infusion at the dose of 1000 mg on day 0 (15 minutes before epirubicin) and at the dose of 500 mg on days 1–4. Before amifostine injection, hydration was administered with normal saline (1000 mL, IV), and ondasetron (8 mg, IV). During amifostine infusion, blood pressure was monitored every 3–5 minutes, and serum ionized calcium levels were monitored on

**Table 1.** Clinical characteristics of the patients at the time of the study

| Patient | Age (years) | Sex | Diagnosis | Previous chemotherapy | Interval without chemotherapy (months) | Previous radiotherapy |
|---|---|---|---|---|---|---|
| 1 | 40 | F | BC | — | NA | — |
| 2 | 33 | M | Sarcoma | — | NA | — |
| 3 | 41 | F | BC | CMFEV × 4, PLF × 3 | 21 | — |
| 4 | 40 | F | BC | CMF × 13, PLF × 4 | 11 | Yes |
| 5 | 54 | F | BC | CMF × 12 | 5 | Yes |
| 6 | 37 | F | BC | GIP × 4 | 0.5 | — |
| 7 | 47 | F | Sarcoma | — | NA | — |
| 8 | 50 | F | BC | GIP × 6 | 1 | — |
| 9 | 49 | F | BC | CMF × 10 | 1 | — |
| 10 | 53 | F | BC | CMF × 6, TAX + VNRB × 9 | 1 | — |

*BC, breast cancer; CMF, cyclophosphamide + methotrexate + 5-fluorouracil; CMFEV, cyclophosphamide + methotrexate + 5-fluorouracil + epirubicin + vincristine; GIP, gemcitabine + ifosfamide + cisplatin; NA, not applicable; PLF, cisplatin + 5-fluorouracil + folinic acid; TAX, taxol; VNRB, vinorelbine.*

a daily basis. In all patients, at both cycles, complete blood counts, number of $CD34^+$ cells, and progenitor cell assays were performed daily on days 0 to 10.

## Flow cytometry

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood was incubated for 30 minutes on ice with 10 µL fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies. The following antibody combinations were used: CD4-FITC/CD8-PE, CD45-FITC/CD14-PE, CD34-PE. Each fluorescence analysis included a double negative isotype control ($IgG_1$-FITC/$IgG_1$-PE). Antibodies and isotype controls were purchased from Becton Dickinson (San Jose, CA). After red blood cell lysis with FACS Lysing Solution (Becton Dickinson), samples were washed in Dulbecco's phosphate buffered saline without $Ca^{2+}$ and $Mg^{2+}$ and acquired in listmode. Threshold was set on the forward scatter (FSC) to exclude cellular debris. A minimum of 40,000 events and a minimum of 100 $CD34^+$ events were acquired. Analysis was performed by setting two gates. The first gate was set on a plot of CD45 vs. CD14 and drawn to include lymphocyte and monocyte cells. The second gate was set on the plot of FSC vs. side scatter (SSC) to include lymphocyte and monocyte cells and was activated to generate a plot of SSC vs. anti-CD34 fluorescence intensity. The percentage of positive cells was determined by subtracting the percentage of fluorescent cells in

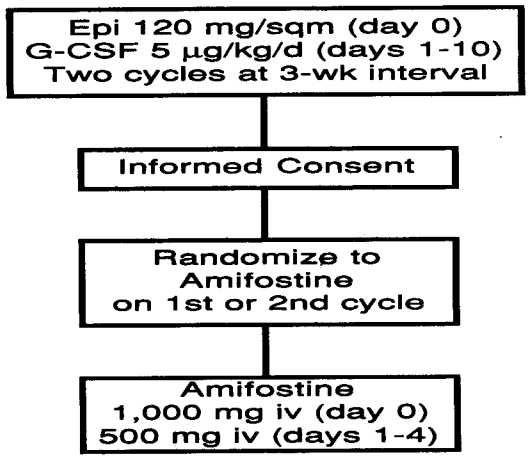


**Figure 1.** *Outline of the study design.*

the control from the percentage of cells positively stained with the anti-CD34. Phenotypic analysis was performed with a FACSort flow cytometer (Becton Dickinson). Data were processed with a Macintosh Quadra 650 computer (Apple, Cupertino, CA) using CELLQuest Software.

## CFU-Mix, BFU-E, and CFU-GM assay

The assay for multipotent colony-forming units (CFU-Mix), erythroid burst-forming units (BFU-E), and granulocyte-macrophage colony-forming units (CFU-GM) was carried out as described elsewhere.[40] Briefly, 1 to $10\times10^4$ nucleated cells were plated in 35-mm petri dishes in 1-mL aliquots of Iscove's modified Dulbecco's medium (IMDM) containing 30% fetal bovine serum (FBS) (Stem Cell Technologies, Vancouver, Canada); $10^{-4}$ M 2-mercaptoethanol (Gibco, Grand Island, NY); and 1.1% (wt/vol) methylcellulose. Cultures were stimulated with IL-3 (10 ng/mL; Sandoz, Basel, Switzerland), G-CSF (10 ng/mL; Amgen, Thousand Oaks, CA), GM-CSF (10 ng/mL; Sandoz), and erythropoietin (3 U/mL; Amgen). Progenitor cell growth was evaluated after 14–18 days of incubation (37°C, 5% $CO_2$) in a humidified atmosphere. Four dishes were set up for each individual data point per experiment. CFU-Mix, BFU-E, and CFU-GM were scored from the same dish according to previously published criteria.[40]

## LTC-IC assay

The long-term culture–initiating cell (LTC-IC) assay was performed according to Sutherland et al.[41] Briefly, test cell suspension ($5\times10^6$ nucleated cells) was

seeded into cultures containing a feeder layer of irradiated (8000 cGy) murine M2-10B4 cells ($3\times10^4$/cm$^2$, kindly provided by Dr. C. Eaves) engineered by retroviral gene transfer to produce human IL-3 and human G-CSF. Test cells were resuspended in complete medium consisting of α-medium (Gibco) supplemented with FBS (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. The total number of clonogenic cells (i.e., CFU-Mix plus BFU-E plus CFU-GM) present in 5-week-old LTC provides a relative measure of the number of LTC-IC originally present in the test suspension. 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, according to limiting dilution analysis studies reported by others.[41]

### Statistical analysis

Four plates were scored for each data point per experiment and the results were expressed as the mean ± 1 standard error (SE). Statistical analysis was performed with the statistical package Statview (BrainPower, Calabasas, CA) run on a Macintosh Performa 6300 personal computer (Apple). The two-tailed Student's *t* test for unpaired data was used to test for significance of differences between samples.

## RESULTS

The effect on PBPC mobilization of adding amifostine to a conventional epirubicin + G-CSF chemotherapy regimen was investigated in 10 patients (Table 1) with solid tumors who were scheduled to receive two consecutive cycles of epirubicin + G-CSF and were randomized to receive amifostine in conjunction with the first or second cycle of epirubicin. Side effects related to amifostine administration were nausea and hypotension, which never required drug discontinuation.

The kinetics of white blood cell and platelet counts were not affected by amifostine administration. In striking contrast, the mobilization kinetics of $CD34^+$ cells (Fig. 2A), and CFU-GM (Fig. 2B) were significantly affected by amifostine. Similarly, amifostine significantly increased the mobilization of CFU-Mix and BFU-E. Peak values of $CD34^+$ cells and progenitor cells after epirubicin + G-CSF or epirubicin + G-CSF + amifostine were detected on days 9 and 10, respectively.

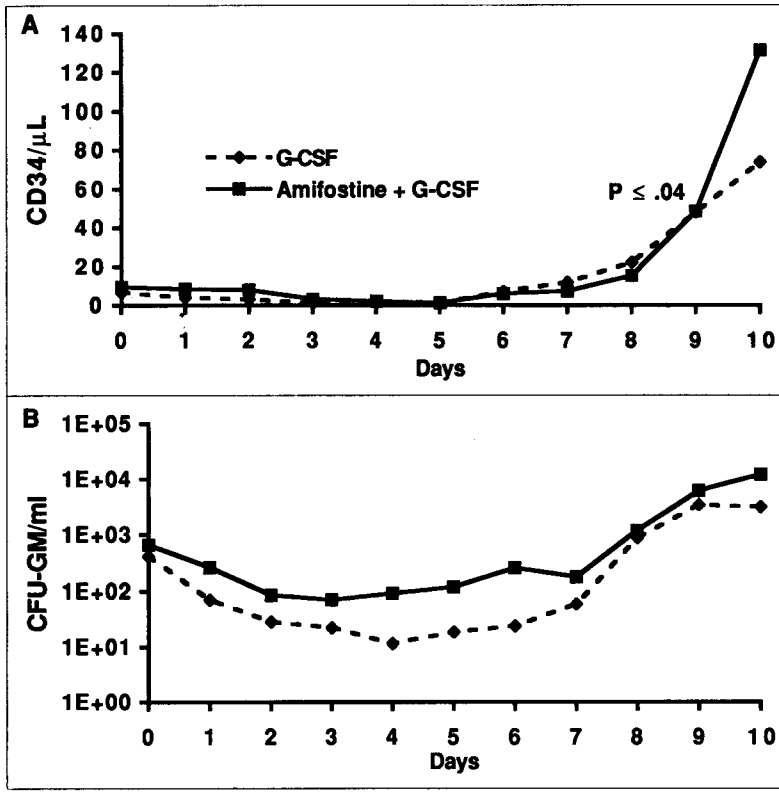


**Figure 2.** *Kinetic of mobilization of CD34⁺ cells (A) and CFU-GM (B) in patients receiving epirubicin + G-CSF (◆) or epirubicin + G-CSF + amifostine (■).*

Addition of amifostine to epirubicin + G-CSF resulted in a statistically significant ($P<0.05$) increase in the peak incidence (mean ± SE) of CD34⁺ cells/mL (131 ± 26 vs. 74 ± 19), CFU-Mix/mL (466 ± 175 vs. 139 ± 38), BFU-E/mL (6363 ± 1479 vs. 2678 ± 1235), and CFU-GM/mL (12,236 ± 3059 vs. 3522 ± 1314). Amifostine also affected the mobilization of primitive hematopoietic progenitors by inducing a threefold increase of LTC-IC growth.

When peak values of progenitor cells were divided by day 0 values, epirubicin + G-CSF + amifostine vs. epirubicin + G-CSF resulted in significantly higher fold increases for CFU-Mix (73 vs. 6), BFU-E (67 vs. 29), and CFU-GM (156 vs. 73). Amifostine-induced enhancement of PBPC mobilization was evident in patients receiving the drug at both the first or second cycle of chemotherapy (data not shown). At the phenotypic level, CD34⁺ cells mobilized with amifostine were not different from those mobilized without amifostine in terms of CD38, HLA-DR, and CD45RA expression.

## DISCUSSION

The lack of optimal regimens for PBPC mobilization associated with the wide individual variability in mobilization yields are major factors supporting the need for the development of mobilization protocols aimed at improving the quantity and quality of collected PBPC.

This randomized study demonstrates that addition of amifostine significantly increases the mobilization of $CD34^+$ cells, primitive LTC-IC as well as lineage-restricted progenitors, in cancer patients receiving a regimen consisting of epirubicin + G-CSF. The lack of phenotypic differences between $CD34^+$ cells mobilized without or with amifostine associated with the enhanced mobilization of LTC-IC demonstrates that amifostine-mediated release of $CD34^+$ cells is not merely due to an increase of mature $CD34^+$ cells. Interestingly, the enhancing effect of amifostine on mobilization was evident even in heavily pretreated patients. Similarly, enhanced mobilization was also evident in patients randomized to receive amifostine at the second cycle of epirubicin + G-CSF, thus suggesting a potential role of amifostine in poor mobilizers.

A variety of mechanisms are likely to be involved in the transient release of progenitors into peripheral blood. Growth factors modulate cell adhesion molecule expression, extracellular matrix metabolism and ligand-receptor systems supporting progenitor cell adhesion to stroma. Chemotherapy is known to induce the disruption of marrow endothelial cell barriers. Different mobilization patterns displayed by chemotherapy-based regimens with different cytoreducing potential suggest an agent- and dose-dependent mobilization effect via the degree of induced myelosuppression. The selective mobilization of LTC-IC and clonogenic progenitors by chemotherapy-based regimens suggests that marrow aplasia might enhance PBPC mobilization potential, likely by affecting cell cycle induction and rate of primitive cells. Moreover, possible toxic effects of mobilizing agents or doses on the stromal/stem cell compartment might be relevant in determining the mobilization pattern of primitive progenitors.

The mechanisms involved in amifostine-mediated enhancement of PBPC mobilization remain a matter of hypothesis. The well-known chemoprotection exerted by amifostine on progenitor cells following injection of a number of different chemotherapeutic agents[26–33] might play a major role in increasing the release of primitive and committed colony-forming cells. In fact, $CD34^+$ cells and hematopoietic progenitors spared by amifostine could be released in the blood during the recovery phase after epirubicin + G-CSF. However, a modulating effect of amifostine on adhesion molecules could also be involved in improving mobilization and is currently under investigation.

In the present study, amifostine was injected before epirubicin and for 4 days thereafter. While the rationale of injecting amifostine before chemotherapy is

immediately evident and relies on the sparing action of amifostine on hematopoietic progenitors, injection of the drug after chemotherapy was based on the hypothesis that prolongation of amifostine injection might eventually result in an in vivo stimulation of hematopoiesis, as has been shown in patients with myelodysplastic syndromes.[39] We are currently testing whether post-chemotherapy amifostine administration could be started on day 7 after chemotherapy to further improve the mobilizing efficacy.

In conclusion, this randomized study demonstrates that addition of amifostine to epirubicin + G-CSF significantly increases the mobilization of $CD34^+$ cells and primitive and committed progenitors in pretreated cancer patients. We are currently investigating the mechanism(s) of action of amifostine as well as the efficacy of different schedules.

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# CHAPTER 8
# AUTOIMMUNE DISEASE

# Autologous Stem Cell Transplantation for Autoimmune Diseases: Clinical Indications

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A number of autoimmune rheumatic diseases are known to cause substantial morbidity and mortality despite current therapy. With significant improvements in the technology of hematopoietic stem cell transplantation (HSCT), it is increasingly possible to apply this modality to the treatment of these disorders. A consensus report written on behalf of the European League Against Rheumatism (EULAR) and the European Group for Blood and Marrow Transplantation (EBMT)[1] have suggested that only diseases with an increased risk of mortality should be considered. This list of rheumatologic disorders included progressive systemic sclerosis (scleroderma) (PSS), autoimmune pulmonary hypertension, necrotizing vasculitis, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), antiphospholipid antibody syndrome, cryoglobulinemia, and several pediatric disorders, such as scleroderma, dermatomyositis, and necrotizing vasculitis. I will confine my remarks to patients with RA, SLE, and PSS.

The rationale for considering HSCT in the rheumatologic diseases listed above stems not only from their considerable morbidity and their substantial economic burden on society but also their mortality.[2] RA has a significant morbidity with almost 60% of patients unable to work within 10 years of disease. Life expectancy is reduced on average about 10 years. With SLE, the 10-year survival rate is approximately 75–90%. PSS has a 5-year mortality rate of 40%. With a mortality rate now associated with autologous stem cell transplantation (autoSCT) of approximately 5% and a morbidity of less than 15%, the time is right for examining this form of treatment for these serious life-threatening rheumatic disorders.

Until now there are only a few case reports[3] published concerning autoSCT in RA, SLE, and PSS utilizing either peripheral blood or bone marrow stem cells. The results were promising, with improvement or remission lasting in some cases as long as 30 months. In almost all patients, improvement was temporary. The problem with data generated to date is that, in most cases, autoSCT was performed with

unmanipulated grafts. Recent data has supported the use of lymphocyte depletion using positively selected $CD34^+$ cells with or without anti–T cell treatment.[4]

There are a number of factors currently affecting patient selection for autoSCT. First, over the past 10–15 years there has been a substantial improvement in therapy, particularly for patients with RA. The prognosis of RA, SLE, and PSS has improved as a consequence of better overall supportive care (e.g., antihypertensive medication and antibiotics). Earlier diagnosis and more aggressive therapy have also contributed to a better outcome. More aggressive treatment earlier in disease has been particularly utilized in patients with a poor prognosis. This approach has been made possible by the generation of a considerable amount of data concerning the clinical and laboratory features associated with a poor prognosis for these rheumatic disorders. One caveat here, however, is that the prognostic indicators are relatively imprecise. Thus, the positive predictive value for these factors is nowhere near the value required to accurately predict the prognosis for an individual patient. On this account, one must view with caution the selection of patients based predominantly on prognostic indicators.

In concert with the consensus report from EULAR and EBMT,[1] patients should be selected for autoSCT who have severe disease and are refractory to conventional therapy. The presence of poor prognostic indicators would provide additional support. In selecting patients, consideration should be given to the balance between disease severity and organ damage. Thus preexisting dysfunction of kidneys, liver, and lung is associated with increased mortality from transplant regimens. In this regard, autoSCT performed earlier in disease is more likely to be successful given that there will usually be less organ damage and fewer toxic treatments pretransplantation.

Application of the selection criteria suggested above for RA would generate the following set of criteria. Patients should have severe active arthritis, defined as the presence on physical examination of at least 20 swollen joints and significant disability such that only self-care is possible.[2] Refractory therapy implies failure of prednisone and disease-modifying drugs including parenteral gold, combination therapy with methotrexate, salazopyrine, and hydroxychloroquine, as well as the soon-to-be-released tumor necrosis blockers. These latter agents have been shown to have substantial efficacy in RA, particularly in combination with methotrexate. The presence of poor prognostic indicators such as early erosions on x-ray, rheumatoid factor, and HLA-DRb1*0404/0401 would be supportive.

Recently, Hahn outlined selection criteria appropriate for ASCT in SLE.[5] Consideration was given to patients with life-threatening disease who exhibited an inadequate response to standard therapy of 3 months' duration including high-dose glucocorticoids and cytotoxic drugs. Adequate function of all major organs to tolerate the preparative conditioning and transplantation was considered essential. Prognostic indicators suggesting a poor outcome in SLE include renal dysfunction,

hypertension, anemia, low C3, and possibly central nervous system involvement and thrombocytopenia. Patients with these prognostic features have a considerably shortened life span. Thus, the 10-year survival of patients without the features is estimated to be 86% compared with 60% of patients with these features.

Scleroderma would appear to be an ideal rheumatic disease for consideration of autoSCT. Currently, there is no adequate conventional therapy for this disorder. Moreover, there is a high mortality rate, particularly in patients who have poor prognostic features. However, significant pulmonary involvement is a feature of scleroderma, precluding autoSCT in the most severely involved patients. Poor prognostic factors include diffuse skin involvement, pulmonary dysfunction (forced vital capacity [FVC] between 50 and 70%, severe restrictive disease, and a diffusing capacity [DLCO] of <70%), cardiac involvement (pericardial effusion, cardiomegaly, LVH, CHF, and infarct), and renal dysfunction. Given the factors listed above, it has been suggested that consideration be given to patients with scleroderma who have diffuse skin involvement, <3 years of disease, a skin score of >20 (modified Rodnan method), and visceral organ dysfunction (≥1).[6]

In conclusion, preclinical and early studies of autoSCT in human autoimmune disease are promising although long-term efficacy remains to be determined. Improved therapy and hence prognosis of patients with autoimmune disorders limits patient selection to some extent. Until more definitive data concerning the effectiveness of autoSCT is available, it should be considered in patients with severe refractory disease. Scleroderma appears to be an ideal indication for autoSCT.

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# Autologous Blood Stem Cell Therapy for Progressive Multiple Sclerosis

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## ABSTRACT

There is strong evidence supporting an immunopathogenic basis for multiple sclerosis (MS), an incurable, severely disabling disease of the central nervous system (CNS). It usually responds to treatment initially, but for the rapidly evolving and the chronic progressive (pMS) forms, there is no effective therapy. Based on the encouraging results of experimental transplantation in animal autoimmune disease models, including allergic encephalomyelitis, we have conducted two consecutive phase I/II trials to investigate the effect of blood stem cell transplantation (SCT) in a total of 24 patients with advanced pMS (median EDSS score 6; range 4.5–8) and evidence of active disease, i.e. gain of 1 EDSS point in the year preceding SCT. In both trials, blood SCs were mobilized with cyclophosphamide (CY) at 4 g/m$^2$ and granulocyte colony-stimulating factor (G-CSF) without serious toxicity or flare of the disease. The BEAM regimen (BCNU, etoposide, cytosine arabinoside, melphalan) was employed for conditioning at doses used in lymphomas. Antithymocyte globulin (ATG) at one-third the dose used to treat aplastic anemia was given after stem cell infusion, along with soluble methylprednisolone. In the second trial, the graft was CD34$^+$-selected, which yielded a 3- and 1.8-log reduction of T and B cells, respectively. Toxicity included infections, mild veno-occlusive disease (VOD) (one case), thrombotic microangiopathy (TTP) (one case) necessitating plasma exchange, and one death from aspergillosis (transplant-related mortality [TRM], 4%). Some neurotoxicity developed in 10 patients early post–SC infusion, but it was mild and transient. Median follow-up time is 21 months (range 6–33). Twenty patients were stabilized (10) or improved (10) on the EDSS score. Relapses (disease exacerbations) occurred in five patients. The cumulative hazard of progression at 3 years is 14%. These results indicate that SCT is feasible in MS and may also have a beneficial effect. Well-designed phase II trials are needed to confirm a positive result of SCT in MS, in which efficacy of a treatment is notoriously difficult to assess.

## INTRODUCTION

Multiple sclerosis is a severely disabling, incurable disease caused by a T cell–mediated destruction of myelin in the central nervous system. Affected areas are characterized by disruption of the blood–brain barrier (BBB) and infiltration with lymphocytes and macrophages. This leads to myelin breakdown and focal neurologic dysfunction. The disease runs a relapsing-remitting course initially, but after 10 years becomes chronic progressive (pMS) in 50% of the cases. Disability accumulates more rapidly at this stage of secondary progression and, 5 years later, more than half of the patients are unable to walk unaided.[1] In 15% of the cases, MS is progressive from onset, with or without relapses (progressive-relapsing or primary pMS, respectively). So-called malignant forms with very short survival exist, too (1–3%). Life expectancy is reduced by ~10 years, compared with an age-matched healthy population.[1] Treatment involves immunosuppressive or immunomodulating agents, which appear to be active in the relapsing-remitting form, whereas in the progressive forms all proposed treatments have very little, only transient, or no effect.[2]

The rationale for using SCT in autoimmune diseases (AD) including MS is based on experimental data and also on clinical observations in patients with AD and concomitant malignancies treated with SCT.[3,4] In experimental allergic encephalomyelitis, an animal model of induced MS, prevention or regression of disease can be achieved by allogeneic, syngeneic, or autologous SCT.[5–8] In the clinical setting, cures of AD have been seen after alloSCT[4] and also prolonged remissions have been noted after autoSCT.[4,9] Relapse rates in experimental SCT depend on residual autoreactive clones surviving high-dose therapy or on high lymphocyte content of reinfused grafts (autoSCT).[8] In clinical SCT, while relapses occur after allo- or autoSCT, they are more frequent after autoSCT.[4,10] It is postulated that alloSCT can cure AD through immune ablation, replacement of the aberrant immune system, and also a graft-vs.-autoimmunity effect.[5] In autoSCT, however, total immune ablation is an impossible goal, but the patient may benefit from the profound immunosuppression of the conditioning regimen. One could also envisage a sort of reeducation of the immune system through induction of suppressor mechanisms or recapitulation of lymphocyte ontogeny.[11–13]

Although more reasonable, an allogeneic transplant may be too toxic for the treatment of AD. We have, therefore, used autoSCT to treat 24 patients with pMS in two consecutive pilot phase I/II trials, in which we studied the medical and neurologic toxicity of the protocol and also its efficacy in terms of improvement or stabilization of the disease. In both trials, the same protocol was used with the exception that CD34 selection was employed only in the second trial. Early results of trial 1 have already been published (14). In this chapter, we provide an update and also present the results of the second trial.

**Table 1.** Patient characteristics

| | *Trial 1* | *Trial 2* |
|---|---|---|
| No. patients | 15 | 9 |
| Age (years) | 37 (24–54) | 43 (22–51) |
| Age <40 years | 9 | 3 |
| Progressive disease: all patients | | |
| Primary | 6 | 2 |
| Progressive/relapsing | 2 | 1 |
| Secondary | 7 | 6 |
| Duration of disease (years) | 10 (2–28) | 11 (4–25) |
| Duration of progressive phase (years) | 6 (2–17) | 7 (4–8) |
| Kurtzke EDSS score | 6 (5–7.5) | 6 (4.5–8) |
| Scripps NRS score | 42 (33–62) | 69 (54–80.5) |
| Gadolinium enhancement | 2 | 1 |
| Follow-up (months) | 24 (21–33) | 12 (6–18) |

## PATIENTS AND METHODS

### Patients

The first trial was initiated in April 1995 and closed 2 years later having included 15 patients. In July 1996, we started the second trial and treated nine patients until January 1998. Patient characteristics are shown in Table 1. All patients had MS in the progressive phase with considerable disability and with evidence of active disease. Scores on the Kurtzke Expanded Disability Status Score (EDSS) ranged from 4.5 (patient can walk unaided some 300 meters) to 8 (patient essentially restricted to chair or bed). Scores on the Scripps Neurologic Rating Scale (SNRS) ranged from 33 to 80 (normal, 100). The patients had failed to respond to previous therapies, i.e., steroids, ACTH, azathioprine, mitoxantrone, intravenous immunoglobulin (IVIG), plasma exchange, and FK506. All had gained (worsened by) 1 EDSS point over the year preceding enrollment and two had gadolinium-enhancing areas on magnetic resonance imaging (MRI). The median follow-up is 2 years for trial 1 and 1 year for trial 2, a period that could be considered only relatively adequate to judge the effect of a treatment in this chronic and often unpredictable disease.

### Stem cell mobilization

Peripheral blood stem cells were used for autografting. Mobilization was performed with cyclophosphamide (CY) at 4 g/m$^2$ and G-CSF or granulocyte-macrophage (GM)-CSF at 5 μg/kg body weight (Table 2) as previously described.[14] In trial 2, Cy at the same dose and G-CSF at 10 μg/kg were employed

**Table 2.** Stem cell mobilization and toxicity

| | *Trial 1* | *Trial 2* |
|---|---|---|
| Mobilization | | |
| CY | 4 g/m$^2$ | 4 g/m$^2$ |
| G-CSF | 5 μg/kg | 10 μg/kg |
| GM-CSF | 5 μg/kg | |
| Toxicity | 7/15 patients | 5/9 patients |
| Allergy | 4 (CY, GM-CSF) | |
| Fever | 2 (grade 2) | |
| Bronchial spasm | 1 (grade 3) | |
| Hypotension | 1 (grade 4) | |
| Oral | 3 (grade 1) | |
| Infections | 9 | 4 |
| Fever | 8 (grade 2) | 3 (grade 2) |
| Bleeding | 1 (grade 3) | |
| UTI | 1 (grade 1) | 1 (grade 1) |
| Herpes | | 1 (grade 1) |
| Epileptic seizure | | 1 |

to conform to the guidelines of the EBMT/EULAR Consensus[15] (Table 2). There were no failures of mobilization. The time from mobilization to transplantation ranged from 16 to 52 days (median, 33) in trial 1 and 29 to 156 days (median, 81) in trial 2. In the latter, CD34$^+$ cell selection of the graft was performed on the Seprate SC System (CellPro). The procedure resulted in 3- and 1.8-log reductions of CD3$^+$ and CD20$^+$ cells, respectively.

## Transplant procedure

The BEAM regimen was administered for conditioning in both trials, at doses used in SCT for lymphomas: BCNU 300 mg/m$^2$ on day –6, etoposide 200 mg/m$^2$ on days –5 to –2, cytosine arabinoside 200 mg/m$^2$ on days –5 to –2, melphalan 140 mg/m$^2$ on day –1. The infused doses of the CD34$^+$ cells/kg were $11.6\times10^6$ (±1.2) and $3.8\times10^6$ (±1) in trials 1 and 2, respectively. The respective doses of CD3$^+$ cells/kg were $96.8\times10^6$ (±27) and $0.07\times10^6$ (±0.04). In trial 2, the mean dose of CD20$^+$ cells/kg was $0.12\times10^6$ (±0.08).

On days 1 and 2, to deplete lymphocytes in vivo, rabbit ATG (thymoglobulin; Merieux) or horse ATG (Atgam; Upjohn) was administered at a total dose of one-third the recommended dose for treatment of aplastic anemia; i.e., patients received 10 mg/kg or 60 mg/kg over 2 days, respectively. Soluble methylprednisolone (S-M, 0.5 g/d) was also given with ATG to prevent adverse manifestations due to cytokine release. As described previously,[14] for prevention of infection the patients

were taking oral ciprofloxacin and fluconazole or itraconazole. IVIG was also given every 2 weeks for a total of four doses, starting on day –7.

### Neurologic evaluation

Assessments were made by two neurologists using two scoring systems (EDSS, SNRS), as previously described.[14] Evaluations were done at entry, after mobilization, or before SCT, 1 and 3 months after stem cell infusion, and at 3-month intervals thereafter. Serial MRIs were also performed along with the clinical assessments. For definition of responses, we have modified the criteria we described previously.[14] Improvement in disability was indicated by reduction of EDSS of ≥1 points, or a gain of at least 10 SNRS points, persisting for at least 3 months. Progression of the disease was defined as a gain of ≥1 EDSS points if the baseline score was below 5.5, or as a gain of only 0.5 point if the baseline score was above 5. These changes should be shown to persist for as long as 6 months. A change of <1 point if the patient's initial score was below 5.5 was considered stable disease. Exacerbations of the disease, usually of isolated symptoms (e.g., visual aggravation, deterioration of ataxia) were regarded as relapses and were treated with S-M at 1 g intravenously for 5 days.

## RESULTS

### Toxicity

Toxicity during mobilization is shown in Table 2. Allergic reactions were more frequent with GM-CSF, which caused severe hypotension in one case. In general, compared with an age-matched population with malignant lymphomas treated with SCT over the same time period, MS patients developed allergic manifestations and fever of unknown origin more frequently ($P<0.05$ and $P<0.01$, respectively; data not shown). There was no serious neurotoxicity during mobilization, apart from an epileptic seizure in a patient with known epilepsy in trial 2.

After stem cell infusion, infections, mainly bacteremias, were the most problematic toxicity (Table 3). Toxicity in trial 2 appeared more serious, including one case of TTP necessitating plasma exchange; one case of VOD; and one fatal case of invasive aspergillosis (died on day 65). Viral infections (e.g., cytomegalovirus [CMV] reactivation detected by polymerase chain reaction [PCR]) were also more frequent in trial 2. Compared with toxicity in lymphomas, MS patients had more allergic reactions, more blood infections ($P<0.0001$ and $P<0.01$, respectively; data not shown) but less oral toxicity, especially grade 3 and 4 ($P<0.001$; data not shown). Treatment-related mortality was similar in MS and lymphoma patients, 4.2% (one of 24) and 5.4% (two of 37), respectively.

**Table 3.** Toxicity after stem cell infusion

| | *Trial 1* | *Trial 2* |
|---|---|---|
| Early toxicity, until day 30 | | |
| Allergy | 14/15 | 9/9 |
| Erythema | 4 (grade 1) | 1 (grade 1) |
| Fever | 9 (grade 1–2) | 4 (grade 2) |
| Hypotension | 2 (grade 4) | 2 (grade 4) |
| Bronchial spasm | 2 (grade 3) | |
| Anaphylaxis | 2 (grade 4) | 2 (grade 4) |
| Sserum sickness | | 1 |
| Oral | 7 (grade 1–3) | 5 (grade 1–2) |
| Bleeding | 1 (grade 3) | 2 (grade 3) |
| Elevated liver enzymes | 3 (grade 1) | 4 (grade 1–3) |
| VOD | | 1 mild |
| Infection | 13/15 | 8/9 |
| Fever | 1 (grade 2) | 1 (grade 2) |
| Bleeding | 9 (grade 3) | 4 (grade 3–4) |
| Pneumonia | 3 (grade 3) | 1 (grade 3) |
| UTI | 2 (grade 2) | 1 (grade 2) |
| Fungal | 1 (grade 3) | 1 (grade 4, death) |
| Late complications, days 30 to 100 | | |
| Infection | 3/15 | 6/9 |
| Fever | 1 (grade 2) | |
| Bleeding | 1 (grade 2) | 1 (grade 2) |
| Sinusitis | 1 (grade 2) | |
| UTI | | 1 (grade 2) |
| Viral | 1 (grade 3) | 6 (grade 3) |
| Pericarditis | | 1 (grade 3) |
| TTP | | 1 plasma exchange |
| Late complications, beyond day 100 | | |
| Viral infection | | 2 (grade 2–3) |
| Hashimoto disease/hypothermia | | 1 |

Ten patients, six in trial 1 and four in trial 2, had evidence of neurologic decompensation (Table 4), especially during the periods of infection, with increases in EDSS scores. However, that was mild and only transient, and all those patients resumed their previous scores within 30 to 60 days after stem cell infusion.

Hematologic recovery was rapid, and there was no secondary graft failure. The median time to 500 neutrophils/μL was 10 (8–11) and 11 (10–13) days in trials 1 and 2, and the median time to 50,000 platelets/μL was 26 (19–36) and 28 (21–65) days. The average number of days spent in hospital for SCT was 28 (±1.3) in trial 1 and 32 (±5) in trial 2 ($P<0.001$).

**Table 4.** Transplantation-related neurotoxicity

| | *Trial 1* | *Trial 2* |
|---|---|---|
| No. of patients with toxicity | 6/15 | 4/9 |
| Visual aggravation | 2 | |
| Headache | 2 | |
| Confusion-disorientation | 1 | 1 |
| Vertigo | 1 | 1 |
| Deterioration of ataxia | 1 | |
| Epileptic seizures | | 2 |
| Weakness | | 1* |
| Transient gain of 0.5 EDSS | 3/15 | 3/8 |

**Gain of 1.5 EDSS points at 30-day assessment.*

## Efficacy

In this chapter, changes in the SNRS and in MRI scans are not presented. Changes in the Kurtzke EDSS are shown in Tables 5 and 6. If we consider the patients altogether, improvement in disability has so far been detected in 10, and stabilization has occurred in 10 of 23 patients (43%). Patients with MS progressive from disease onset responded equally well as patients with secondary pMS. Improvements on the EDSS were more frequent in patients <40 years of age and in patients with short disease duration (data not shown). In three patients (13%), the disease did not respond to therapy and progressed. Actuarial progression-free survival is 86% at 30 months (Fig. 1). Four patients had relapses, as shown in Table 7. None of these four has had evidence of generalized disease progression, as defined above.

Some evidence of less disease on the MRI at 1 year post-SCT was provided by MRI analysis in 11 patients with secondary pMS who had no missing data over the follow-up period: five active lesions were counted in two patients at baseline (11 scans) but, at 1 year, only four active lesions were found in two (other) patients (66 scans; data not shown).

## DISCUSSION

The results indicate that autoSCT is feasible treatment for patients with active, advanced-stage MS. Stem cell mobilization did not cause serious adverse events or exacerbations of the disease. CSFs have been reported to induce disease flares when given to patients with AD to treat neutropenia.[9,16,17] This is probably prevented by CY, which can also be given at lower doses for mobilization to avoid hematologic toxicity—unless an additive immunosuppressive effect by the high-

**Table 5.** Trial 1: changes in the Kurtzke EDSS

| | | Post-SCT (months) | | | | |
|---|---|---|---|---|---|---|
| *Patient no.* | *At entry* | *6* | *12* | *18* | *24* | *30* |
| Progressive MS from onset of disease | | | | | | |
| Improved | | | | | | |
| 1. Primary | 5 | 3.5 | 3 | 2 | — | 2 |
| 5. Relapse | | 5 | 3 | 3.5 | 3.5 | 4 |
| 13. Relapse* | 7 | 5.5 | — | —† | | |
| 14. Primary | 7.5 | 6.5 | 6.5 | 6.5 | | |
| Unchanged/stable | | | | | | |
| 8. Primary | 6 | 6 | — | — | — | |
| 12. Primary | 6.5 | 6.5 | 6.5 | 6.5 | 7‡ | |
| 15. Primary | 6 | 6 | 6 | 6.5‡ | | |
| Worsened | | | | | | |
| 3. Primary | 6 | 6.5 | 6.5 | 6.5 | 7 | |
| Secondary progressive MS | | | | | | |
| Improved | | | | | | |
| 2. | 7 | 6 | 6 | 6 | 6 | 6 |
| 7. | 5 | 2.5 | 2.5 | 3 | 3.5 | 3.5 |
| 11. | 7 | 6.5 | 6 | 6 | 6.5 | |
| Unchanged/stable | | | | | | |
| 4. | 6.5 | 6.5 | — | 6.5 | — | |
| 6. | 6.5 | 6 | 6 | 6 | 6 | |
| 10.* | 6 | 5.75 | — | 5.5 | 6 | |
| Worsened | | | | | | |
| 9. | 5 | 6 | 6 | 6 | — | |

**Gadolinium enhancement on MRI at entry; †no gadolinium enhancement; ‡will be considered worsened if confirmed at next assessment.*

dose CY is desirable. After stem cell infusion, toxicity did not seem to differ from the toxicity occurring in patients undergoing autoSCT for lymphomas or solid tumors. Treatment-related mortality appears to be the same, around 5%, but even this may be unacceptable in patients suffering from a nonmalignant disease. Therefore, selection of patients with particularly adverse prognostic signs is of critical importance, although to define the pattern of deterioration in this disease is not easy.[1]

Milder conditioning regimens not carrying a high mortality risk could possibly be used instead of the BEAM, BUCY, or TBI. However, less intensive regimens are associated with high relapse rates in EAE[5] and also in human rheumatoid arthritis.[18] Moreover, it is necessary to perform some sort of adequate T cell depletion because high lymphocyte doses[8] or unmanipulated grafts[10] are also

**Table 6.** Trial 2: changes in the Kurtzke EDSS

| | | *Post-SCT (months)* | | | |
|---|---|---|---|---|---|
| *Patient no.* | *At entry* | *3* | *6* | *12* | *18* |
| Improved | | | | | |
| 1. Secondary | 8† | 6 | 6 | 6 | 6 |
| 4. Secondary | 6 | 6 | 5.5 | 6 | 5* |
| 8. Primary | 5 | 5* | | | |
| Stable | | | | | |
| 3. Secondary | 6.5 | 6.5 | 6.5 | 6 | 6‡ |
| 5. Secondary§ | 4.5 | 4 | 4 | 4 | |
| 7. Primary/relapse | 6 | 6 | 6 | | |
| 9. Secondary | 5 | 4.5 | 4.5 | | |
| Worsened | | | | | |
| 2. Primary | 6.5 | 7 | 6.5 | 7 | 7 |
| Dead | | | | | |
| 6. Secondary | 5.5(6) | X | | | |

**To be confirmed at next assessment; †sustained relapse of secondary pMS; ‡15 months post SCT; §gadolinium enhancement on MRI at entry.*

associated with relapses. We chose BEAM for conditioning because it is less organotoxic than BUCY or TBI but is still myelotoxic and immunotoxic. Moreover, it comprises drugs that can cross the blood–brain barrier. It has recently been shown effective as conditioning for autoSCT in refractory lupus[19,20] and also in MS patients treated in other institutions in Europe (personal communications). A 3-log T cell depletion is recommended for autoBMT in AD[8]; therefore, in blood SCT, greater depletion of T cells seems to be necessary. On the other hand, the T cell depletion caused by ATG given at the time of stem cell infusion may equal the effect of ex vivo lymphocyte depletion. Consequently, when ATG is used, other ex vivo manipulations for lymphocyte depletion may be superfluous and could only add to posttransplant toxicity,[21] as is shown in the present study. Nevertheless, the question of lymphocyte depletion of the graft is still open.

AutoSCT induced unexpected and striking improvements in the patients, even in cases of primary pMS, which is notoriously refractory to treatments. Neurologists are usually reluctant to admit any clinical improvements in chronic pMS, one of the most difficult diseases in which to judge the effect of a treatment. In addition, the unpredictability of the disease course can be argued against favorable and durable responses. It is, however, improbable that the responses (improvements in particular) observed in this study occurred spontaneously and to such a degree, since patients with active pMS usually deteriorate steadily with time.[1] Placebo groups in trials enrolling patients with pMS have a failure rate of 24

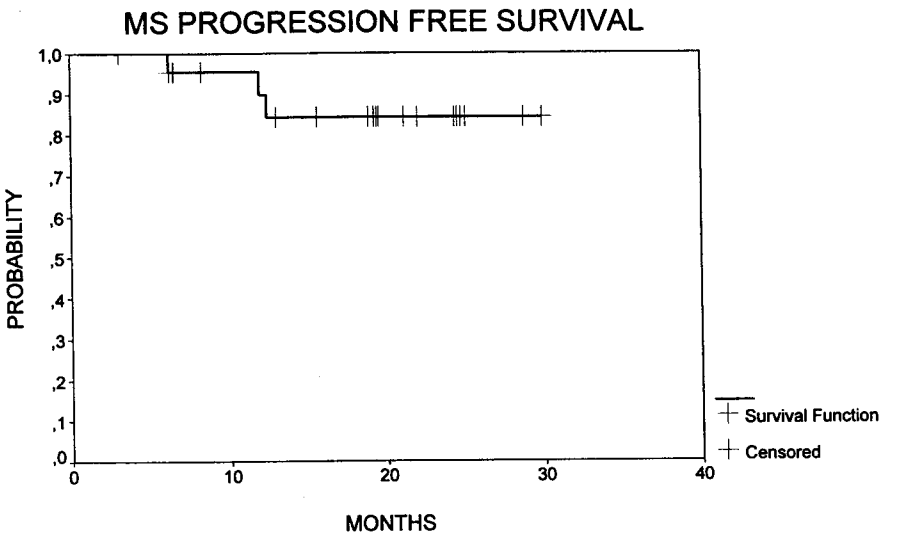


**Figure 1.** *Progression-free survival of MS patients submitted to SCT. The patient who died on day 65 is not included. Cumulative hazard of progression at 30 months is 14%*

to 80% over periods of 2 to 4 years (Comi G et al., manuscript in preparation). In the present study, the cumulative hazard of progression at nearly 3 years is only 14%, which is another indication of the efficacy of autoSCT. A beneficial effect of autoSCT has been observed, too, in three rapidly progressive cases of pMS, recently published.[22] The patients received the combination of CY (120 mg/kg) and

**Table 7.** Disease exacerbations (relapses) post-SCT

| *Patient no.* | *Time of occurrence (months)* | *Symptoms aggravated* | *Therapy* | *Outcome* |
|---|---|---|---|---|
| Trial 1 | | | | |
| 5. Primary/relapse* | 3 | Visual | 2-cdA | No effect |
| | 24 | Visual | S/medrol | No effect |
| 6. Secondary† | 12 | Spasticity Ataxia | No | |
| | 24 | Visual | No | |
| 4. Secondary† | 18 | Weakness in lower limbs | No | |
| Trial 2 | | | | |
| 1. Secondary* | 15 | Visual | S/medrol | Slight improvement |

**Improved on EDSS post SCT; †stable on EDSS post SCT.*

TBI (1200 cGy) for conditioning, which was well tolerated, and ex vivo T cell–depleted grafts. Functional improvement occurred in all three patients.

AutoSCT cannot possibly provide definite cure of MS. Three of our patients continued to deteriorate and, on MRI analysis, four new active lesions were found to have developed in two patients who had evidence of active MRI lesions at enrollment. However, it seems that autoSCT may induce prolonged stabilization, or may bring the disease to a lower level of activity, in the four patients who had disease flares without generalized progression. Longer follow-up of the patients is necessary, not only to confirm any beneficial effect but also to determine long-term toxicity. At present, it would seem reasonable to conclude that autoSCT in MS warrants further study, with well-designed phase II and, eventually, comparative trials.

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# Hematopoietic Stem Cell Transplantation of Systemic Lupus Erythematosus, Rheumatoid Arthritis, and Multiple Sclerosis

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## INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is being used as a treatment for severe autoimmune diseases (SADS).[1–4] Although this approach remains experimental and unproven, results from phase I trials are encouraging.[5–11] Besides being a potential therapy for patients with high-risk autoimmune diseases, HSCT offers an opportunity to study mechanisms of tolerance and autoimmunity. Initial studies on hematopoietic stem cell therapy for autoimmune diseases began in animal models.[12–21] These models continue to provide tools to analyze immunity and tolerance.

## RELAPSING EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (R-EAE)

R-EAE is an inflammatory demyelinating disease of the central nervous system (CNS) initiated by $CD4^+$ T lymphocytes.[22] Affected animals have a relapsing-remitting course that is clinically and histologically similar to relapsing-remitting multiple sclerosis. Disease is initiated by active immunization with myelin peptides homogenized in complete Freund's adjuvant or by adoptive transfer of lymphocytes from immunized mice. R-EAE is an autoimmune disease that can be induced in a wide variety of species including mice, rats, guinea, pigs, monkeys, and even humans. The relapsing-remitting course suggests that the immune system is not static but dynamic, with wide fluctuations in regulation of autoreactive clones. Disease progression is accompanied by an orderly recruitment of clones responsive to new myelin epitopes.[23,24] The most prevalent myelin protein is proteolipid protein (PLP). If disease is initiated to PLP 139–151 epitope, during each relapse lymphocytes become responsive to other myelin epitopes including PLP 178–191 as well as determinants in myelin basic protein (MBP), another protein within the lipid bilayer of myelin.

Syngeneic transplantation of the marrow from a normal mouse resulted in marked improvement in the neurologic disability and histology of mice with R-

EAE.[12,16] Because lymphocytes from a normal mouse do not respond (proliferate or release cytokines) when presented with PLP or MBP epitopes, we assumed that myeloablation with total-body irradiation and rescue with nondiseased syngeneic marrow would result in a return to myelin-specific immune unresponsiveness. However, despite clinical and histologic improvement, there was no improvement in proliferative responses. Improvement correlated with myelin-specific delayed type hypersensitivity but not proinflammatory cytokine profile or proliferative responses.[16] This indicates either a posttransplant dissociation between lymphocyte proliferative and cytotoxic effector pathways or presence of a posttransplant regulatory or suppressor subset of lymphocytes. HSCT of R-EAE appears to result in a posttransplant "pro-tolerance environment." The mechanisms and milieu of a posttransplant tolerogenic environment are unclear. We are currently studying this effect by retroviral mediated gene marking studies.

## THEILER'S MURINE ENCEPHALOMYELITIS VIRUS (TMEV)

TMEV is an enteric murine picornavirus that causes a CNS biphasic disease marked by an initial viral cytopathic infection followed by autoimmune-mediated demyelination. The disease has a chronic primary-progressive course similar to primary-progressive multiple sclerosis. The importance of the immune system in clearing virus and controlling the initial cytopathic effect is supported by clearance of TMEV from the CNS of disease-resistant murine strains, persistence of CNS infection in disease-susceptible strains, and abrogation of resistance by use of immunosuppressive total-body irradiation.[25–27] The importance of the immune system in mediating progressive demyelination is supported by correlation of demyelination with both virus-specific and myelin peptide–specific delayed type hypersensitivity reactions and prevention of demyelination in disease-susceptible strains of mice after treatment with immunosuppressive agents such as cyclophosphamide, antithymocyte globulin, or irradiation.[28,29]

Immune-mediated delayed-type hypersensitivity (DTH) responses to both virion proteins and myelin autoepitopes indicate simultaneous immunity to virus and autoimmunity to myelin epitopes. Immune-mediated destruction of myelin could arise by innocent bystander destruction of myelin after immune-mediated attack directed toward virus epitopes within myelin sheaths. However, approximately 4 weeks after disease onset, T cell responses to myelin epitopes arise in an ordered temporal progression.[29] Lack of crossreactivity between TMEV and myelin epitopes indicates that CNS autoimmunity arises by epitope spreading similar to R-EAE.[29]

Syngeneic transplantation from uninfected donor mice into mice with TMEV results in a high early mortality with an increased CNS viral titer (unpublished data). Allogeneic transplantation from uninfected disease-resistant donor strains

into infected disease-susceptible recipient strains results in an equally high mortality from neurologic disease. Allogeneic transplantation using healthy but previously infected disease-resistant donors into infected disease-susceptible recipient strains results in a significantly lower mortality. Therefore, in a viral-mediated autoimmune disease, the immune system is a double-edged sword. Temporary ablation of the immune system may unleash viral cytopathic effects. However, in those animals that survive transplant, immune-mediated demyelination is diminished. Further advances in HSCT of viral-associated autoimmune diseases should recognize the importance of controlling viral cytotoxicity after transplantation with either peritransplant antiviral drugs and/or viral specific adoptive immunotherapy at the time of graft infusion. HSCT in animal models of viral-induced autoimmunity may help understand the interrelationships between viral immunity, autoimmunity, and tolerance.

## SYSTEMIC LUPUS ERYTHEMATOSUS

The mortality from SLE improved with the introduction of better antihypertensive medications and after general acceptance of more aggressive immunosuppressive therapy with National Institutes of Health (NIH) short-course cyclophosphamide (500–1000 mg/m$^2$ monthly $\times$ 6 months, then quarterly as necessary).[30] However, as a rule of thumb, for all patients with SLE, the mortality is still 1% per year.[31–35] High-risk patients for early mortality are those with visceral organ involvement who have active disease despite corticosteroids and at least six cycles of cyclophosphamide (500–1000 mg/m$^2$). We consider these patients to be candidates for autologous HSCT.

Since SLE is responsive to cyclophosphamide, we choose a cyclophosphamide (200 mg/kg) and antithymocyte (90 mg/kg) conditioning regimen that is commonly used for allogeneic transplantation of aplastic anemia. This regimen also avoids the pulmonary and late carcinogenic side effects of total-body irradiation. Since patients are generally referred with active and refractory disease, we mobilize stem cells with cyclophosphamide (2 g/m$^2$) and granulocyte colony-stimulating factor (G-CSF) (10 μg/kg). The cyclophosphamide mobilization ameliorates disease activity, may help prevent a potential G-CSF flair of disease, and theoretically provides a partial in vivo purge of lymphocytes. Lymphocytes are further purged ex vivo by immunoadsorption for CD34$^+$ progenitor cells using the CellPro CEPRATE device.

For patients with SLE, end organ dysfunction is not necessarily a contraindication for transplantation provided a biopsy demonstrates reversible active disease, not just chronic fibrosis. This is exemplified by activity and chronicity indices on renal biopsy. For example, a patient with a creatinine of 5.0 mg/dL may still have significant improvement if the activity indices are high and chronicity score low.

Since cyclophosphamide is metabolized by the liver, we do not reduce mobilization dose or conditioning cyclophosphamide based on renal function.

We have performed autologous HSCT on four patients with SLE. The longest follow-up is a patient who is now >1 year after transplantation.[8,11] This patient has remained without evidence of active disease since transplantation. The creatinine has improved from 5.0 to 1.9 mg/dL; 24-hour urine protein from 3.6 to 0.2 g/d. The anti-dsDNA and complement (C3 and C4) have normalized. The patient's C3, C4, and serologies had been abnormal since disease onset 13 years ago. She is also currently off all medications including corticosteroids and antihypertensive drugs. Before transplant, she was corticosteroid dependent and had failed hydroxychloroquine, plasmapheresis, multiple courses of cyclophosphamide, azathioprine, and methotrexate and required four different antihypertensive medications for marginal control of blood pressure. The other three patients with SLE are earlier after transplant but are showing similar improvement.

There is no consensus on the definition of a complete remission in SLE. However, since transplantation, this patient has had no evidence of active disease, all previously abnormal parameters have normalized or markedly improved, and cushingoid habitus has resolved. The mechanism of posttransplant remission remains vague. Flow cytometric studies demonstrate an early decrease in $CD4^+$ helper cells, especially CD4/CD45RA (naive helper) cells, increase in natural killer (NK) cells, and decreased CD19 (B cells) but increased CD5/CD19 immature B cells. Within several months, the proportion of CD4/CD45RA cells began to increase but even by 1 year, the CD4/CD8 ratio remains inverted (<1). Horwitz et al.[36] suggest that suppressor $CD8^+$ positive cells that may be upregulated by NK cells exist in individuals with SLE. The posttransplant nonspecific increase in $CD8^+$ T cells and NK cells may provide a supportive environment for generation of lupus-specific regulatory cells.

## RHEUMATOID ARTHRITIS

Mortality from rheumatoid arthritis can be predicted by the number of involved joints and limitations in activities of daily living (ADL).[37,38] Patients with >30 involved joints or significant limitations in several of a number of daily activities such as opening a carton of milk have a 5-year survival of approximately 40%. We consider these patients candidates for transplantation if they have failed corticosteroids and at least two disease modifying drugs (e.g., penicillamine, gold, methotrexate, hydroxychloroquine). We define failure as more than six swollen joints and either >30 involved joints (swollen, tender, painful, deformed) or answering less than 15 of 20 activities of daily living questions "with ease."

Treatment of disease is continually evolving, and soluble tumor necrosis factor receptor (TNFR) is a new and promising therapy for RA.[39] Whether TNFR failure

should be considered a criterion to be considered a candidate for HSCT, or if phase III studies should compare HSCT to TNFR therapy, are debatable questions. Finally, subclinical pulmonary function abnormalities, especially low diffusing capacity (DLCO), are probably more common in RA than currently appreciated. We currently view these abnormalities not as contraindications to enrollment, but at least in some cases resulting from active rheumatoid interstitial pneumonitis that may respond to therapy.

The American College of Rheumatology criteria for complete remission requires that five or more of the following be fulfilled for at least two consecutive months: *1*) duration of morning stiffness not exceeding 15 minutes, *2*) no fatigue, *3*) no joint pain (by history), *4*) no joint tenderness or pain on motion, *5*) no soft tissue swelling in joints or tendon sheaths, *6*) erythrocyte sedimentation rate less than 30 mm/h for a female or 20 mm/h for a male.[40] Of interest, a complete remission does not depend on rheumatoid factor (RF) or normalization or improvement in joint radiographs. The significance of RF, an antibody against the Fc region of immunoglobulin, is uncertain. RF may conceivably be protective.

We have now treated four patients with RA. The first patient we treated with the same mobilization and conditioning regimen (cyclophosphamide and antithymocyte globulin) used for SLE had marked improvements for >1 year after transplantation.[11] Sedimentation rate normalized, swollen and tender joint count markedly improved, and corticosteroids and all other medications except hydroxychloroquine have been discontinued. Interestingly, RF has remained elevated. Although this patient's results are encouraging, durability of response is unknown.

## MULTIPLE SCLEROSIS

Of the autoimmune diseases we have mentioned, multiple sclerosis is the most difficult in which to identify patients with early mortality. Although most patients with MS have a normal life expectancy, severe neurologic disability lowers survival. In general, patients confined to a wheelchair have a 10-year survival of only 30%. The histopathology of multiple sclerosis is multifactorial, with components of acute inflammation, chronic demyelination, and neuronal degeneration. Intense immunosuppressive conditioning and autologous hematopoietic stem cell support would be anticipated to affect the inflammatory but not degenerative stages of MS. In progressive MS, transplantation, even if effective, may only result in stabilization of disease progression without functional improvements. If transplantation prevents progression of disease, its greatest benefit would therefore be in patients with relapsing-remitting disease at high risk of developing progressive and severe disability. Possible candidates are patients with more than three to five severe relapses in 1 year. However, as a phase I

toxicity study, we chose patients with primary or secondary progressive disease who are unable to walk without assistance, including use of a wheelchair.

We have performed transplants in nine patients with multiple sclerosis. Four patients are >1 year out from transplantation. The mobilization regimen is G-CSF (10 μg/kg/d) with apheresis beginning on day 5. The conditioning regimen is cyclophosphamide (120 mg/kg divided over 2 days) and total-body irradiation (1200 cGy divided b.i.d. over 4 days). TBI is given in the AP/PA position with a 40% lung block. Nonhematopoietic toxicity has been minimal. Disease activity is followed by neurologic rating scales, Kurtzke extended status disability scale (EDSS) and Scripps neurologic rating scale (NRS), relapse rate, and MRI. To date, all patients have demonstrated either no progression or slight improvements (>10 point improvement in the Scripps NRS).

## SUMMARY

Hematopoietic stem cell transplantation has become a therapy for autoimmune diseases. A few questions can be answered. For example, there are patients with autoimmune disorders whose disease is life-threatening. In these patients, the risk benefit ratio of a potentially lethal therapy such as HSCT is justified provided it is done in an experimental study. Also, in our phase I trials, HSCT was done with minimal non-hematopoietic toxicity. Efficacy of this procedure is suggested but not proven by these early phase I studies.

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# Autologous Stem Cell Therapy in Autoimmune Disease: Follow-Up of Cases and Issues Raised by the EBMT/EULAR Databank

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## INTRODUCTION

In severe autoimmune diseases (ADs), conventional therapy can be insufficient to halt progression, organ damage, and death. Side effects are common, especially bone marrow toxicity from cytotoxic agents and cumulative glucocorticosteroid effects on bone, skin, blood vessels, and other organs. Over the past two decades, several reports of individual cases and small series noted an improvement in coincidental AD when bone marrow transplantation was performed for a conventional indication such as malignancy or aplastic anemia.[1] This raised the possibility that bone marrow transplantation could be an option for AD alone, a concept supported by animal models.[2] Specifically, the reduced morbidity of autologous stem cell transplantation (autoSCT) together with the realization that the middle- and long-term morbidity of severe ADs is considerable, allowed this approach to be considered in the setting of ADs.

The diffuse form of systemic sclerosis (SSc), especially when rapidly progressive and with involvement of internal organs, and the limited form with pulmonary hypertension, have a bad prognosis.[3] In both forms of SSc, treatment of the autoimmune process with conventional immunosuppression and supportive measures has been of limited benefit in severe cases.[4] We therefore performed SCT with autologous reconstitution in two patients primarily to treat their AD, to achieve more substantial immunosuppression.

## FOLLOW-UP OF CASES

A 47-year-old female patient with limited SSc[3] developed dyspnea on exertion, which was attributed to pulmonary hypertension. Her functional status continued to deteriorate in spite of conventional therapy with corticosteroids and monthly pulses of cyclophosphamide. By the time her estimated mean pulmonary arterial pressure (PAP) had increased to 60 mmHg, she was short of breath while walking slowly on

an even surface. A lung transplant was discussed with the transplantation team, but was refused because of her underlying AD. Her antinuclear antibodies (ANA) were positive at a titer of 1:640, and anti-centromere antibodies were present at a titer of 1:10,240. The option of SCT was discussed with the patient and performed after approval by the ethics committee. Priming was with cyclophosphamide 2 × 3, 300 mg, followed by granulocyte colony-stimulating factor (G-CSF) 600 μg qd subcutaneously. Harvested cells were positively selected for the $CD34^+$ fraction with Ceprate column, and T and B cells were depleted with combined anti-CD2/3 (Dako) and anti-CD19/20 (Baxter) antibodies using a Maxsep selection device. Ten days later, conditioning with 50 mg/kg cyclophosphamide daily for 4 days was given. Then $10.1 \times 10^6$/kg T and B cell–purged autologous stem cells were infused. The infusion was without incident but antibiotics were given on day 5, a hypotensive episode occurred on day 6, cholecystitis was diagnosed on day 9, and a pleural effusion, on day 14. On day 15, an emergency cholecystectomy was performed because of the cholecystitis. Twenty-two days after the transplant, the patient was discharged in fair condition and experienced an unremarkable further course, except for an episode of dyspnea due to pulmonary emboli when her anticoagulation became insufficient. Thirty months after the transplant, the patient has markedly improved exercise tolerance with no limitation in her daily routine. She has remained off all therapy except for anticoagulation since the transplant. During this time, her ANA and anti-centromere antibodies have decreased markedly, and her mean estimated PAP has decreased to 38 mmHg.

A second female patient with diffuse SSc,[4] aged 37, had rapidly progressive skin involvement, contractures of peripheral joints, myositis, and developing pulmonary fibrosis as demonstrated by high-resolution computed tomography (CT) scan. Her ANA was positive at a titer of 1:5120, and she had anti-topoisomerase II antibodies at a titer of >1:1000. Within 6 months, her skin score increased to 41, and she was severely limited in her daily activities due to contractures of her hands, elbows, and shoulders. Her breathing was restricted by skin involvement of the chest. The possibility of SCT was discussed with the patient, who agreed to the procedure. Priming was with cyclophosphamide using two doses of 2 g/m$^2$ and G-CSF 5 μg/kg daily. On day 10, nucleated cells were harvested from the peripheral blood. The autograft was purged by positive selection for $CD34^+$ cells (Ceprate R), and T cells were depleted with anti-CD3 antibodies (Miltenyi). Complications included a mild, self-limiting hemorrhagic cystitis and an episode of herpes zoster, which resolved with acyclovir. After 6 months, the skin score had improved to 34, and after 24 months to 11. The restriction of her respiration resolved, muscle strength normalized, and the joint contractures improved markedly. No progression of pulmonary involvement was noted, and the patient has not had any immunosuppression since her transplant. She is again able to take care of her child and plans to return to work.

**Table 1.** Patients who received SCT primarily for AD

| *Autoimmune disease* | *No. of patients transplanted* |
|---|---|
| Multiple sclerosis | 36 |
| Scleroderma family | 18 |
| Rheumatoid arthritis | 13 |
| Juvenile chronic arthritis | 8 |
| Systemic lupus erythematosus | 6 |
| Cryoglobulinemia | 3 |
| Pure red cell aplasia | 4 |
| Other cases | 8 |

These two cases are evidence that intense immunosuppression followed by reconstitution with autologous stem cells can be effective treatment for ADs unresponsive to conventional immunosuppression and supportive measures. They raise several questions concerning therapeutic value and long-term outcome. Had conventional therapy failed because the degree of immunosuppression was inadequate, and if this is the case, what constitutes adequate immunosuppression? Would priming alone be enough? What are the reversible and irreversible components of clinically manifest pathology? Is long-term outcome influenced by whether an autologous or an allogeneic graft is performed? Will the disease recur and, if it does, in which form and how severely?

## THE EBMT/EULAR DATABANK

As of July 1998, a total of 96 patients who had received SCT primarily for their AD and not for a traditional indication of neoplasia were registered with the European Group for Blood and Marrow Transplantation/European League Against Rheumatism (EBMT/EULAR) databank (Table 1). The main diseases for which SCT was used were multiple sclerosis and the scleroderma group. The stem cell source was peripheral blood in the overwhelming majority of cases. Mobilization was primarily with cyclophosphamide and G-CSF, followed by G-CSF alone and cyclophosphamide and granulocyte-macrophage (GM)-CSF.

Of all cases, only two were not transplanted after mobilization, one due to lethal infection during mobilization and one due to a cardiac event in which the relationship to mobilization remains unclear. A further six deaths were reported, of which two were unrelated to the procedure. The four transplant-related deaths occurred in a patient with SSc with pneumonia and cardiac failure immediately after mobilization; a patient with multiple sclerosis and invasive aspergillosis 58 days after treatment; a patient with SSc with central nervous system (CNS) bleeding 40 days later; and a patient with SSc and influenza pneumonia.

B and/or T cell depletion was performed in only about a third of all cases. There was prolonged CD4 cytopenia in those cases for which data were reported. During the transplantation procedure, there were no major disease flares, and there was a tendency toward improvement in a significant number of cases.

The available data indicate that mobilization does not seem to be a major problem, even in patients previously treated with prolonged conventional immunosuppression. Aplasia after conditioning was similar to cases with traditional indications for the procedure. Transplant-related mortality was similar to that seen in a cohort of lymphoma patients treated during the same period, possibly due to the underlying disease processes and preexisting systemic tissue damage.

Issues that need to be resolved are criteria for patient selection, especially in view of prospective trials to determine the benefit of SCT for ADs. Without such trials, it will be impossible to define the patient population in whom SCT is beneficial without exposing patients to undue risk. Such trials will also help to establish the extent of immunosuppression needed to control the disease and to minimize the risk of relapse.

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# Autologous Hematopoietic Stem Cell Transplantation: An Alternative for Refractory Juvenile Chronic Arthritis

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## ABSTRACT

*Background.* In adults, autologous hematopoietic stem cell transplantation (autoHSCT) has been described recently as a possible treatment for severe autoimmune disease refractory to conventional treatment. We here report the four first children with severe forms of juvenile chronic arthritis (JCA) treated with autoHSCT. *Methods.* We studied three children with systemic JCA and one with polyarticular JCA. Unprimed bone marrow was harvested 1 month before autoHSCT. T cell depletion of the graft was performed with CD2 and CD3 antibodies. We used a preparative regimen of antithymocyte globulin (ATG, 20 mg/kg), cyclophosphamide (Cy, 200 mg/kg), and low-dose total body irradiation (TBI, 4 Gy). Methotrexate and cyclosporin A (CsA) were stopped before autoHSCT, and prednisone was tapered after 2 months. *Findings.* After AHSCT our patients showed a follow-up free of anti-inflammatory drugsof 6 to 18 months with a marked decrease in joint swelling, pain, and morning stiffness. Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and hemoglobin (Hb) returned to near normal values within 6 weeks. Despite T cell depletion, there was very rapid immune reconstitution. Two patients developed limited varicella Zoster virus (VZV) eruptions, which were treated with acyclovir.

## INTRODUCTION

Juvenile chronic arthritis is the most common autoimmune disease of childhood. Although the overall prognosis for most children with chronic arthritis is good, 5–10% of children with the systemic and polyarticular onset form are refractory to conventional therapy, consisting of combinations of nonsteroidal anti-inflammatory drugs (NSAIDs) and immunosuppressive drugs such as methotrexate and corticosteroids.[1,2] These patients ultimately develop severe joint damage and loss of joint function, while the chronic use of immunosuppressive drugs leads to adverse drug effects. To prevent severe joint damage and loss of joint function, new treatment strategies have been developed.[3] Recently, we

started a program for the treatment of refractory JCA with autologous hematopoietic stem cell transplantation. We here report the 6 to 18 months follow-up in the first four children with longstanding, severe JCA and polyarticular JCA treated with autoHSCT.

## Patients

*Inclusion criteria.* Children with systemic chronic arthritis and polyarticular chronic arthritis refractory to combinations of NSAIDs and immunosuppressive drugs such as methotrexate, CsA, and corticosteroids, and with a disease course longer than 6 months, were included in this study.

*Bone marrow harvest and T cell depletion.* Hematopoietic stem cells were obtained by conventional bone marrow aspiration under general anesthesia without priming. The graft was purged by two cycles of T cell depletion with CD2 and CD3 antibodies.[4] Finally, a suspension of $0.5–6.5\times10^6$ CD34 positive cells/kg and less than $0.5\times10^5$ CD3 cells/kg was stored in liquid nitrogen.

*Conditioning.* The conditioning regimen included 4 days of ATG (IMTIX, Pasteur Mérieux, France) in a dosage of 5 mg/kg daily from day –9 to –6, cyclophosphamide (Cy) in a dose of 50 mg/kg daily from day –5 to –2, and low-dose (4 Gy) single-fraction TBI on day –1.

## RESULTS

The clinical characteristics of the four patients are depicted in Table 1. They were characterized by a refractory chronic arthritis with the occurrence of erosions and growth disturbances of the joints. Laboratory investigations showed increased ESR and a moderate to severe anemia in all patients. Methotrexate and CsA were stopped before autoHSCT, and corticosteroids were tapered and stopped 2 months after autoHSCT. NSAIDs were continued, but in two patients medication could be stopped completely within 6 months.

The recovery of the bone marrow took 20 to 30 days for neutrophils ($>0.5\times10^9$/L) and 16 to 35 days for the platelets ($>20\times10^9$/L). The number of T cells and mitogen responses normalized within 3–6 months.

Within 2 weeks of autoHSCT, all patients showed a marked improvement as measured by a decrease in joint swelling, pain, and morning stiffness. ESR, CRP, and Hb normalized within 8 weeks. As measured by child health assessment questionnaires (CHAQ), EPM-ROM, juvenile arthritis functional scores (JAFAS), there was a marked improvement of the functional activities of all children. Because of the longer follow-up in the first two patients, a favorable effect of autoHSCT on their growth could already be seen (Table 1). In general, the autoHSCT was well tolerated, and there were few complications (Table 2).

**Table 1.** Clinical characteristics of the JCA patients before autoHSCT

| | *Patient 1* | *Patient 2* | *Patient 3* | *Patient 4* |
|---|---|---|---|---|
| Sex | Female | Female | Male | Female |
| Age at disease onset | 1 year | 3 years | 3.5 years | 5.25 years |
| Onset form of JCA | Systemic | Polyarticular | Systemic | Systemic |
| ANA | – | – | – | + |
| RF | – | – | – | – |
| Treatment | NSAIDs, corticosteroids, IVIG, methotrexate, CsA | NSAIDs, corticosteroids, methotrexate, CsA | NSAIDs, corticosteroids, methotrexate, CsA | NSAIDs, oral gold, ATG, azathioprine methotrexate, CsA, pulses methyl-prednisolone |
| Clinical characteristics | Erosions, growth disturbance | Erosions, growth disturbance | Erosions, growth disturbance | Erosions, growth disturbance |
| age at AHSCT | 6 years, 7 months | 7 years, 9 months | 11 years, 2 months | 11 years |

## DISCUSSION

The effect of autoHSCT on signs and symptoms of chronic joint inflammation in four patients was very favorable. The initial improvement could be explained by the immune suppression of the conditioning regimen, but the duration of the remission, even after recovery of the T cells and T cell function, is indicative of an immune modulatory effect of autoHSCT. However, the follow-up period is too short for definite conclusions. An interesting phenomenon is the occurrence of a benign, oligoarticular synovitis in two of the four patients. This occurred simulta-

**Table 2.** Complications after autoHSCT in children with refractory arthritis

| |
|---|
| Patient 1 |
| Varicella Zoster at 3 months, transient oligoarticular synovitis at 4 months follow-up |
| Patient 2 |
| Varicella Zoster at 6 months follow-up |
| Patient 3 |
| Transient oligoarticular synovitis at 6 months follow-up |
| Patient 4 |
| Throat infection with hemolytic streptococci B at 4 months follow-up |

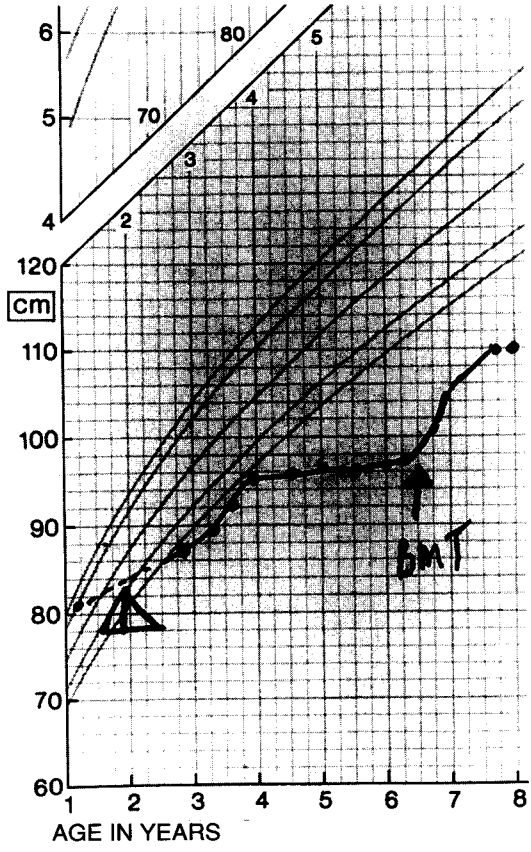


**Figure 1**

neously with T cell recovery. The self-limiting character of the synovitis favors the concept of a beneficial immunomodulatory effect post-HSCT. Whether the T cell depletion of the graft is necessary to sustain remission is not known. Early recurrences of systemic lupus erythematosus (SLE) after autoHSCT with unmanipulated grafts suggest that T cell depletion may be essential.[5] The use of TBI in our conditioning regimen is questionable. TBI is very effective in an animal model of arthritis,[6,7] so we included a low-dose TBI in our protocol. By using a low dose, we avoid the negative effects of TBI on growth and maturation,[8,9] although it is not known whether low-dose TBI increases the risk for secondary malignancies.[1] Careful evaluation of these patients is necessary to balance the risk of autoHSCT against conventional therapy with regard to outcome measurements and toxic side effects of treatment. In this respect, AHSCT must still be regarded as an experimental therapy for children with severe refractory JCA and other autoimmune diseases. Therefore, we need consensus on inclusion on criteria, conditioning

regimen, and graft handling. However, the short-term results of autoHSCT in our four patients are very promising, and although the follow-up period is short, the first impression is that autoHSCT could be an important weapon in the battle against refractory juvenile chronic arthritis.

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# CHAPTER 9
# LONG-TERM EFFECTS

# Presence in Pretransplant Marrow of Abnormal Progenitor Cell Clones That Give Rise to Posttransplant Myelodysplasia: Implications for High-Dose Chemoradiotherapy With Autologous Hematopoietic Cell Transplantation

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## INTRODUCTION

The myelodysplastic syndromes (MDS) are a group of hematologic disorders characterized by ineffective myelopoiesis, multilineage dysplasia, peripheral blood cytopenias, and in some cases, eventual evolution to acute myeloid leukemia (AML). Although the pathogenesis of these disorders is poorly understood, many authors favor a multistep process initiated by a clonal "transforming event" at the early progenitor cell level. This is followed by subsequent clonal evolution and dysregulation of protooncogene and/or tumor suppressor gene expression and cytokine pathways, leading to accelerated proliferation and apoptosis.[1] Studies of the clonality of hematopoietic progenitors, however, have yielded varying results, with some suggesting monoclonality of early progenitors,[2,3] and others suggesting that hematopoiesis remains polyclonal at the early progenitor cell level.[4,5]

Myelodysplastic syndromes may occur de novo, or may be the result of exposure to DNA-damaging agents, most commonly alkylating antineoplastic agents. With increasing application of high-dose chemotherapy and chemoradiotherapy with autologous bone marrow or peripheral blood stem cell transplantation in the treatment of a variety of malignant and nonmalignant disorders, the potential for development of myelodysplastic syndromes posttransplant has become cause for concern. Others have reported cumulative incidences of 2–31% at 4–5 years

posttransplant[6–7]; at the higher end of this range, posttransplant myelodysplastic syndromes become a significant limiting factor in the successful application of dose-intensive therapies. It remains to be determined, however, whether the development of posttransplant MDS is the result of the high-dose chemoradiotherapy or events occurring in association with the transplant process itself, or whether it stems from the use of the conventional-dose chemotherapy that most patients receive in the months or years preceding the transplant process. The answer to this question has significant implications for the use and methodology of autologous marrow and stem cell transplantation, and for the application of this therapy to newer, nonmalignant indications such as autoimmune disorders.

In 30 to 50% of MDS cases, nonrandom cytogenetic abnormalities are apparent in karyotypic analyses of bone marrow cells; this proportion may reach 80% in patients with chemotherapy-induced (secondary) MDS.[8] The most common abnormalities are deletions of 5q, 7q, or 20q, monosomies of 7 or Y, and trisomy 8. These abnormalities, once identified, can be used as markers to study the appearance and progression of the MDS clone(s). Here we summarize the results of two investigations of myelodysplastic syndromes using fluorescence in situ hybridization (FISH) to study the origin and clonality of MDS progenitors in de novo and posttransplant MDS. We then discuss their implications with respect to HDC/autologous marrow or stem cell transplantation.

## MATERIALS AND METHODS

### Study 1: FISH analysis of pretransplant progenitor cells from patients with posttransplant MDS

In this study, we identified patients from three institutions with posttransplant MDS. Twelve patients with clonal, MDS-related cytogenetic abnormalities for whom a FISH probe was available and from whom pretransplant marrow or stem cell specimens were available were selected for further analysis. Patient characteristics are shown in Table 1.

FISH probes corresponding to each patient's karyotypic abnormality were used to analyze cryopreserved pretransplant marrow or peripheral blood stem cell specimens ($n$=8) or archival bone marrow smears obtained immediately before transplantation ($n$=4). Two hundred cells were examined on each slide by two independent observers, and the mean of these determinations is reported.

### Study 2: FISH analysis of immunomagnetically sorted CD34-positive progenitors in patients with MDS

In this study, marrow aliquots in heparin were obtained from patients with known or suspected MDS. If a diagnosis of MDS was confirmed histologically, and

**Table 1.** Study 1 patient characteristics

| *Category* | *n* | *Category* | *n* |
|---|---|---|---|
| *n* | 12 | FAB category | |
| Age (years) | 42 (25–66) | RA | 3 |
| Sex | | RARS | 1 |
| Male | 5 | RAEB | 6 |
| Females | 7 | RAEBt | 2 |
| Prior therapy | | Transplant indication | 2 |
| Chemotherapy | 11 | Breast cancer | 4 |
| Radiotherapy | 0 | Non-Hodgkin's lymphoma | 4 |
| None | 1 | Hodgkin's lymphoma | 3 |
| Transplant type | | Melanoma | 1 |
| Bone marrow | 8 | | |
| Bone marrow/PBSC | 1 | | |
| CD34-selected PBSC | 1 | | |
| mAb-purged bone marrow | 1 | | |
| PBSC | 1 | | |

*mAb, monoclonal antibody; PBSC, peripheral blood stem cell; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; RAEBt, refractory anemia with ringed sideroblasts in transformation.*

if conventional G-banding cytogenetic analysis confirmed the presence of an MDS-related clonal cytogenetic abnormality for which a FISH probe was available, mononuclear cells were isolated by density-gradient centrifugation and sorted into CD34-positive and CD34-negative fractions using an immunomagnetic system (mini-MACS; Miltenyi Biotec). Successful sorting was confirmed by light microscopy, immunoperoxidase staining, and when sufficient cells were available, flow cytometry. FISH analysis was then carried out on both fractions using probes corresponding to each patient's known karyotypic abnormality.

The characteristics of the evaluable patients are summarized in Table 2.

## RESULTS

### Study 1: Analysis of pretransplant progenitors

Data from the 12 evaluable cases are summarized in Fig. 1. In nine of 12 cases, the cytogenetic abnormality detected posttransplant (at the time of diagnosis of posttransplant MDS) using conventional cytogenetic analysis could also be detected in pretransplant marrow or stem cell specimens using FISH. The percentage of analyzed cells belonging to the abnormal clone ranged from 20 to 46% in the

**Table 2.** Study 2 patient characteristics

| | |
|---|---|
| *n* | 12 |
| Age (years) | 56 (7–77) |
| Sex | |
| Males | 7 |
| Females | 5 |
| FAB category | |
| RA | |
| RAEB | |
| RAEBt | |
| CMML | 3 6 2 1 |
| De novo/secondary | |
| De novo | 7 |
| Secondary | 5 |
| Posttransplant | 2 |

*CMML, chronic myelomonocytic leukemia; RA, refractory anemia; RAEB, refractory anemia with excess blasts; RAEBt, refractory anemia with excess blasts in transformation.*

pretransplant specimens. One patient's specimen was analyzed using FISH probes for two different karyotypic abnormalities, with similar results for each.

In three cases, the number of abnormal cells identified in the pretransplant specimen fell below our conservative 10% threshold, indicating that the clonal karyotypic abnormality may not have been present in these cases before HDC/autologous transplantation. This group included the only patient who had received no antineoplastic therapy before HDC/autologous transplantation.

## Study 2: Analysis of immunomagnetically sorted CD34-positive progenitors

Data from the 12 evaluable patients are summarized in Fig. 2. In seven of 12 cases (group 1), the patient's clonal marker segregated preferentially with the CD34-negative cell fraction, suggesting at least partial sparing of the CD34-positive fraction. In the other five cases (group 2), there was no difference in the proportions of abnormal cells in the CD34-negative and CD34-positive fractions. Among the 12 specimens were pretransplant specimens from two patients with posttransplant MDS also included in study 1 above; one fell into each group with respect to CD34 selection results.

There were no obvious differences between the two groups of patients, although those whose marker segregated with the CD34-negative fraction had somewhat less advanced and/or lower risk MDS when compared with the group whose markers did not segregate with CD34 expression.

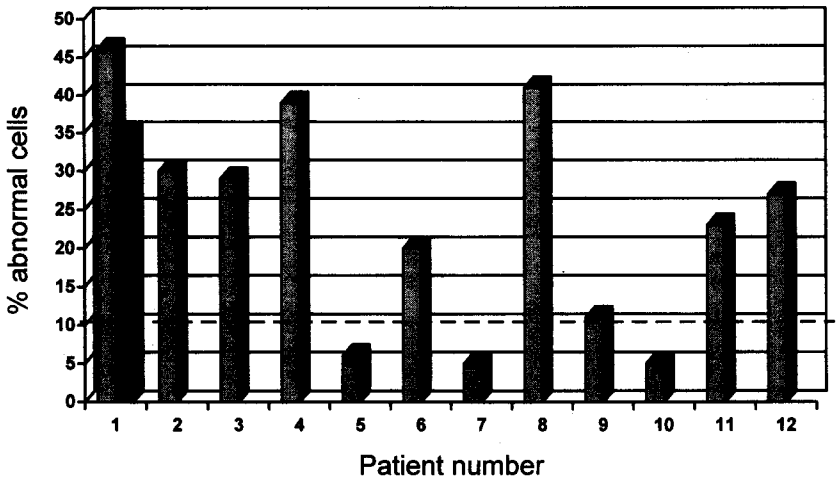


**Figure 1.** *FISH analysis of pretransplant bone marrow or peripheral blood stem cell specimens from patients developing posttransplant MDS. Dotted line indicates threshold for a positive sample. The specimen from patient 1 was analyzed using two separate markers.*

## DISCUSSION

High-dose chemotherapy or chemoradiotherapy is now part of the standard therapeutic approach to a variety of high-risk malignancies, and its use is now being investigated in the management of a variety of nonmalignant disorders as well.[9] Expanded application of this technology has been accompanied by increasing recognition of the potential for development of secondary myelodysplasias. However, despite the common use of the term "posttransplant MDS" in reference to these conditions, it is possible that the clonal "transforming event" leading to posttransplant MDS may originate in the prior conventional-dose therapy rather than in the course of the HDC/autologous transplant process itself, since most patients undergoing HDC/autologous transplantation have previously received conventional-dose chemotherapy and/or radiotherapy.

Most previous analyses have addressed this issue indirectly, using X-chromosome inactivation patterns or comparisons of the extent and duration of conventional-dose chemotherapy in patients who developed posttransplant MDS with those who did not.[10–12] Others have used conventional cytogenetic analysis to study small numbers of patients with posttransplant MDS.[6] Results of these analyses have been conflicting; most authors have suggested that posttransplant MDS results from the patient's prior conventional-dose therapy,[7,10–12] whereas the authors of a recent large study of breast cancer patients concluded that the high-dose chemotherapy was the causative factor.[6]

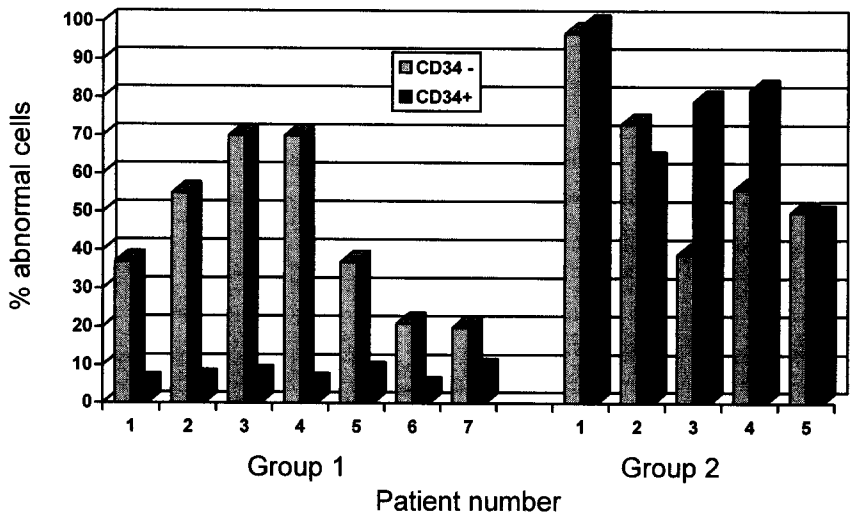


**Figure 2.** *FISH analysis of CD34-positive and CD34-negative bone marrow cells from patients with MDS.*

It also remains to be determined at what point in hematopoietic differentiation the clonal transforming event occurs. Previous studies of protooncogene mutations,[13] colony-forming potential,[3,5] X-chromosome inactivation patterns,[2,4,13] or molecular cytogenetics[14] have yielded conflicting results. Consequently, the maturation level of the initially transformed cell (and of later-evolving subclones) remains uncertain[15]; it is possible that it varies among patients in a fashion similar to that of acute myeloid leukemia.

Our studies show that, in most cases, the transforming event leading to development of posttransplant MDS occurs before the patient undergoes HDC/autologous transplantation. The presence in our series of one patient with no prior chemotherapy and normal pretransplant cells by FISH documents that HDC/autologous transplantation may itself be followed by development of MDS; nevertheless, in the majority of cases the clonal abnormality antedated the transplant process. In these cases, the transforming event was likely related to the patient's prior, conventional-dose therapy, a conclusion supported by the frequency in this group of karyotypic abnormalities associated with secondary MDS. This does not necessarily mean that the HDC/autologous transplant process played no role in the development of MDS in these patients. The pathogenesis of MDS may be a complex multistep process, and it is possible that the evolution of the condition from a presymptomatic genetic abnormality to a clinically relevant defect in hematopoiesis may somehow be permitted or accelerated by HDC/autologous

transplantation, perhaps through the use of growth factors or as a result of the unique cytokine milieu that predominates posttransplant.

Our studies also show that the CD34-positive progenitors of approximately half of MDS patients contain predominantly cells that do not show the MDS-associated clonal chromosomal abnormality. Again, this does not prove that CD34-positive progenitors are not part of the myelodysplastic process; the clonal karyotypic abnormalities studied may be later acquisitions in the evolution of the myelodysplastic process. Nevertheless, these abnormalities appear to be acquired or expressed predominantly in the CD34-negative population in a substantial fraction of patients. If the acquisition of these abnormalities is an integral part of the evolution to a clinically relevant disorder of hematopoiesis, then it is conceivable that this distinction could be exploited clinically in the prevention and treatment of MDS.

These results have therapeutic implications. Most importantly, they would suggest that strategies intended to prevent the development of posttransplant MDS should focus primarily on the pretransplant, conventional-dose therapy. Such strategies might include limiting the extent or duration of pretransplant therapy, avoidance of alkylating agents during the pretransplant period, or early marrow or stem cell harvesting before administration of the bulk of conventional-dose therapy. It is also possible that one could use molecular cytogenetic techniques, such as FISH, to screen for common MDS-related clonal karyotypic abnormalities pretransplant in high-risk populations such as heavily pretreated lymphoma patients. In such high-risk situations, the finding that the CD34-positive progenitor compartment is spared in many patients suggests that the use of CD34-selected autografts might prevent or delay onset of posttransplant MDS by preventing reinfusion of cells belonging to the dysplastic clone. In reality, such an approach is unlikely to be curative or completely preventive, since such selection is not 100% effective. Moreover, earliest cells affected by the transforming event may exist in the CD34-positive compartment and may not have acquired the karyotypic abnormalities that characterize more advanced subclones. Nevertheless, such an approach might delay onset of MDS by removing the majority of cells belonging to late-evolving, more aggressive subclones that have acquired the karyotypic abnormalities characteristic of clinically manifest MDS.

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# Importance of Marrow Doses and Modelization of Engraftment and Outcome in 229 Patients With Acute Leukemia Autografted With Marrow Purged by Mafosfamide in a Single Institution

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## INTRODUCTION

Since its introduction in the late 1970s, autologous bone marrow transplantation (autoBMT) has been extensively studied for high-dose consolidation in patients with acute leukemia (AL).[1–4]

Results from numerous institutions,[5–21] international data registries, and more recently, prospective randomized trials[22–25] have established the superiority of autoBMT over conventional chemotherapy (CT) in acute myeloid leukemia (AML). In these studies, however, no attempt has been made to treat the autologous marrow infused in an effort to purge it from contaminating leukemic cells. Data from animal models,[26,27] transplant registries (Miller CB, Smith D, Horowitz MM, Lazarus HM, Jones RJ: Autologous stem cell transplant in acute myelogenous leukemia after relapse. Ninth International Symposium on Autologous Blood and Marrow Transplant),[28,29] and gene transfer marking experiments[30,31] have accumulated in favor of purging, but the benefit of purging on the patient outcome has remained unproven and the field is still controversial.

As opposed to AML, the role of autoBMT in ALL is not established and is still being investigated.

A considerable variety of purging means have been assessed. Regarding chemotherapeutic agents, cyclophosphamide derivatives (i.e., 4 hydroperoxycyclophosphamide [4HC])—used essentially in North America—and its congener mafosfamide—used essentially in Europe—have been the most used. 4HC is no longer available in the United States outside the frame of randomized prospective trials, and efforts to build such trials have failed.

Since 1982, all adult patients in our institution with either AML or ALL fulfilling criteria for high-dose intensification in complete remission (CR) have been autografted with marrow purged by mafosfamide. We report here on the results in a series of 229 consecutive patients. The large number of patients treated has enabled

us to identify several prognostic factors to model prognostic groups for engraftment and outcome. In our study, the doses of marrow infused evaluated before and after purging with mafosfamide appeared to be of major importance in predicting for engraftment, transplant-related mortality (TRM), relapse incidence (RI), and leukemia-free survival (LFS). The characteristics of the marrow infused, therefore, are critical and, we believe, should be carefully monitored before autografting.

## MATERIALS AND METHODS

### Patients

The study protocol was approved by the St-Antoine Human Subject Review Committee (Paris). A total of 229 adult consecutive patients (165 AML, 61 ALL, and three undifferentiated AL) entered the study from January 1983 to December 1997 (median year of study 1989). The sex distribution was 140 males and 89 females. The median age was 36 years (range 9 to 65). A total of 123 AML, 46 ALL, and two undifferentiated AL were autografted in CR1. 32 AML, four ALL, and one undifferentiated AL were autografted in CR2. Twenty-one patients were autografted in more advanced status.

### Collection of marrow, incubation with mafosfamide and cryopreservation

All patients underwent bone marrow (BM) collection while in CR. BM collected was divided into two parts: a backup BM corresponding to $0.75\times10^3$ nucleated BM cells per kg that was saved and directly cryopreserved and a BM treated with mafosfamide to be used for ABMT.

To purge the BM with mafosfamide, we used two techniques. In the first period, until January 1990, we adjusted the doses of mafosfamide (AD) to the individual sensitivity of the normal granulocyte-macrophage colony-forming units (CFU-GM) in each patient in an effort to reach the highest tolerable dose that would achieve a maximum antileukemic activity without jeopardizing BM engraftment.[8,9,32–35] This dose was defined as the CFUGM LD95 on buffy-coat BM cells, sparing 5 ± 5% CFU-GM. A total of 126 patients had their BM treated according to this technique.

In the second period, from January 1990, 103 patients had their BM Ficoll-Hypaque separated; the mononuclear cell fraction was adjusted to a final concentration of $10^7$/mL and treated with a constant unique dose (UD) of 50 μg/mL mafosfamide. Whatever the dose, the BM suspension was incubated with mafosfamide for 30 minutes in a water bath at 37°C and then immediately cooled and centrifuged at 4°C to block the action of the drug abruptly. After two washes, the BM cells were then resuspended in irradiated (40 Gy) autologous plasma and

TC 199 medium and finally frozen with 10% dimethylsulfoxide in Teflon-Kapton DF 1000 Gambro bags (Gambro Dialysatoren, GMBH, Germany), following our freezing technique as previously published. The purged BM was then stored in the gas phase of liquid nitrogen at a temperature constantly below −190°C.[36] Cell counts and CFU-GM evaluations were performed at all steps of the procedures. The numbers of residual progenitor cells after incubation with mafosfamide were known for each individual patient before autoBMT. To evaluate the cryopreservation efficiency, a small aliquot of unpurged marrow was frozen in parallel with the purged marrow and thawed 48 hours later. The cryopreservation efficiency was expressed as the CFU-GM percentage recovery.

### High-dose consolidation and transplantation

The standard regimen for consolidation combined cyclophosphamide (CY) (60 mg/kg for two doses along with 2-mercaptoethane, sodium sulfate at 60% of the dose of CY) and total body irradiation (TBI). It was used in 190 patients.

Patients with contraindications for CY + TBI (>55 years old, poor cardiac function by echography and isotopic evaluation of ejection fraction) received alternative pretransplant regimens: 22 received the BAVC combination consisting of BCNU 800 mg/m$^2$, M-AMSA and VP16 450 mg/m$^2$ each, and cytosine arabinoside 900 mg/m$^2$.

## RESULTS

### Engraftment

The median time to recovery of polymorphonuclear cells (PMN) to 500/mm$^3$ was 27 days (10–153). The median time to recovery of platelets to 50,000/mm$^3$ was 63 days (15–1054). Variables possibly linked to faster engraftment as identified in univariate analyses included characteristics of the patient and the disease—younger age, diagnosis of ALL, transplant in CR1 (Figs. 1 and 2)—and characteristics of the graft—richer marrow as evaluated pre- but not postpurging, better cryopreservation efficiency. Regarding mafosfamide purging, the adjusted dose technique and a higher (and not a lower) dose of mafosfamide for purging appeared possibly associated to faster engraftment; this last observation concerned both the global population of patients, with the two purging techniques combined (Table 1), and the population of patients treated according to the AD technique only (Table 2). After inclusion of these variables in the Cox proportional hazard model, factors found to significantly influence the speed of engraftment (Table 3) were as follows:

*1*) Initial diagnosis: patients with ALL engrafted significantly more rapidly than patients with AML (Table 4). Recovery of PMN in ALL was 20 days (13–136) vs.

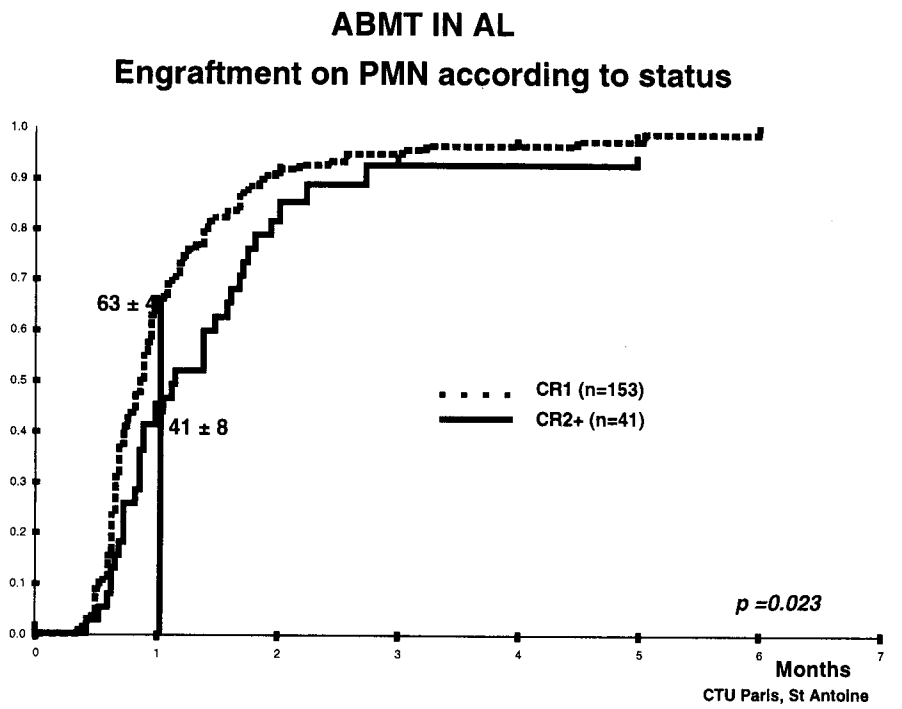


**Figure 1**

29 days (10–153) in AML. Recovery of platelets was 39 days (15–766) vs. 70 days (18–1054).

*2*) Doses of marrow submitted to purging with mafosfamide. Patients receiving higher doses engrafted more rapidly (Table 5): at 30 days posttransplant, 66 ± 5% patients receiving the higher marrow doses were successfully grafted vs. 51 ± 5% in those receiving the lower doses. Similarly, the probabilities for engraftment of platelets at 6 months were 78 ± 5% vs. 65 ± 6% (Table 6).

*3*) Use of the adjusted-dose technique for purging. Patients receiving marrow purged with this technique engrafted more quickly on platelets than those receiving marrow purged with the constant dose of 50 μg/mL (Fig. 3).

*4*) Cryopreservation efficiency: patients receiving marrow "well" cryopreserved (efficiency above median value) had a higher probability of engraftment for PMN at day 30 (73 ± 5% vs. 51 ± 6%). There was no impact on platelet engraftment (Table 7).

## Outcome

The marrow dose submitted to purging (the graft itself) influenced TRM, LFS, and overall survival (OS) (Fig. 4), but not the relapse incidence. The technique of

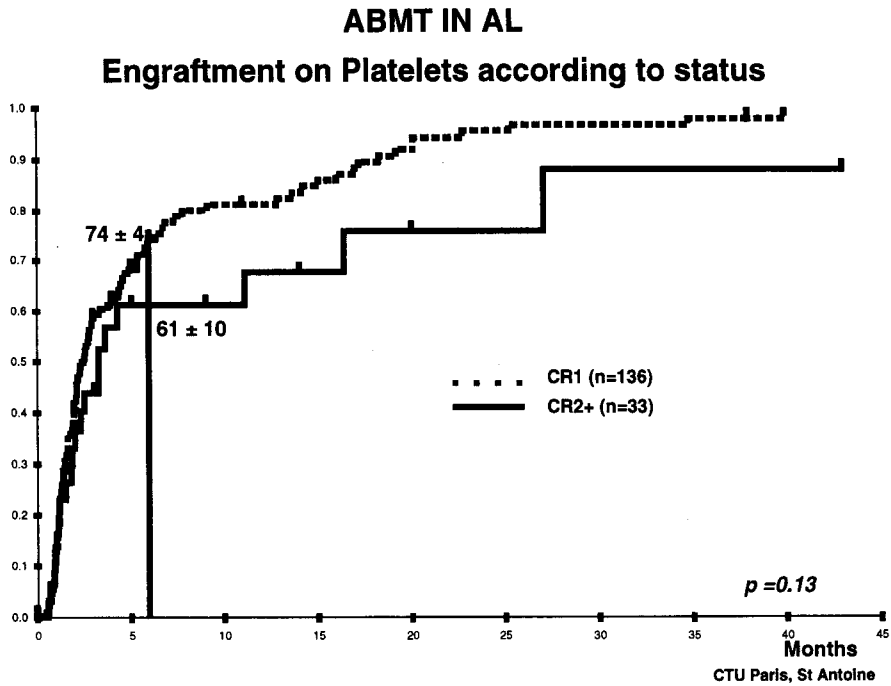


**Figure 2**

purging and the dose of mafosfamide used had no impact. The CFU-GM dose recovery postpurging and the dose actually infused influenced the relapse incidence, a lower incidence being observed for a lower CFU-GM residual dose postpurging (Fig. 5). After inclusion of these variables in the Cox proportional hazard model, only four factors appeared significant, the dose of marrow submitted to purging, the dose of marrow postpurging actually infused, the CFU-GM recovery postpurging, and the age of the patient (Table 8).

**Table 1.** Treatment with mafosfamide ($n$=195)

| | *Fraction of patients engrafted* | | | | |
|---|---|---|---|---|---|
| | *PMN* | | *Platelets* | | |
| | *30 days* | *60 days* | *6 months* | *12 months* | *P value* |
| Higher mafosfamide dose | 50 ± 5 | 88 ± 3 | 64 ± 6 | 74 ± 5 | 0.03 |
| Lower mafosfamide dose | 76 ± 5 | 89 ± 3 | 83 ± 4 | 87 ± 4 | 0.01 |

**Table 2.** Treatment with adjusted-dose mafosfamide ($n$=98)

| | *Fraction of patients engrafted* | | | | |
|---|---|---|---|---|---|
| | *PMN* | | *Platelets* | | |
| | *30 days* | *60 days* | *6 months* | *12 months* | *P value* |
| Higher mafosfamide dose | 51 ± 7 | 85 ± 5 | 71 ± 7 | 71 ± 7 | 0.05 |
| Lower mafosfamide dose | 71 ± 7 | 95 ± 3 | 92 ± 4 | 96 ± 3 | 0.003 |

**Table 3.** Factors favorably influencing engraftment: multivariate analyses

| | *Engraftment of neutrophils* | | *Engraftment of platelets* | |
|---|---|---|---|---|
| *Factor* | *P* | *RR* | *P* | *RR* |
| ALL | 0.012 | 1.7 (1.12–2.63) | 0.0002 | 2.12 (1.42–3.22) |
| Dose of marrow treated | 0.0009 | 1.91 (1.3–2.8) | 0.05 | 1.43 (1–2.05) |
| Mafosfamide dose adjustment | NS | | 0.003 | 1.6 (1.1–2.3) |
| Good cryopreservation efficiency | 0.014 | 1.6 (1.1–2.3) | NS | |

**Table 4.** ASCT for acute leukemia (CTU Paris, St. Antoine): kinetics of engraftment

| *Population* | *PMN recovery (days)* | *Platelet recovery (days)* |
|---|---|---|
| Global | 27 (10–153) | 63 (15–1054) |
| AML | 39 (15–766) | 70 (18–1054) |
| ALL | 20 (13–136) | 29 (10–153) |

## Prognostic classification

*Engraftment of neutrophils.* Initial diagnosis, dose of marrow prepurging and cryopreservation efficiency defined three groups: a good-risk group with fast engraftment (100% engraftment probability at 30 days), a poor-risk group with slow/delayed engraftment (37 ± 7%), and an intermediate group (67 ± 5%).

The good-risk group consisted of patients fulfilling the following three criteria: a diagnosis of ALL, a higher stem cell dose prepurging, and good cryopreservation. The poor-risk group included patients with AML and a lower stem cell dose. The intermediate group combined AML patients receiving higher marrow doses and ALL patients not fulfilling the three criteria that included them in the standard-risk group.

*Engraftment of platelets.* Initial diagnosis, dose of marrow prepurging, and the mafosfamide technique used defined three groups: a good-risk group with fast

**Table 5.** Engraftment according to mafosfamide dose

| | *Fraction of patients engrafted* | | |
|---|---|---|---|
| | *By day 30* | *By day 60* | *P value* |
| Treated in vitro | | | |
| Lower mafosfamide dose | 51 ± 5 | 87 ± 4 | 0.03 |
| Higher mafosfamide dose | 66 ± 5 | 91 ± 3 | |
| Actually infused | | | |
| Lower mafosfamide dose | 57 ± 5 | 88 ± 3 | 0.5 |
| Higher mafosfamide dose | 61 ± 5 | 90 ± 3 | |

**Table 6.** Platelet recovery according to mafosfamide dose

| | *Fraction of patients engrafted* | | |
|---|---|---|---|
| | *By 6 months* | *By 12 months* | *P value* |
| Treated in vitro | | | |
| Lower mafosfamide dose | 65 ± 6 | 72 ± 6 | 0.04 |
| Higher mafosfamide dose | 78 ± 5 | 83 ± 4 | |
| Actually infused | | | |
| Lower mafosfamide dose | 65 ± 5 | 73 ± 5 | 0.2 |
| Higher mafosfamide dose | 78 ± 5 | 82 ± 5 | |

engraftment (97 ± 3% engraftment probability at 6 months), a poor-risk group (55 ± 7%), and an intermediate group (75 ± 6%). The good-risk group consisted of patients with ALL receiving marrow purged with mafosfamide with the adjustment technique. The poor-risk group included AML patients receiving lower doses of marrow and ALL patients receiving marrow purged with mafosfamide according to the unique-dose technique. The intermediate group consisted of AML patients receiving higher doses of marrow.

*Transplant-related mortality.* Age and stem cell dose prepurging defined four groups. The best one, with a TRM of 2 ± 2%, consisted of younger patients receiving higher doses of marrow. The worst one, with a TRM of 42 ± 9%, consisted of older patients receiving lower doses of marrow. Two intermediate groups were defined for older patients receiving higher doses of marrow (intermediate/good: 11 ± 5%) and for younger patients receiving lower doses of marrow (intermediate/poor: 26 ± 6%).

*Leukemia-free survival.* With the dose of stem cells prepurging and the residual dose postpurging, three groups were identified. The best (LFS 70% at 10 years with

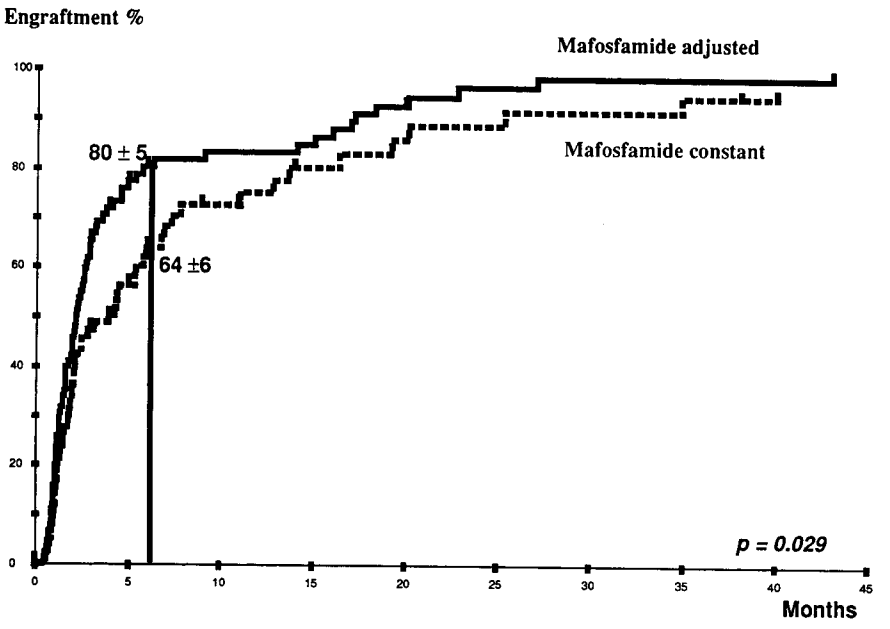


**Figure 3**

a plateau starting at 24 months) consisted of patients receiving higher marrow doses evaluated prepurging and lower residual doses postpurging. Patients with the reverse (lower doses prepurging, higher residual doses postpurging) were in the worst category (LFS 23 ± 8%). The intermediate group (LFS 45 ± 5%) combined patients receiving higher doses prepurging but also higher residual doses postpurging and those receiving lower doses prepurging and also lower residual doses postpurging.

*Overall survival.* Age, stem cell dose prepurging, and stem cell dose postpurging defined four prognostic groups (Fig. 6). The best group (group 1: OS 77 ± 7% at 5 years with a plateau starting at 25 months) consisted of patients

**Table 7.** Engraftment according to cryopreservation efficiency

| | *Fraction of patients engrafted* | | | | |
|---|---|---|---|---|---|
| | *PMN* | | *Platelets* | | |
| *Cryopreservation efficiency* | *30 days* | *60 days* | *6 months* | *12 months* | *P value* |
| 56 | 51 ± 6 | 85 ± 4 | 68 ± 6 | 76 ± 6 | 0.02 |
| >56 | 73 ± 5 | 90 ± 3 | 77 ± 5 | 85 ± 54 | 0.12 |

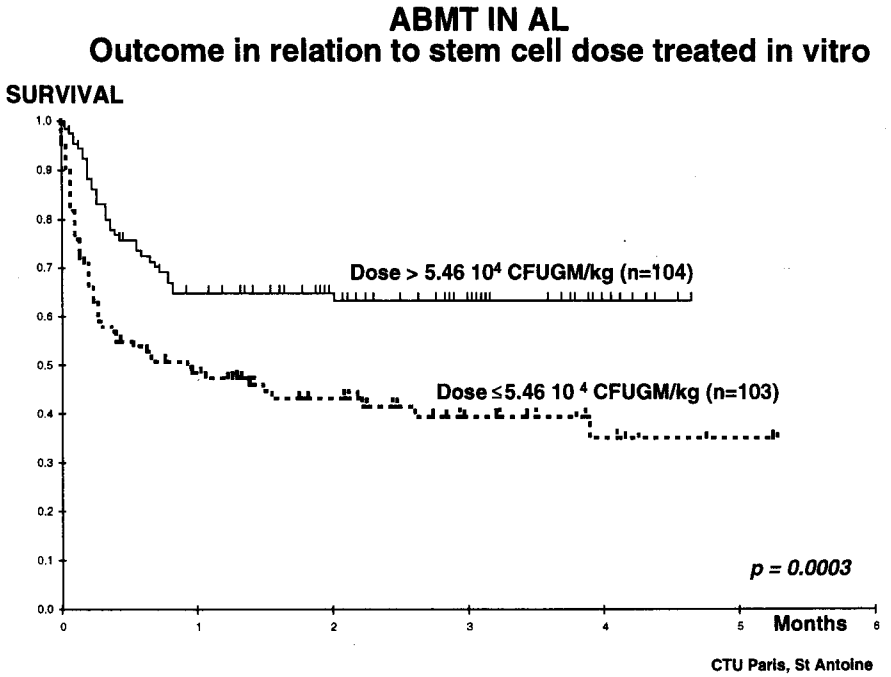


**Figure 4**

receiving higher marrow doses evaluated prepurging and lower residual doses postpurging. Patients with the reverse were in the worst group (group 3: OS 22 ± 8%). In the intermediate group (group 2), one of the two marrow dose characteristics was nonoptimal (lower marrow dose prepurging or higher residual marrow dose postpurging); younger patients, however, had a better OS (intermediate/good: 57 ± 7%) than older patients (intermediate/poor: 44 ± 7%).

## CONCLUSION

Tables 9 and 10 summarize factors predicting for faster engraftment and factors predicting for better outcome. With these factors a model was built.

This model led to simple and useful messages: it clearly showed that by combining the infusion of a rich marrow aggressively purged by mafosfamide within safe margins as ensured by the AD technique and carefully monitored for optimal cryopreservation, secure engraftment can be obtained and outcome improved with less TRM and better LFS and OS. Initial diagnosis, patient age, marrow richness, purging intensity, and cryopreservation efficiency are the key factors to consider before starting an autograft. As a consequence of this study, we

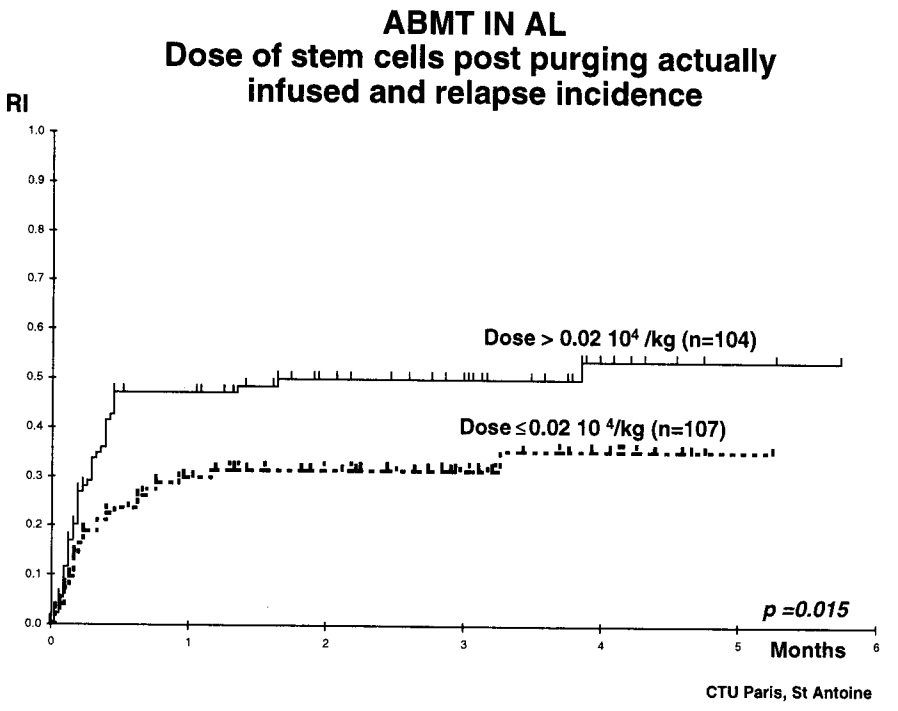


**Figure 5**

**Table 8.** Factors influencing outcome: multivariate analyses

| | *Outcome* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *TRM* | | *RI* | | *LFS* | | *OS* | |
| *Factor* | *RR* | *P* | *RR* | *P* | *RR* | *P* | *RR* | *P* |
| Higher dose of marrow submitted to purging | 0.11 (0.03 to 0.51) | 0.005 | NS | | 0.5 (0.28 to 0.8) | 0.005 | 0.40 (0.23 to 0.7) | 0.0011 |
| Lower dose of marrow postpurging (infused) | NS | | NS | | 0.57 (0.34 to 0.96) | 0.04 | 0.44 (0.25 to 0.77) | 0.004 |
| Lower CFU-GM recovery (%) postpurging | NS | | 0.51 (0.28 to 0.91) | 0.003 | NS | | NS | |
| Patient age ≤36 y | 0.18 (0.1 to 0.81) | 0.02 | NS | | NS | | 0.57 (0.33 to 0.97) | 0.04 |

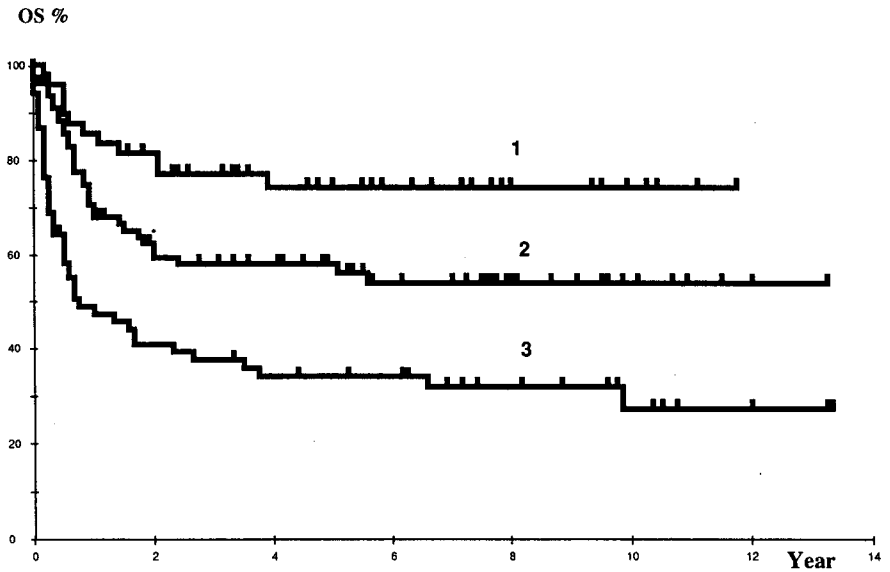


**Figure 5**

have developed a pilot study for peripheral blood stem cell purging with mafosfamide at the adjusted level aimed at marrow and blood combination in an effort to increase the size of the graft in all patients without any reduction in the purging intensity.

**Table 9.** Factors predicting for faster engraftment

- SCD treated
- Diagnosis of ALL
- Mafosfamide adjustment
- Cryopreservation efficiency

**Table 10.** Outcome (multivariate): factors predicting for a better outcome

| |
|---|
| Younger age: lower TRM, higher OS |
| Higher SCD treated: lower TRM, higher EFS, higher OS |
| Lower SCD actually infused: lower RI, higher LFS, higher OS |

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# Long-Term Follow-Up of Autologous Transplant Patients: London Experience

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The Royal Marsden Hospital prospective database identified 74 patients who had survived >10 years after autologous bone marrow transplant for leukemia and myeloma. Thirteen of these patients were selected for in-depth analysis because of close proximity to our institution. Survival was between 10 and 13.7 years (median 13.3). Three had been conditioned with melphalan 110 mg/m$^2$ and TBI 9.5–10.5 cGy single fraction. The remainder had received melphalan alone 200 mg/m$^2$. Patients had been evaluated for performance status, cataracts, lung function, secondary malignancies, psychosexual problems, and the social impact of their transplant on work, marriage, and their offspring. Laboratory investigations have included biochemistry, hematology, thyroid function, lymphocyte subsets, and their relationship to infection and autoimmune profile, and an attempt has been made to assess biological age relating to chronological age. Quality of life data has prospectively been generated in this group of patients. University College, London, identified 42 survivors (18 AML, two ALL, seven NHL, 15 HD) who were alive >10 years. One AML patient and one Hodgkin's patient have relapsed. Pregnancies have occurred but not without assisted conception.

# Long-Term Observations Following Hematopoietic Cell Transplantation

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Currently, about 30,000 patients annually receive an autologous or allogeneic marrow or peripheral blood stem cell transplant. For numerous indications of transplantation, the success rate has increased progressively, and as a result there is a rapidly growing population of surviving patients who are at risk of developing delayed complications. Most published data deal with results after allogeneic transplantation; only a few reports on observations in autologous transplant recipients have been presented.

We reviewed results in 3675 patients who survived at least 1 year following transplantation for various indications at the Fred Hutchinson Cancer Research Center in Seattle. Among these, 2245 had received a transplant from a related allogeneic donor, 469 from an unrelated allogeneic donor, 127 from a syngeneic donor, and 834 had received autologous marrow or peripheral blood stem cells. Among autologous recipients surviving beyond 1 year, 625 were observed for at least 3 years, 205 for 5, and 37 for 10 years or longer.

The major difference between allogeneic and autologous transplant recipients was, of course, the presence of chronic graft-vs.-host disease (GVHD), the most frequent complication following allogeneic transplantation, which also contributed substantially to other long-term problems. In contrast, for autologous recipients the major cause of failure was persistence or relapse of the underlying disease, which accounted for 55% of all deaths that occurred more than 2 years posttransplant compared with 35% in allogeneic related transplant recipients. However, many of the other late complications were seen both in allogeneic and in autologous recipients. Some of the problems after autologous transplantation are listed in Table 1. Depending on the conditioning regimen, pulmonary function impairment and the development of a sicca syndrome were not uncommon. Neuropsychologic problems were frequent and, depending on the instrument used for testing, might take 3 to 5 years to resolve. Changes in skin and endocrinologic function were clearly related to the conditioning regimen, in particular to the use of total-body

**Table 1.** Late effects after autologous marrow/stem cell transplants

| | |
|---|---|
| Pulmonary fibrosis | Endocrinological |
| Sicca syndrome | Hypothyroidism |
| Ocular | Adrenal insufficiency |
| Oral | Osteoporosis |
| Vaginal | Cardiovascular |
| Neuropsychological | Hypotension |
| Memory loss | Edema |
| Depression | Hepatic/gastrointestinal |
| Neuropathy | Anorexia |
| Fatigue | Cirrhosis |
| Skin/appendages | Cataracts |
| Dyspigmentation | Cytopenias |
| Alopecia | New malignancies |

irradiation. Osteoporosis may, in addition, be affected by the low level of activity of many of those patients. The etiology of cardiovascular complications, in addition to the effect of irradiation, was not clear. Cataracts were related to the use of irradiation and, less frequently, steroids. Hepatic and gastrointestinal dysfunction were probably multifactorial in etiology; infection with hepatitis C presumably contributed to the development of cirrhosis. Cytopenias were of multifactorial etiology, possibly related to damage inflicted on the stem cells pretransplant, the microenvironment, and the development of new dysfunctions including autoimmune phenomena posttransplantation. The development of malignancies was related in part to factors that have been identified as risk factors for malignancies in allogeneic transplant recipients (use of cytotoxic and potentially mutagenic therapy as well as immunosuppression) in addition to factors that were related to the use of autologous stem cells that had been exposed to various toxic agents before harvesting for transplantation. At present, we are aware of 14 malignancies in patients followed at our institution that have developed after autologous transplantation, 10 in patients given irradiation as part of the conditioning regimen, and four in patients who did not receive irradiation. These data are briefly summarized in Table 2.

Thus, these very preliminary data indicate that although many patients do very well following autologous transplantation, a proportion of them is likely to develop delayed effects. Considerable systematic work is necessary to better understand the risk factors involved and to define the kinetics of development such that interventions can be planned.

**Table 2.** Secondary malignancies after autologous transplantation

| *Secondary malignancy (no. of cases)* | *Original disease** |
|---|---|
| Adenocarcinoma (2) | NHL; NHL |
| Squamous cell carcinoma (2) | NHL; HD |
| MDS/AML (3) | ALL; HD; MM |
| Malignant melanoma (1) | NHL |
| Myxoid chondrosarcoma (1) | ALL |
| Glioblastoma (1) | NHL |
| Basal cell carcinoma (4) | AML (2); NHL; breast carcinoma |

*ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; HD, Hodgkin's disease; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma.*

## ACKNOWLEDGMENTS

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# CHAPTER 10
# GRAFT MANIPULATION

# Gene Marking to Assess Tumor Contamination in Stem Cell Grafts for Acute Myeloid Leukemia

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## ABSTRACT

We have been using gene transfer to investigate the biology of autologous transplantation in children with acute myeloid leukemia (AML). For the past 7 years, we have used retroviral gene marking of autologous marrow to discover whether infused marrow contributes to relapse, whether genes may be transferred into cells that produce long-term repopulation, and how these cells respond to ex vivo manipulation. In first-generation studies, four of 12 patients relapsed and the marker gene was definitively detected in three. In second-generation studies comparing purging techniques, three of 15 patients relapsed and all relapses were negative for the marker gene. Marker signal has been detected in peripheral blood granulocytes and T and B cells for up to 6 years at a level of 0.01 to 1%. The marker gene was also detected in marrow clonogenic assays at a level one log higher than peripheral blood with no apparent trend for reduction in signal strength with time. Our results show that retrovirally transduced genes remain detectable for at least 6 years after transfer to otherwise unmanipulated marrow, and that even after purging with agents that remove all colony-forming cells, long-term engraftment still occurs. These data provide an approach by which the long-term consequences of other stem cell manipulations may be determined in vivo.

## INTRODUCTION

Although the role of autologous bone marrow transplantation (autoBMT) in patients with AML in first remission remains contentious,[1–3] several recent studies suggest the procedure does reduce the risk of relapse and improve long-term outcome.[4,5] The major cause of failure after autologous transplant remains relapse. We initially used gene marking to address the question of whether leukemic cells in the infused marrow may contribute to relapse.[6] Detection of both the transferred

marker and a tumor-specific marker in the same cells at the time of relapse provides unequivocal evidence that the residual malignant cells in marrow are a source of leukemic recurrence. In follow-up studies, we have used discriminative gene marking with two vectors to evaluate purging techniques. A second, longer-term purpose of these studies was to learn more about gene transfer into human hematopoietic cells, to determine whether retroviral vectors transduce long-lived, repopulating progenitor cells, and to ascertain whether separate populations of hematopoietic stem cells (HSCs) could be tracked with distinguishable vectors.

## PATIENTS AND METHODS

### Patients

Patients diagnosed with de novo AML at St. Jude Children's Research Hospital between 1991 and 1996 were treated on the frontline institutional study AML-91.[7] All patients received one to two courses of 2-chlordeoxyadenosine (2-CDA) followed by one to two courses of daunomycin, cytosine arabinoside, and etoposide.[7] All patients attaining remission who did not receive matched sibling BMT were eligible for autoBMT. Conditioning was with busulfan 16 mg/kg and cyclophosphamide 200 mg/kg. AutoBMT recipients were eligible to participate in two successive marking studies approved by the Institutional Review Board, Recombinant DNA Advisory Committee of the National Institutes of Health (NIH) and the Food and Drug Administration (FDA).[8,9] During the time these gene-marking studies were open, from September 1991 to December 1996, 44 patients with AML in first remission received autologous transplants. Eleven were entered on the first-generation gene marking study and 15 on the second-generation study. Of the remaining 18 patients, three declined the gene marking component and 15 were transplanted at times when clinical grade vector was not available.

### First-generation marking studies

The initial marking study in patients with AML began in September 1991 and closed in March 1993.[10] Nucleated bone marrow ($>1.5\times10^8$ cells/kg body wt) was harvested, and two-thirds were cryopreserved without manipulation. Mononuclear cells were separated from the remaining third and transduced with either the LNL6 or G1Na retroviral vector in a simple 6-hour transduction protocol in the absence of growth factors. Both vectors encode the *neo* gene, which can be detected in transduced cells either phenotypically because it confers resistance to the neomycin analog G418, or genotypically by polymerase chain reaction (PCR). At the time of transplant, the patient received both the transduced and unmanipulated marrow cells. The transgene was subsequently detected in marrow progenitor cells by

clonogenic assays in the presence or absence of G418,[11] or in marrow colonies or peripheral blood cells by PCR.[11]

### Second-generation marking studies

Second-generation studies that used double discriminative gene marking to compare two different purging techniques[9] enrolled patients between September 1993 and November 1996. The two vectors used in these studies, G1N and LNL6, differ in 3′ noncoding sequence, so primers can be designed that will result in amplification of fragments of different sizes. In this protocol, at least $10^8$ marrow cells/kg were frozen without manipulation as a safety backup. The remaining marrow mononuclear cells were divided in half and randomized to marking with G1Na or LNL6. The aliquots were then randomly assigned to the two purging techniques under evaluation. We initially compared the pharmacologic "gold standard" hydroperoxycyclophosphamide (4HC) with an immunologic purge by culture with interleukin (IL)-2[12] (in collaboration with Dr. H. Klingemann). Later, we used CD15 antibodies[13] (provided by Dr. E. Ball) instead of IL-2. Patients received both purged aliquots at transplant and the unmanipulated fraction if engraftment was delayed. In patients who relapsed, marrow and peripheral blood specimens were evaluated for presence of both marker genes.

## RESULTS

### First-generation gene marking studies

Twelve patients were enrolled (11 with AML in first remission and one with AML in second remission), and follow-up now ranges from 68 to 84 months. Four patients have relapsed. In three of these patients, malignant cells identified by a tumor-specific marker have contained the marker gene. The fourth patient was uninformative, as his blasts did not have a leukemia-specific marker, so it was not possible to determine if the PCR signal was arising from normal or malignant cells. These data show definitively that marrow harvested from patients with AML in apparent remission may contain residual leukemic cells with the potential to contribute to a subsequent relapse. These observations suggest that effective purging of marrow might remove this source of leukemic cells and potentially improve the outcome of autoBMT.

These studies also allowed assessment of the efficiency of gene transfer to normal hematopoietic cells. Four patients in this cohort relapsed and two died (one sepsis, one myocarditis), but the remaining six have now been followed for 68–84 months. The marker gene has continued to be detected in these patients for up to 7 years in both clonogenic assays performed on marrow cells and PCR assays

performed on circulating peripheral blood mononuclear cells. The level of detection was highest in marrow clonogenic assays, where it ranged from 1 to 15%. Of interest, the level of transfer in clonogenic assays was around 1 log higher than in peripheral blood, an observation also found in other gene transfer studies.[14] In peripheral blood cells, expression was higher in granulocytes than in mononuclear cells and higher in T lymphocyte lines than in B lymphocyte lines. These observations show that the infused product contributes to both short- and long-term hematopoietic and immune reconstitution after autologous transplantation.

## Second-generation marking studies

Fifteen patients were treated in the second-generation study protocol between October 1993 and October 1996. In five patients, aliquots of marrow were purged with both 4HC and IL-2, and in three patients, aliquots were purged with 4HC and CD15. 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 25 to 58 months with a median of 48 months. Two patients relapsed early at 2 and 3 months and were noninformative, as marked malignant cells were not detected. A third patient relapsed at 20 months, and his malignant blasts were also negative.

The *neo* gene has been detected in normal hematopoietic and immune system cells at a lower level than in the studies using unpurged marrow. PCR studies on peripheral blood granulocytes and mononuclear cells have consistently shown a stronger signal from the 4HC-purged fraction than that from the IL-2-purged fraction, regardless of which vector was used for marking each aliquot. These observations suggest that the 4HC-purged fraction is making a greater contribution to hematopoietic reconstitution than the IL-2-purged fraction. We therefore discontinued purging with IL-2 and substituted CD15 antibodies.

## Safety of gene marking

Patient follow-up is now >1100 patient months in this group, and no adverse effects attributable to gene marking have occurred. Patient samples have been analyzed for replication-competent retrovirus (RCR) at intervals requested by the FDA, and all results have been negative. We have compared survival curves of patients with AML in first remission enrolled in marking studies with patients treated with the same chemotherapy and autoBMT protocol who received unmarked marrow due to unavailability of clinical grade supernatant or refusal of marking component. The survival curves shown in Fig. 1 show no significant difference in leukemia-free survival between recipients of marked marrow (59% at 3 years) or unmarked marrow (39% at 3 years). The overall survival is 70% at 3

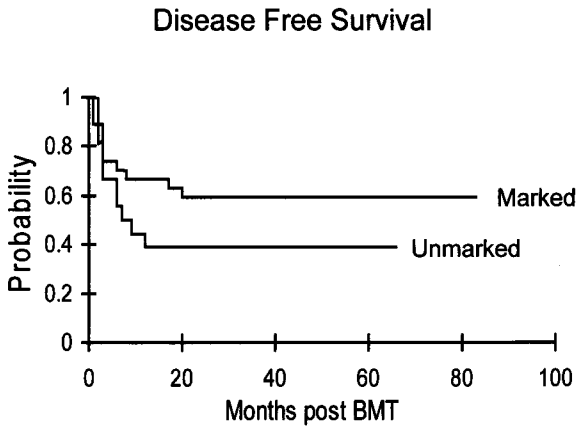


**Figure 1.** *Leukemia-free survival in patients with AML receiving autografts. Leukemia-free survival was estimated using the log-rank test and Kaplan Meier plots. Patients with AML in first remission who received an autoBMT with marked marrow were compared with contemporary control patients who received unmanipulated marrow after the same chemotherapy and transplant regimen. There is no significant difference in leukemia-free survival between recipients of marked marrow (59% at 3 years) and unmarked marrow (39% at 3 years).*

years for the patients who received marked marrow and 54% for the patients who received unmarked marrow. Similarly, the cumulative incidence of relapse is not significantly different between recipients of marked marrow (30% at 3 years) and unmarked marrow (59% at 3 years) ($P$=0.06).

## DISCUSSION

Transfer of marker genes to autologous marrow has shown that residual malignant cells contaminating the product may be one source of subsequent leukemic relapse in patients with AML. A similar result was seen in a study of adult patients with CML,[15] in which marked cells coexpressing *bcr-abl* and *neo* were found at the time of relapse. Marked malignant cells were not found in adult patients relapsing after autograft for AML or acute lymphoid leukemia (ALL).[16] However, only ~10% of the marrow was marked in that study, and gene transfer to normal cells was very low.

There are no data yet in the second-generation study on the efficacy of purging. A potential problem with this approach is that only a positive result is definitive. Hence, a relapse with marked leukemic cells indicates that the purging technique employed does not eradicate all leukemic cells. However, a negative result may reflect either a low number of residual leukemic cells in marrow, which were not marked, or a relatively small contribution to relapse from the infused marrow as

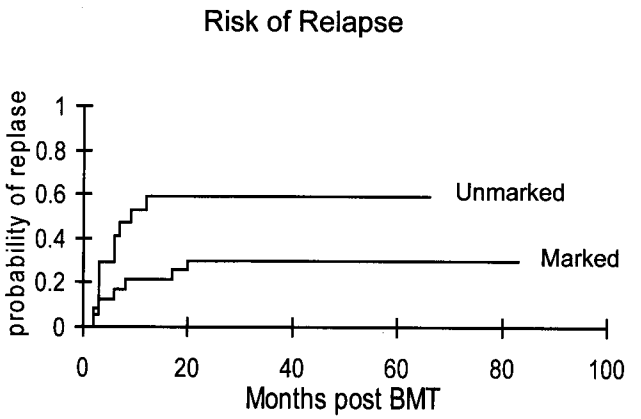


**Figure 2.** *Relapse risk in patients with AML receiving autografts. Cumulative incidence of relapse in children with AML who received autologous transplantation with either marked or unmarked marrow shows no significant difference in risk of relapse (P=0.06).*

well as efficacy of purging. This double-marking study has, however, allowed us to compare hematopoietic reconstitution by the two purged aliquots. In patients who received both 4HC and IL-2-purged aliquots, the contribution from the 4HC-purged fraction was consistently greater than that from the IL-2-purged portion. This approach using discriminative marking may also be used to compare different sources, different subsets, or differentially treated aliquots of HSCs.[17]

One intriguing observation from these studies is that the percentage of positive colonies is consistently higher than the proportion of marker-positive cells in the periphery, raising the possibility that the *neo* gene may be toxic to dividing cells by either a biochemical or immunologic mechanism. Whatever the explanation, these data suggest that *neo* may have some adverse effect on cell growth and may therefore not be the optimal marker gene. A number of alternatives to *neo* are now available, including truncated NGFR receptors[18] or GFP proteins.[19] While the products of these genes are all easier to detect, as either internal or surface markers, and do not appear to impede function in animal models, they may be immunogenic in humans. For the moment, perhaps the best marker is *neo*-less, a vector in which *neo* is removed or disabled and only the integrated sequence is found. However, detection of expression with this system is problematic.

## ACKNOWLEDGMENTS

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# Enrichment of Tumor Cells From Autologous Transplantation Grafts From Breast Cancer Patients

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## ABSTRACT

The presence of low numbers of tumor cells (so-called minimal residual disease [MRD]) in autologous stem cell transplantation (autoSCT) grafts from breast cancer patients is reported to be of clinical relevance. In the high-dose chemotherapy (HDC) autoSCT setting, the presence of MRD in the hematopoietic graft is associated with reduced disease-free and overall survival. However, MRD detection assays vary considerably in terms of sensitivity and specificity. A potential way to increase the ease, sensitivity, specificity, and efficiency of MRD assays is to capture the desired tumor cells in a highly concentrated cell fraction. We have developed a laboratory-scale, monoclonal antibody (mAb)-based tumor enrichment column (the CellPro TEC system) for the selective capture of tumor cells from blood, marrow, and apheresis specimens from cancer patients. In preclinical and clinical testing experiments, the CellPro TEC system has consistently demonstrated increased tumor detection sensitivity for breast cancer MRD detection. The application of this technology to various MRD assays will assist researchers in improving MRD assay sensitivity and specificity.

## INTRODUCTION

Several recent studies of breast cancer patients treated with HDC/autoSCT have concluded that the presence of tumor cells in blood and marrow is associated with poor posttransplant prognosis and reduced disease-free and overall survival.[1–4] Yet other studies report no correlation of MRD with post-HDC/autoSCT clinical outcome.[5,6] It is possible that these equivocal results are due, in part, to variability in tumor detection by the variety of MRD assays that were used. Indeed, the routine use of tumor detection assays in HDC/autoSCT patients to address the role of MRD in posttransplant clinical outcome has been hampered by the difficulty of detecting

low numbers of tumor cells in hematopoietic tissues.[7–9] Reported numbers of micrometastatic tumor cells range from an average of 5–10 tumor cells per $10^5$ to $10^6$ hematopoietic cells in bone marrow to 1 tumor cell per $10^5$ to $10^6$ in autoSCT grafts.[9–11] Therefore, MRD detection assays should have a detection sensitivity of at least 1 tumor cell in $10^6$. MRD detection assays must also be highly specific. False-positive results due to nonspecific crossreactivity, coexpressed antigens, and/or user variability severely limit the clinical value and applicability of any tumor detection assay.[4–9] In addition, the assay should be of limited complexity, so that its ease of use will enable widespread use in research and clinical settings.

Several investigators have explored the use of immunologic or immunomagnetic cell capture systems in an effort to improve tumor detection sensitivity.[14–17] While these various systems have met with success, some procedures are labor-intensive and others may be limited in application by disruption of cell viability and integrity. A tumor enrichment technology that maintains cell integrity and is rapid and simple to perform would likely assist transplanters to measure occult tumor cells in autoSCT grafts more efficiently and more accurately.

Thus, the sensitive detection of hematogenous micrometastases in the HDC/autoSCT treatment setting may be a powerful prognostic tool in selecting those patients who are tumor-negative and may have a better clinical outcome after HDC/autoSCT. In the autologous graft engineering area, the enhanced detection of hematogenous micrometastases may also assist transplant physicians in selecting autoSCT products that are tumor-free or identifying those grafts that are tumor-contaminated and may require additional tumor cell purging. We report on the development of a laboratory-scale tumor enrichment column that incorporates avidin-biotin cell selection technology for the enrichment of tumor cells from blood, marrow, and stem cell apheresis products.

## MATERIALS AND METHODS

### Tumor cell seeding experiments

Breast cancer cell line CAMA was seeded into normal donor bone marrow (BM) and peripheral blood progenitor cell (PBSC) specimens at concentrations ranging from $1{:}10^3$ to $1{:}10^8$. A pre-TEC portion of each seeded sample was reserved for immunocytochemical (ICC) staining, and the remaining sample was processed on the TEC as follows. The seeded samples were incubated with an anti-epithelial-cell capture antibody (NeoRx, Seattle, WA) for 30 minutes at room temperature, then washed with 1% bovine serum albumin (BSA). Each sample was resuspended in up to 3 mL of 5% BSA and processed on the TEC. The flow-through (FT) fraction (i.e., nonenriched cells) was collected and reserved, and the

post-TEC fraction was collected after release of the bound cells by manual disruption of the column bed. Cytospin preparations of the entire pre-TEC fraction for each sample were made. Cytospin preparations from the pre-TEC, post-TEC, and FT fractions were immunostained with the cocktail of anti-cytokeratin mAbs for the presence of tumor cells as detailed below.

A minimum of two experiments was performed for each seeding concentration. For the following tables, these calculations were used to express tumor cell recovery and enumeration:

$$\%\ \text{recovery of total tumor cells} = \frac{(\text{number tumor cells in post-TEC fraction}) \times 100\%}{\text{number tumor cells seeded in pre-TEC sample}} \quad (1)$$

$$\text{fold enrichment} = \frac{\%\ \text{of tumor cells in post-TEC fraction}}{\%\ \text{tumor cells in pre-TEC sample}} \quad (2)$$

$$\log \text{enrichment} = \log (\text{fold enrichment}) \text{ (e.g., log of 500 = 2.69)} \quad (3)$$

$$\text{total tumor cells in TEC (FT)} = \frac{(\text{tumor cell count})\ (\text{volume of FT fraction})}{\text{mL}} \quad (4)$$

## Patient specimens

Bone marrow and PBSC apheresis specimens were obtained from researchers at collaborating institutions. Both fresh and cryopreserved specimens were analyzed. Specimens were split, with $5.0\times10^6$ cells reserved for standard pre-TEC ICC analysis. A minimum of $1.0\times10^8$ cells were incubated with the biotinylated TEC mAb and processed as described above.

## ICC staining

The pre-TEC, post-TEC, and TEC FT specimens from both tumor cell seeding and patient specimens were prepared as cytospins and fixed in a mixture of Histochoice (AMRESCO, Solon, OH) and 4% paraformaldehyde for 30 minutes at 4°C and rinsed thoroughly in phosphate-buffered saline (PBS). The slides were then loaded on the TechMate 500 (Ventana Medical Systems, Tucson, AZ) immunostainer and placed in blocking solution for 25 minutes. They were then rinsed in PBS and incubated for 20 minutes in an anti-cytokeratin mAb cocktail. The slides were then rinsed in PBS, incubated for 20 minutes in the secondary antibody, and incubated for an additional 20 minutes in the ABC-alkaline

phosphatase reagent. Next, the slides were placed in three washes of BioTek red chromagen reagent, rinsed thoroughly in PBS, and counterstained in hematoxylin. All ICC reagents are manufactured by Ventana/BioTek Systems. Alternatively, some ICC experiments were performed with the CellPro ImmunoCytoChemistry Staining Kit (CellPro, Bothell, WA) according to manufacturer's instructions. ICC methods were comparable for tumor detection sensitivity and specificity (unpublished observations). All ICC results were read by three independent reviewers. Equivocal ICC results were resolved by consultation, restained and reexamined, or recorded as unevaluable.

## RESULTS

### Tumor cell seeding experiments

Results from the tumor cell seeding experiments showed that use of the TEC column resulted in tumor cell enrichment at all seeding levels tested (Table 1). Even at the lowest tumor cell concentration ($1:10^8$), the TEC system was able to enrich tumor cells in each experiment. Further, enriched tumor cells were capable of sustained growth in vitro (data not shown). ICC staining revealed intact tumor cells, with no interference with anti-cytokeratin immunoreactivity.

### Patient specimens

A total of 139 marrow and PBSC patient specimens were analyzed. Twenty-one specimens were unevaluable due to invalid TEC runs or equivocal ICC staining results. Data are summarized in Tables 2–5.

In five cases, tumor cells were detected by ICC pre-TEC, but no tumor cells were detected post-TEC (Table 3). These data suggest that in one of the samples (#193) the TEC capture antibody did not bind to the patient's tumor cells. In the

**Table 1.** Mean log enrichment of breast cancer cell-seeded samples

| *Seed level* | *$1:10^3$* | *$1:10^5$* | *$1:10^6$* | *$1:10^7$* | *$1:10^8$* |
|---|---|---|---|---|---|
| Sample type | | | | | |
| Bone marrow | 2.80 ± 0.12 ($n$=3) | 2.65 ± 0.15 ($n$=3) | 2.27 ± 0.12 ($n$=3) | ND | ND |
| PBSC | 2.45 ± 0.0 ($n$=2) | 2.48 ± 0.48 ($n$=22) | 2.49 ± 0.37 ($n$=20) | 2.32 ± 0.35 ($n$=27) | 2.50 ± 0.25 ($n$=6) |

*Results of tumor cell-seeding experiments with breast cancer cell line CAMA in normal donor marrow and PBSC specimens. Reported values are mean log enrichment for cell-seeding concentrations ranging from $1:10^3$ to $1:10^8$. ND, not done.*

**Table 2.** Results of tumor enrichment in bone marrow or PBSC apheresis collections from breast cancer patients

| *Patient stage (n)* | *ICC results (before/after TEC)* | | | |
|---|---|---|---|---|
| | –/– | +/– | –/+ | +/+ |
| II (96) | 63 | 3 | 12 | 3 |
| III (1) | 0 | 0 | 1 | 0 |
| IV (42) | 14 | 2 | 4 | 16 |

*Tumor enrichment data from breast cancer patient specimens. Data reflect numbers of patients in whom results were obtained.*

remaining cases, either tumor cells were captured in the TEC avidin column and not released, or Poisson distribution of rare event detection may have been a factor.

Seventeen patient specimens were ICC-negative for tumor cells pre-TEC but ICC-positive for tumor cells post-TEC. As illustrated in Table 4, both fold and log enrichment of tumor cells were significantly increased post-TEC. Further, greater numbers of tumor cells were observed in post-TEC specimens from stage IV breast cancer patients than from stage II or III patients. These data suggest that tumor contamination of autoSCT grafts can be missed with standard ICC techniques.

Nineteen patient specimens were ICC-positive for tumor cells pre-TEC and post-TEC (Table 5). These data illustrate that a greater number of tumor cells were available for ICC analysis after TEC enrichment, which resulted in considerable time savings for the examiners reviewing the slide.

Both cryopreserved and fresh patient specimens were examined. No differences were noted in TEC column performance, tumor cell capture, or ICC staining results.

**Table 3.** Tumor enrichment results on +/– specimens

| *Specimen # (patient stage)* | *Tumor cells before TEC (/2.5×10⁶)* | *Tumor cells after TEC (/5.0×10⁵ to 1×10⁶)* | *Tumor cells flow-through (/2.5×10⁶)* |
|---|---|---|---|
| 021 (II) | 1 | 0 | ND |
| 068 (II) | 1 | 0 | 0 |
| 103 (II) | 2 | 0 | 0 |
| 111 (IV) | 2 | 0 | 0 |
| 193 (IV) | 2 | 0 | 6 |

*Tumor enrichment results on patients who had tumor cells detected before but not after TEC.*

**Table 4.** Tumor enrichment results on -/+ specimens

| Clinical stage (n) | Mean no. tumor cells: Before TEC (/2.25×10⁶) | Mean no. tumor cells: After TEC (/1×10⁴–4.5×10⁶) | Mean no. tumor cells: Flow-through (/2.25×10⁶) | Fold enrichment | Log enrichment |
|---|---|---|---|---|---|
| II (12) | 0 | 9.2 (1–35) | 2.6 (0–15) | >81.6 (9.8–262) | >1.8 (0.99–2.42) |
| III (1) | 0 | 21 (NA) | 4 (NA) | >159 (NA) | >2.2 (NA) |
| IV (4) | 0 | 79 (1–308) | 1.5 (0–6) | >60.1 (0.5–122) | >0.9 (0.3–2.09) |

*Tumor enrichment results on patients who had no tumor cells detected before TEC but had tumor cells detected after TEC.*

## DISCUSSION

The experiments with both tumor cell seeded and patient marrow and PBSC specimens showed that the TEC system is effective in enriching tumor cells for subsequent ICC analysis. Even at tumor cell concentrations as low as $1:10^8$, tumor cells were captured and released for subsequent ICC analysis. Thus, tumor detection with TEC is capable of superior detection sensitivity that surpasses the most sensitive PCR assays.[3,19] Further, as the cells are intact and viable, subsequent morphologic or functional analyses are not impaired.

Seventeen specimens converted from tumor-negative to tumor-positive after TEC enrichment, and 19 tumor-positive specimens showed considerable enrichment of tumor cells for subsequent ICC analysis. This will likely result in more sensitive ICC assays for occult tumor detection. However, in certain cases (e.g., ≤2 tumor cells per total of $3.0\times10^8$ nucleated cells run through the TEC column), tumor cells may be missed. One patient's results (#193, Table 3) suggest

**Table 5.** Tumor enrichment results on +/+ specimens

| Clinical stage (n) | Mean no. tumor cells: Before TEC (/2.25×10⁶) | Mean no. tumor cells: After TEC (/1×10⁴–4.5×10⁶) | Mean no. tumor cells: Flow-through (/2.25×10⁶) | Fold enrichment | Log enrichment |
|---|---|---|---|---|---|
| II (3) | 3 (1–3) | 94 (5–211) | 0.3 (0–1) | 278.5 (34.4–643) | 2.1 (1.5–2.8) |
| IV (16) | 272.2 (1–1255) | 110,731 (1–~555,000) | ND | 390.7 (3.97–~1000) | 2.1 (.6–3) |

*Tumor enrichment results on patients who had tumor cells detected both before and after TEC. ND, not done.*

that the capture antibody used in the TEC system may not bind to all tumor cells, thereby resulting in false-negative TEC results. For these reasons, ongoing experiments are analyzing the addition of additional antitumor cell antibodies to the existing formulation.

Other groups have recently reported on a variety of methods to accomplish tumor enrichment.[14–17] These methods vary considerably with regard to efficiency of cell capture, sensitivity and specificity, and ease of use. For example, Naume et al.[16] reported the enrichment of epithelial-derived tumor cells using mAbs coupled to magnetic beads. While efficient in cell capture, the adherence of the beads to the cells can cause difficulty in subsequent immunostaining and morphologic verification. Bead removal procedures that rely on enzymatic treatments often severely disrupt the cell integrity, thereby rendering the cells nonviable and morphologically disrupted. These problems are also encountered in methods that use magnetic particle separation technology against intracellular components such as cytokeratins.

It is possible to enrich tumor cells by using magnetic ferrofluids followed by multiparameter fluorescence activated cell sorting.[17] However, due to the limits of cell detection sensitivity and specificity in the cell sorting procedure, a significant level of false-positives in normal donor specimens has been reported.

Another approach has been to isolate tumor cells by negative depletion of hematopoietic cells.[16] This method has the advantage of leaving the tumor cells free of capture antibody, magnetic particles, and presumably viable. However, if hematopoietic cell depletion is efficient, the possibility exists that the few remaining tumor cells may be difficult to capture for subsequent analysis.

For these reasons, we have found that the avidin-biotin selection system, when used in conjunction with an epithelial-associated antibody, is easy, efficient, and reproducible for the enrichment of breast cancer cells. The use of such a system may result in the more accurate assessment of MRD in autoSCT grafts from patients with breast cancer.

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# Combining Positive/Negative Selection in Stem Cell Transplantation: Current Approaches and New Avenues

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## ABSTRACT

Manipulation of hematopoietic stem cell grafts has become a very exciting research area due to the improved accessibility of hematopoietic progenitor cells using apheresis procedures compared with bone marrow harvesting. This development in graft engineering relates also to further refined approaches to eliminate contaminating tumor cells from autografts or to ex vivo expanded hematopoietic progenitors and postprogenitor cells. Such sophisticated procedures rely on the positive selection (i.e., enrichment) of a given target cell population using either immunoaffinity approaches or density gradient centrifugation procedures. Negative selection methods (i.e., depletion) of nontarget cells (e.g., tumor cells, GVH-reactive cells) also use immunoaffinity approaches. Cell depletion can be achieved by pharmacologic agents, cell-specific induction of apoptosis, or genetic cell manipulation. In the future, it will be important to design purging strategies that do not rely solely on the physical elimination of tumor cells but also aim at the functional impairment of tumor cells, thereby preventing their tumorigenicity in vivo. In this study, we describe the current approaches of combining positive and negative cell selection using immunomagnetic selection techniques and present data showing that adenovirus-mediated suicide gene transfer into breast cancer cells is a highly efficient purging technique in vitro and in vivo.

## INTRODUCTION

Transplantation of autologous hematopoietic progenitor cells after high-dose chemotherapy has found widespread application as a major novel therapeutic strategy and has led to a worldwide rapid increase. Despite this dramatic development, there

are a number of unresolved problems in autologous transplantation. One of the most intriguing and relevant issues is the contribution of minimal residual disease to the development of relapse after high-dose chemotherapy. This refers to contaminating tumor cells within the autologous stem cell graft as well as to residual tumor cells within the patients who survive after myeloablative chemotherapy. Clinical trials[1,2] as well as gene marking studies[3,4] have suggested that contaminating tumor cells within the autograft might contribute to relapse after high-dose chemotherapy. During the last 15 years, different purging strategies have been designed and employed for the depletion of tumor cells from autografts.[5–7] Apart from pharmacologic and other tumor cell purging techniques, in vitro manipulation of $CD34^+$ hematopoietic progenitor cells recently has found widespread application in autologous transplantation because the procedure is simple, fast, and without any detrimental effect on graft quality.[8–10] Furthermore, this approach leads to decreased infusional toxicity of the cryoprotectant dimethyl sulfoxide (DMSO) as a result of smaller infusion volume.[11] Another advantage of this approach apart from the improved accessibility of CD34 cells for genetic manipulations, is the passive depletion of tumor cells.[8] However, it has been demonstrated by different groups including ours that despite highly efficient CD34 selection, tumor cells can still be detected within the CD34 fraction.[12,13] CD34 enrichment, however, might be complicated by CD34 antigen expression on tumor cells, leading to a potential enrichment of tumor cells. Thus, it might be necessary to combine different purging techniques to obtain a tumor-free graft. Recently, efforts have been undertaken by a number of investigators to improve on CD34 selection-mediated depletion of nontarget cells (e.g., tumor cells or T cells). Different methodologic approaches have been tried to design a more efficient purging procedure: CD34 enrichment using density Percoll gradient centrifugation,[14] immunoaffinity selection followed by treatment with immunotoxins,[15] complement-mediated lysis,[16] immunomagnetic purging,[7] and tetrameric antibody-complexes binding tumor cells and dextran.[17] New strategies to enhance purging efficiency after positive selection of stem cells might rely on the functional impairment of tumor cells to engraft after transplantation, thus losing their tumorigenicity. The aim of this clinical pilot study was to evaluate a combined positive/negative purging system based on immunomagnetic cell selection with regard to feasibility and purging efficiency. An adenovirus-mediated suicide gene transfer method was also tested for purging efficiency in vitro and in vivo.

## MATERIALS AND METHODS

### Patients and treatment

After patients gave informed consent, human $CD34^+$ peripheral blood progenitor cells were isolated by immunomagnetic selection from leukapheresis

products of patients with nonhematological malignancies using the Isolex 300 device (Baxter Biotech, Munich, Germany) as previously described.[12] $CD34^+$ hematopoietic progenitor cells were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 20% fetal calf serum (FCS), interleukin (IL)-3 (100 U/mL), IL-6 (100 U/mL), and stem cell factor (50 mg/mL). All growth factors were purchased from Promocell (Heidelberg, Germany). Patients were mobilized either with granulocyte colony-stimulating factor (G-CSF) alone (12 μg/kg; Amgen, Thousand Oaks, CA) or in combination with chemotherapy (epirubicin 12 mg/m$^2$ plus ifosfamide 7.5 g/m$^2$) in established therapy protocols.

## CD34 selection and subsequent immunomagnetic tumor cell depletion

Leukapheresis bags were stored in the original cell collection bag of the C4Y collection set (Fresenius) up to 72 hours at 4°C in autologous plasma and ACD-A. Bags stored for 24, 48, and 72 hours had a mean cell concentration of $65.5\times10^6$/mL ($\pm26.14\times10^6$/mL) and $58\times10^6$/mL ($\pm42.67\times10^6$/mL), respectively. Since the Fresenius AS104 cell separator was used for apheresis, cells are directly collected in autologous plasma plus ACD-A in a total volume of 280–360 mL. Trypan blue dye exclusion staining revealed a mean cellular viability of 100 ± 0.7, 99 ± 1, 99 ± 1.5% after 24, 48, and 72 hours, respectively. Median total CFU formation per $2\times10^4$ mononuclear cells (MNC) was 202 ± 88, 247 ± 121 after 24, 48, and 72 hours, respectively. Median viability after the first platelet wash of pooled LP was 98%.

Enrichment of CD34 cells from leukapheresis product (LP) was performed using the ISOLEX 300SA and the ISOLEX 300i device (Baxter Biotech) according to the protocol provided by the manufacturer. For the ISOLEX SA, after washing (3× Dulbecco's phosphate-buffered saline [DPBS], 0.2% sodium citrate [wt/vol], 1% human albumin at room temperature, 200*g* for 10 minutes) cells were incubated with the anti-CD34 mAb 9C5 (0.5 μg/$1\times10^6$ cells for 30 minutes at 4°C). Unbound antibody was removed by washing as mentioned above. Thereafter, sensitized cells were incubated with immunomagnetic beads (4.5 μm) coated with goat anti-mouse antibodies (Cynal AS, Oslo, Norway). Subsequently, cells were captured by a magnet in the ISOLEX 300 chamber. Cells were released from the beads using the peptide release agent (PR34+). After collection and washing of the $CD34^+$ fraction, cell count and viability testing were performed. CD34 selection using the ISOLEX 300I was performed as follows: after storage of leukapheresis products, bags were pooled and a platelet wash was performed. The volume was adjusted to 30 mL with working buffer (PBS, 1% human albumin, 4.5% ACD-A [vol/vol]). Cells were incubated for 15 minutes at room temperature with a human immunoglobulin preparation (5% Gammagard; Baxter) before the enrichment procedure. After priming of the

tubing set, each bag (release agent bag, antibody bag, wash bag) was filled with the appropriate reagent. After washing, one vial of magnetic beads ($4\times10^9$ in 10 mL working buffer) was added to the isolation chamber. The cell container was connected to the tubing set, and another platelet wash was performed. Thereafter, the automatic process of enrichment was initiated. After addition of the release agent, $CD34^+$ cells were recovered in the end product bag. The bag was separated from the tubing set, and cells were washed once more and subjected to negative selection as follows.

The subsequent immunomagnetic purging was performed with the ISOLEX 300 SA device and an ISOLEX 50 chamber using an adapter. The ISOLEX 50 chamber and tubing system were sterilely connected (sterile connection device; Haemonetics, Braintree, MA) to an ISOLEX 300 plastic tubing set. Monoclonal antibodies 520C9, 260F9, and 317G5 (Baxter) directed against epithelial surface antigens of 200, 55, and 42 kD, respectively,[18,19] were directly coupled to Dynal beads. $8\times10^8$ beads were washed twice in 20 mL working buffer and resuspended in 2 mL. Thereafter 40 µg of each mAb was added. The incubation was performed with gentle agitation for 60 minutes at room temperature. After washing, the beads were resuspended in 2 mL. The beads and cells (20 mL) were entered into the ISOLEX 50 chamber. Rosetting was carried out for 30 minutes. Thereafter, the cells were immunomagnetically separated, and the negative fraction was collected. After final washing, samples were drawn for immunophenotype analysis, immunocytochemical tumor cell staining, and colony assays. The selected cell fraction was cryopreserved and stored within the vapor phase of liquid nitrogen according to the guidelines at our institution.

### Immunocytochemical staining and quantitation

Immunocytochemical staining of tumor cells was performed as previously described or using the EpiMet kit according to the manufacturer's advice (MicroMet, Munich, Germany). Both assays used the A45-B/B3 anti-pancytokeratin monoclonal antibody. As previously described, the detection limit of this assay is $1:10^6$.[12] Mononuclear cells from leukapheresis product were isolated by density centrifugation through Ficoll-Hypaque (Seromed, Hamburg, Germany), washed twice with PBS, and resuspended at $1\times10^6$/mL. Thereafter, cells were spun onto the slides using a Shandon cytocentrifuge with $5\times10^5$/slide. After CD34 enrichment, cells were directly spun onto cytocentrifugation slides without density gradient centrifugation. Tumor cells were considered immunocytochemically positive when staining on more than 70% of cell membrane or cytoplasm was observed and cell morphology showed malignant features. Generally, an overall number of at least $2\times10^6$ up to $4\times10^6$ MNC were analyzed, tumor cells were counted, and the total number of tumor cells extrapolated.

### Hematopoietic colony-forming assay and tumor cell clonogenic assay

$CD34^+$ cells ($3\times10^5$/well) or MT-1 cells ($1\times10^5$/well) were seeded as triplicates into a 24-well plate. Cells were infected with AdCMV.CD (20 multiplicity of infection [m.o.i.]), followed by the addition of 1 mM 5-FC (Sigma Chemie GmbH, Germany). Controls were treated as indicated. After 48 hours of incubation, $2\times10^3$ $CD34^+$ cells or $1\times10^3$ MT-1 cells were plated into 1 ml of standard methylcellulose (MethoCult GF H4434, Stem Cell Technologies, Vancouver, Canada) in a 35-mm dish. Colonies were scored after 8 (MT-1 cells) and 14 ($CD34^+$ cells) days of culture.

### Recombinant adenovirus vector

The recombinant adenovirus vector AdCMV.CD (generously provided by Dr. Ronald Crystal, New York) carries the cDNA of the *E. coli* cytosine deaminase driven by the cytomegalovirus (CMV) major immediate promoter.[20] The Ad vector AdCMV.nlsϑ-gal carries the reporter gene ϑ-galactosidase combined with a nuclear localization signal driven by the CMV promoter.[21] The Ad vector AdCMV.p21 (generously provided by Dr. Michael Strauss, Berlin, Germany) carries the cDNA p21 driven by the CMV promoter. All vectors were propagated on 293 cells and purified and stored at –70°C, as previously described.[22]

## RESULTS

### Combined positive and negative selection

We have conducted a phase I/II study in breast cancer patients evaluating the feasibility and purging efficiency of a combined positive/negative purging approach using the ISOLEX 300 device (Baxter, Munich, Germany). Positive selection of CD34 cells was followed by an immunomagnetic tumor cell depletion using a cocktail of monoclonal antibodies directed against epithelial antigens.

Preclinical experimentation had suggested that this combination of CD34 selection and subsequent immunomagnetic purging leads to an improved purging efficiency, resulting in an overall tumor cell depletion of 4.9 log (Table 1). These results could be confirmed in our trial. Thus, after double purging, tumor cells could only be detected in 6.3% of the autografts (1/16) compared with 50% before purging (8/16). A median of >4 log of tumor cell depletion could be achieved (Table 2). Positive/negative purging resulted in excellent results concerning CD34 purity and CD34 recovery leading to a median CD34 purity of 97.2% and a median cell loss of 48.9% after both purging procedures.[23] Furthermore, these cells led to rapid and sustained engraftment after high-dose chemotherapy. Thus, it can be

**Table 1.** Tumor cell spiking experiment: combined positive/negative selection using ISOLEX 300 SA

| | *Start fraction* | *Positive purging* | *Positive/negative purging* |
|---|---|---|---|
| TNC $\times 10^8$ | 146 | 3.78 | 2.80 |
| CD34 purity (%) | 1.8 | 77.7 | 76.8 |
| CD34 recovery (%) | — | 95.5 | 78.8 |
| No. tumor cells/$5 \times 10^5$ | 1500 | 27 | 1 |

*0.7% MCF-7 was spiked into a leukapheresis product.*

concluded that such an extensive purging approach can be safely conducted with acceptable cell loss.

## Purging of breast cancer cells using adenovirus-mediated suicide gene transfer

Moreover, in breast cancer we pursued a purging strategy based on the selective elimination of breast cancer cells using a gene therapeutic approach. The aim of generating selective transfer into micrometastatic breast cancer cells, while sparing hematopoietic progenitors, is achieved by taking advantage of the high transfer efficiency into breast cancer cells (Fig. 1). Consequently, already low dosage of Ad vector and 5-FC administration leads to tumor cell death in vitro, suggesting that tumor cells can be eliminated from stem cell autografts by this approach (Fig. 2). We were able to show that an adenovirus-mediated suicide gene transfer into breast cancer cells completely prevents tumor growth after xenotransplantation of tumor cells in vivo in SCID mice (Fig. 3A and B).[24] Furthermore,

**Table 2.** Purging efficiency

| | *No. tumor cells* $\times 10^4$ | | | |
|---|---|---|---|---|
| *Patient no.* | *Start fraction* | *After positive purging* | *After negative purging* | *Log depletion* |
| 1 | 1.7 | 0 | 0 | >4 |
| 3 | 1.6 | 0 | 0 | >4 |
| 7 | 4.8 | 0.036 | 0.027 | 2.2 |
| 11 | 0.78 | 0 | 0 | >4 |
| 12 | 2.2 | 0 | 0 | >4 |
| 13 | 17.7 | 0.020 | 0 | >4 |
| 15 | 3.7 | 0.052 | 0 | >4 |
| 16 | 4.3 | 0 | 0 | >4 |

*Tumor cells counted per $10^6$ were extrapolated with respect to the total cell number.*

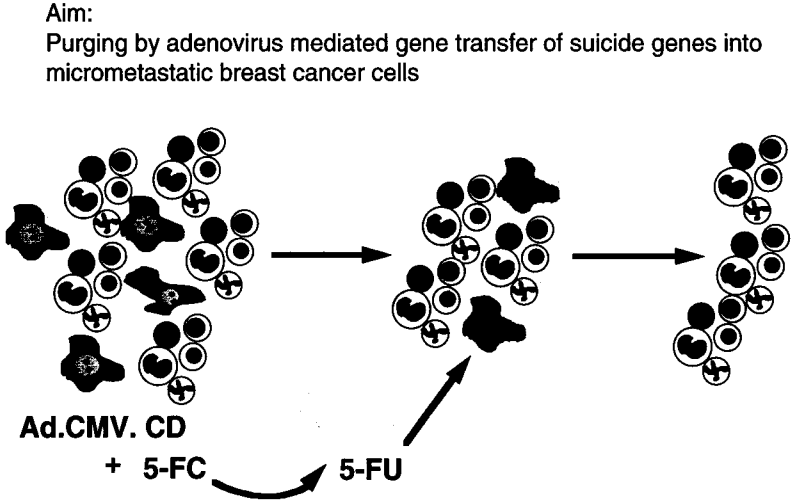


**Figure 1.** *Adenovirus-mediated purging.*

other groups have demonstrated that such an approach does not compromise long-term culture–initiating human hematopoietic stem cells.[25,26]

## DISCUSSION

Concerning combined positive and negative selection, different groups have reported comparable results with regard to CD34 yield, purity, and purging efficiency. Negrin and co-workers reported on a combined purging procedure in patients with non-Hodgkin's lymphoma. As a first step, CD34 cells were enriched using density gradient centrifugation and subsequently purged using antibody-complement lysis.[14] This procedure achieved a median CD34 recovery of 81.2%, albeit with a broad range of 9.9 to 302.6%. Purging efficiency was monitored by fluorescence-activated cell sorting (FACS) analysis and, in some cases of patients with follicular lymphomas, using t(14;18) specific polymerase chain reaction (PCR). They were able to show that combined purging was superior to CD34 enrichment alone. They were able to achieve PCR negativity after combined purging in three of three apheresis products and in two of three bone marrow harvests. However, it must be considered that the defined density gradient for eliminating lymphoma cells might not be applicable to other malignant diseases. Recently, Paulus et al.[27] used a method similar to our procedure in B-CLL patients and were also able to improve purging efficiency considerably, purging beyond the detection limit by flow cytometric analysis. However, clonal B-cells were still detectable using a sensitive CDR-III-PCR.[27] Nevertheless, these results are in line with our observations that

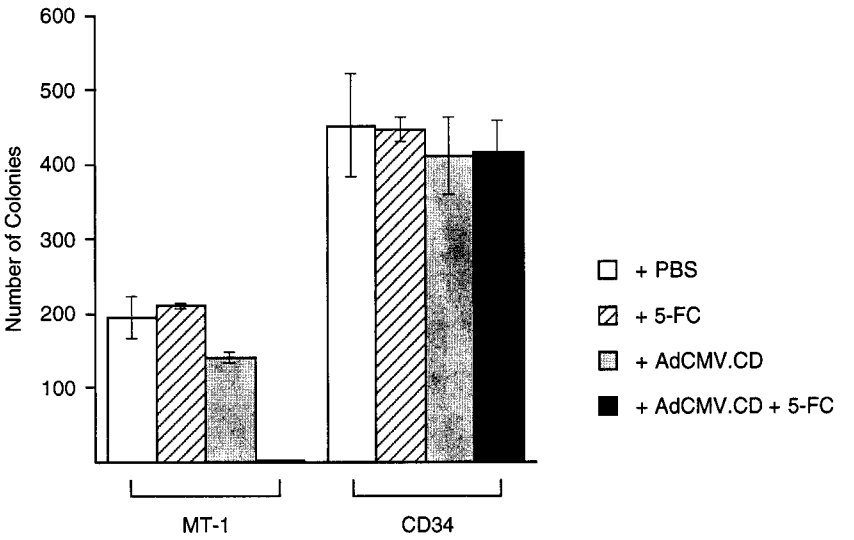


**Figure 2**

positive/negative purging leads to a considerably improved purging efficiency compared with positive selection alone. Nevertheless, tumor cells might evade even this purging approach. Hence, it is desirable to design purging strategies that do not rely solely on the physical elimination of tumor cells, but aim at the additional functional impairment of tumor cells, thus preventing their tumorigenicity in vivo. Therefore, we have evaluated different strategies with this aim by using antibody-mediated apoptosis via the CD20 or CD95 antigen in lymphoma cells or using adenovirus-mediated purging (Mapara et al., manuscript in preparation).[24] Alternatively, residual tumor cells could be targeted by an immunotoxin approach as demonstrated by Spyridonidis et al.[28] and Myklebust et al.[15]

The introduction of a suicide gene by means of gene delivery vectors offers highly interesting new options in graft manipulation. As described above, this technique is highly effective, provided a high gene transfer efficiency can be obtained by the vector. In the case of tumor cell purging, adenoviruses are excellent vectors for transducing breast cancer cells (as reported above) or myeloma cells.[29] Provided a vector is chosen that leads to stable integration of vector DNA into the transduced cells, such an approach would offer the opportunity to treat the recurrent malignant cells carrying the suicide gene in vivo. Furthermore, such an approach could also be applied for the elimination of T cells from allografts in vitro for the prevention of GVHD. A similar approach using herpes simplex thymidine kinase (Hs-Tk) has been successfully applied for the treatment of GVHD in vivo.[30]

In conclusion, combined positive/negative purging leads to a considerably

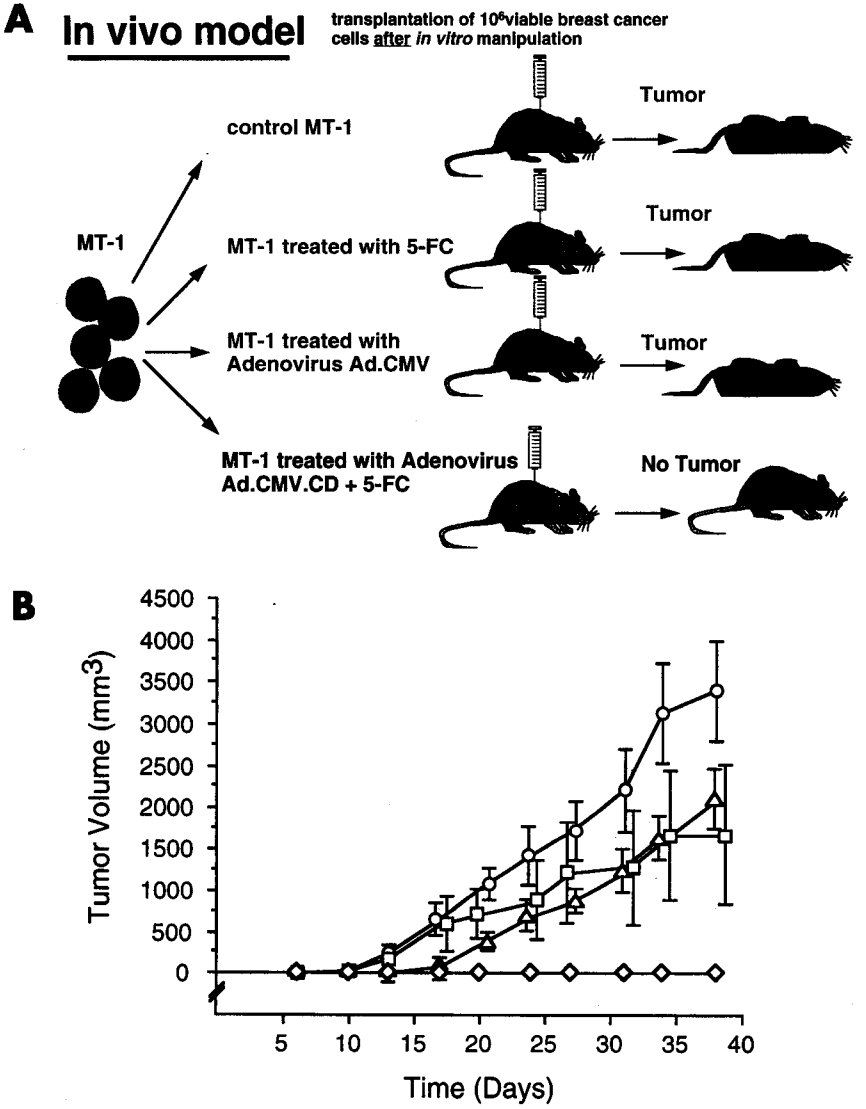


**Figure 3**

enhanced purging efficiency without compromising the quality of the graft. Double purging procedures might be especially relevant in malignancies with a high tumor burden within the autograft. Therefore, for an optimal tumor-free autograft, several approaches could be combined: in vivo purging by induction chemotherapy[31]; careful determination of the optimal stem cell harvest day[32–34]; and application of

ex vivo tumor cell depletion procedures.[23,24,28] Considering the rapid progress in the field of stem cell mobilization and graft engineering, it is conceivable that a tumor cell–free autograft is becoming a reality. Thus, it is now time to perform controlled randomized studies to validate the clinical relevance of such highly efficient and rigorous but time-consuming and cost-intensive purging procedures.

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# Tumor Purging Technologies: More Questions Than Answers

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## INTRODUCTION

Access to hematopoietic stem cell transplantation is limited by the availability of HLA-matched graft donors. Attempts to use matched unrelated and mismatched related donors are complicated by increases in the incidence and severity of graft-vs.-host disease. Depletion of T lymphocytes, or T cell subsets, to overcome this problem has generally resulted in problems of engraftment and an increase in the incidence of relapse, particularly in leukemia patients.[1] An alternative approach has been to use autologous marrow or peripheral blood progenitor cells as the source of the graft.[2] While this potentially could allow any patient to receive a transplant, there has been concern that such grafts may return viable occult tumor cells to the patient, and that these could act as a source of relapse. This concern has lead to the development of multiple strategies to purge tumor cells from autologous grafts ex vivo. The clinical benefit of purging has yet to be determined definitively in a randomized trial. There is, however, indirect evidence from tumor cell marking studies, and from the relapse patterns in patients receiving tumor-infiltrated grafts, that the presence of tumor cells in an autologous graft may be associated with disease relapse posttransplant.[3–5]

The purpose of this article is not to provide the reader with an overview of the technology for purging autologous grafts, but rather to raise some of the issues and controversies associated with the practice.

## THE PURGING DILEMMA

The controversy of whether it is beneficial to purge tumor cells from autologous stem cell preparations has raged for nearly 20 years. It has been fueled by the lack of definitive data that would come from a randomized clinical trial. There are multiple reasons why such a study has never been successfully completed. These include the ethical issue of whether it is acceptable to randomize patients to a study arm where there is a risk that they would receive viable tumor cells in the graft, if there is a safe method available for their removal. Second, the complexity of selecting a disease in

which the benefit of purging could be clearly demonstrated, in a reasonable time, and within a reasonably sized patient population, has often proven too much for even ardent purging advocates. Third, it is recognized that no single purging technology is regarded as the gold standard. This has made it difficult to decide whether the primary purpose of a purging trial should be proof of principle of a depletion technology, of the generic value of purging, or of the efficacy of a particular product. This has been further complicated in the United States by the Food and Drug Administration's evolving regulatory strategy for stem cell products,[6] which has seen purging and positive selection transition from classification as minimal manipulation, to extensive manipulation, and back again. The final status has still to be determined. These impediments to traditional methods to demonstrate that purging is both safe and effective by randomized trials have resulted in the use of other surrogate measurements to demonstrate its potential benefits.

## Pros and cons

Some of the most convincing evidence has come from gene marking experiments in which autologous cells have been transfected with a marker gene in the hope that it would be incorporated into any tumor cells within the graft. The graft is then infused and the patient monitored for signs of relapse. At that time, samples of blood and marrow are taken to determine whether gene-marked cancer cells are present. Since they can only have originated from the graft, their presence suggests that relapse is associated with reinfusion of tumor cells.[3,7] It cannot be definitively shown that these cells caused relapse, and it has been argued that they may simply home to sites of relapse and may not contribute substantially to its etiology. This theory is based on the relative numbers of tumor cells that may be present in the infused graft vs. those that escape the high-dose therapy. Depending on the bulk of the disease at the time of treatment, it would be anticipated that there might be several orders of magnitude more cells remaining in the patient than would be returned with the graft. This fact would tip the balance irrevocably in favor of chemotherapy-resistant cells acting as the origin of relapse. An argument against the theory is that metastatic tumor within the marrow or mobilized into the blood may differ markedly in its potential to cause relapse than cells at other sites of disease. Their removal from the graft could, therefore, have more clinical impact than would be anticipated solely based on their numbers. This possibility has yet to be convincingly demonstrated.

Other evidence in favor of purging has come from transplants in which some grafts were found to be ineffectively purged when examined by highly sensitive molecular methods, such as the polymerase chain reaction (PCR).[4,8] Patients receiving these grafts were found to have decreased disease-free survival in comparison with those who were given grafts purged to PCR negativity. It has been

argued that these two patient populations were inherently different, as was indicated by the failure of a single purging technique to achieve the same level of tumor eradication in both groups. These observations are, however, strengthened by reports of delayed relapse in a variety of patients who received grafts that were purged.[9,10] Unfortunately, careful examination of the literature reveals as many papers demonstrating no benefit from purging as have been published in its favor.[10] Interestingly, it appears that the regulatory bodies have now agreed to accept a surrogate marker of effective ex vivo depletion of tumor cells as demonstration of its potential clinical value, making it all the more likely that a formal randomized clinical trial may never be undertaken. It remains to be established whether such a surrogate marker is acceptable for every disease in which it is possible to demonstrate that tumor cells can be removed from the graft, and that purging as a generic entity will be accepted. Given the numerous factors that can contribute to the risk, pattern, and time to relapse in cancer, the impact of purging is likely to be a highly variable factor. In the era of managed health care, with capitated costs for transplantation, the question of relative contribution to clinical outcome becomes particularly important, especially when considering a procedure that is probably incapable of achieving complete tumor removal, and where a "safe" number of residual tumor cells within the graft may never be established.

## APPROACHES

Most cell selection technologies have their clinical origins in autologous graft purging. For many years, the tumor cell was the primary target, and methods were developed for its elimination in situ or for its physical removal. One of the earliest techniques was incubation of marrow from patients with acute myelogenous leukemia with the cyclophosphamide derivatives.[9,11] This technique could effectively eliminate leukemic cells, but it also affected normal hematopoietic progenitors, with a resulting delay to engraftment. The trend has, therefore, been toward increasing the specificity of purging techniques, an action made possible by the availability of various monoclonal antibodies with reactivity toward tumor-associated antigens. Monoclonal antibodies have been used extensively in combination with complement, toxins, and magnetic particles to achieve varying degrees of tumor removal from marrow and blood-derived grafts. The particular methods have been extensively reviewed elsewhere[10]; however, there are some important points to be made regarding the technology of tumor depletion.

### Residual cells

*Logs vs. numbers.* The tendency has been to quantify purging efficiency in terms of logs of tumor cell depletion. This may give a misleading idea of the

power of a technique, since it can be relatively easy to achieve impressive numbers of logs of depletion by increasing the initial level of infiltration. Under these conditions, many depletion methods will perform efficiently. That may not be true under clinically applicable conditions, where the best chances of affecting outcome are when the tumor burden is low within the graft, and the aim is to achieve complete removal. In that case, the limiting factors to the technique become more apparent and the characteristics of the residual cells provide useful information on how to optimize the purging system. For example, in antibody-mediated purging methods, an evaluation of target antigen density on the cells that escape elimination is invaluable in developing a strategy to improve efficacy. In general, low antigen density cells are usually more difficult to purge, particularly when using antibodies and complement[12]; however, cells with excessively high expression of an antigen may also be able to evade elimination.[13] The use of a clinically appropriate model system in combination with multiple sensitive assays for residual target cells is, therefore, central to the development of an effective purging technology.

*Detection technologies.* Validation of any new purging technology is also critically dependent on the use of a sensitive, well-characterized method for the detection of the target cell population. Molecular techniques are capable of impressive levels of sensitivity but may not provide quantitative information. They will also detect material that has been released from dead or dying target cells, thereby underestimating the purging efficiency. Nonetheless, these assays can still be regarded as the gold standard for evaluating purging methods. Purging to PCR negativity has become the synonym for complete purging in many diseases.[4,8] In some studies, however, the sensitivity and reproducibility of the detection technique is not reported, making it difficult to determine what a negative finding truly represents. It becomes all the more important, under such circumstances, to think in terms of absolute numbers of cells rather than levels of depletion. Based on preliminary purging experiments, and knowing the sensitivity of the detection technique, it should be possible to calculate the probable number of target cells that may remain after purging. The volume and number of samples of the postpurge product that would be required to detect these cells with a high degree of probability can then be determined. Publications still appear in which a single small volume sample of a purged graft is used to demonstrate purging efficacy, under conditions where elementary mathematics and the Poisson distribution would clearly indicate that a negative result would essentially be guaranteed.

*Tumor enrichment.* Recently, there has been a renewed emphasis on using tumor enrichment techniques to improve the sensitivity of tumor cell detection in clinical samples.[14] In most cases, this involves using tumor-directed antibodies and

immunomagnetic particles to identify and concentrate the tumor cells. While this approach is certainly of value for diagnostic specimens, it should be used cautiously in purging applications. The tendency has been to use the same antibodies for purging and for concentrating the residual tumor cells in the purged graft to aid in their detection. This can be self-defeating if the residual cells have escaped purging by their inability to bind those particular monoclonal reagents. An alternative strategy that can be used under these circumstances is to enrich the residual tumor cells by depleting CD45-positive normal leukocytes. Obviously this can only be used in diseases in which the tumor population does not express the CD45 antigen.

Antibody-based detection techniques are generally about an order of magnitude less sensitive than molecular techniques. Although methods are available to allow flow cytometers to detect extremely small numbers of residual tumor cells, the technique of choice has been to use immunocytochemistry.[14,15] This offers the combination of detection by positive staining and visual confirmation of the appropriate morphology of the stained cell. Careful validation of the method may allow limits of detection of about one positive event in 1 million cells; however, as indicated previously, it then becomes crucial to examine a sufficiently large number of cells to obtain accurate information. This endeavor may be facilitated by computerized image analysis, in which many cells can be scanned by the system and positive events recorded and retrieved subsequently for visual examination.[14]

Detection methods that rely on molecular or surface markers do not give any indication of the likely proliferative potential of the tumor cells, which can be obtained from various in vitro colony-forming assays.[16] Again, these suffer from limitations, in that failure to grow in culture may reflect the inadequacy of the culture conditions, rather than the absence of tumor cells. Positive growth is the only result that can be interpreted with any degree of certainty.

Detection technology is, therefore, inextricably linked to purging. Ideally, the combination of molecular, antibody-based, and colony-forming assays should be used, since each provides complementary, if incomplete, information. These assays must also be used within their limits of detection—which seems intuitively obvious but is not always the case in some studies. Purging that achieves a negative result should then be reported as achieving depletion to the level of sensitivity of the assay, with an indication of how many residual cells may still be present in the purged graft. Purging performed on heavily infiltrated grafts is, as discussed above, not representative of most clinical situations, but it may give at least some indication of the tumor removal capacity of a purging technique. When the technique is then used under more clinically appropriate conditions, and achieves elimination to the level of sensitivity of the detection assays, this capacity measurement can provide some idea of the degree of additional tumor removal that may have been achieved.

## BLOOD VS. MARROW

Cytokine-mobilized peripheral blood progenitor cells (PBPC) have largely replaced bone marrow as the source of hematopoietic grafts in many diseases.[17] This allows collection of a graft without the use of general anesthesia and from patients who are unable to undergo a marrow harvest. PBPC show more rapid engraftment kinetics with resulting potential decreases in the cost of transplantation. An additional advantage frequently cited is the lower level of tumor contamination in grafts derived from the blood compared with the marrow.[18] Again, such information must be interpreted with caution, since it must be viewed in the context of what constitutes a graft. Traditionally, marrow graft adequacy has been determined by the total number of nucleated cells, since marrow harvests were first performed when there was no other method to assay progenitor cell content. In most cases, a nucleated cell dose of $1\times10^8$/kg has been regarded as satisfactory, which translates to approximately $1–2\times10^6$ CD34-positive cells/kg. CD34 cell content very rapidly became adopted as the standard measure for the adequacy of PBPC grafts, since, unlike colony-forming assays, it could be used in real time. In spite of certain differences in assay methods, a CD34 dose of $1–2\times10^6$/kg is also thought to be adequate. The collection of this same dose is, however, subject to many more variables.

There is a general consensus that resting peripheral blood shows lower levels of tumor infiltration than marrow from the same patient. This situation has been extrapolated to suggest that the blood would, therefore, provide a graft with a lower risk of tumor involvement. It is important, however, to work in absolute cell numbers. PBPC grafts are usually collected by repeated aphereses until the target CD34-positive cell dose has been reached. To achieve such a dose from heavily pretreated patients, it is sometimes necessary to collect larger numbers of nucleated cells than would be harvested to collect an equivalent CD34-positive cell dose from the marrow. This essentially counteracts the lower level of tumor contamination found in the blood.[18] In addition, there is some evidence to suggest that cytokine administration can mobilize tumor cells into the peripheral circulation, so tumor levels in resting blood may not be a reliable indicator of levels at the time of apheresis.[19] Incorporating a cytotoxic drug into the mobilization regimen may counteract this. These findings have, however, stimulated a number of studies to quantitate the relative tumor contamination in both graft sources.[20]

Ex vivo manipulation of PBPC grafts poses an additional challenge to the purging laboratory, in that there may be multiple collections containing large numbers of cells, with very different handling properties from those of marrow harvests. This challenge has necessitated modifying the purging protocol and developing conditions for holding collections overnight so that sequential aphereses may be combined and purged at one time.[18]

## POSITIVE OR NEGATIVE?

Given all of the aforementioned caveats, it is still evident that purging techniques are available that can substantially reduce the tumor burden within autologous grafts. Antibody-based immunomagnetic techniques probably represent the standard at the moment, although various promising molecular methods are in early clinical trials. The debate that has now surfaced is whether similar or superior results could be achieved by CD34-based enrichment of normal hematopoietic progenitor cells. This approach offers a number of clear advantages. First, it could be used for a number of diseases in which there is clear evidence that the tumor population is CD34-negative, e.g., most solid tumors. In contrast, negative selection techniques usually require multiple, disease-specific monoclonal reagents, which are expensive to prepare and test. Second, the positive selection technique requires the enrichment of only a sufficient number of cells to restore hematopoiesis, whereas the aim in negative selection is to remove every possible tumor cell.

In theory, it is possible to calculate the tumor purging efficiency of positive selection quite easily if it is assumed that the tumor cells in the graft act as passive by-standers during the enrichment, rather than being either selectively enriched or depleted.[21] These calculations reveal that the technique cannot achieve comparable levels of tumor depletion to efficient negative selection methods, unless the purity of CD34 cells in the final product is extraordinarily high. To date, this level of purity has not been achievable for most of the commercially available positive selection systems. Considerable variability in performance has become almost a trademark of CD34 selection, although there has been some trend toward improvement over time. If the aim is to go for maximal tumor removal, then positive selection alone is unlikely, in its present form, to meet the criteria. It does, however, provide a useful method for "de-bulking" the graft, to be used in combination with a negative selection procedure.

## ENGINEERING THE GRAFT

As our understanding of the cell populations involved in hematopoiesis grows, it is becoming obvious that numerous cells within a graft can influence clinical outcome. The dose of hematopoietic progenitor cells is obviously of central importance, and their relative quality[22] and potential proliferative lifespan[23] are emerging as additional major factors. Other numerically small populations may, however, have important roles in facilitating engraftment[24] and eradicating residual disease in the recipient.[25] These are all factors that will ultimately have an impact on graft engineering technologies.

In allogeneic transplantation, the last few years have seen dramatic changes, including the introduction of mobilized blood as a graft source, the emergence of

graft-vs.-leukemia responses, the use of hematopoietic cell subpopulations to facilitate engraftment across histocompatibility barriers, and a rebirth of immunotherapy in the context of posttransplant relapse.[26] In autologous transplantation, we continue to wrestle with the purging issue. Is this worthwhile?

It appears that, in leukemias and solid tumors, it is possible to achieve a potent anticancer immune response directed toward small numbers of residual tumor cells after both allogeneic and autologous transplant.[26–28] Infusion of allogeneic primed or targeted leukocytes may ultimately prove to be the most effective method of preventing or combating relapse posttransplant in autologous transplant recipients. The fact that immunotherapy has worked best under conditions of minimal residual disease supports the hypothesis that the best chance of cure using this modality can be expected when steps are taken to minimize tumor burden.

Purging of the autologous graft provides a method of reducing the tumor burden in the transplant recipient. We still do not know whether purging, in itself, can result directly in significant improvements in disease-free survival. It could, however, contribute to improving the success of immune-based therapy posttransplant. These are extremely difficult hypotheses to test using traditional methods. We may find that we will have to settle ultimately for the explanation that it simply does not seem to make sense to return cancer cells to a patient if there is an efficient, low-risk way of either eliminating them or reducing them to a level that could be eradicated by an immune response.

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# Behavior of Hematopoietic Stem Cells and Solid Tumor Cells During Ex Vivo Culture of Transplants From Human Blood

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## ABSTRACT

Ex vivo culture of human hematopoietic cells has been investigated in the context of blood progenitor cell transplantation to *1*) purge tumor cells, *2*) provide extended time for genetic manipulation of hematopoietic cells, and *3*) selectively amplify progenitor or accessory cells that could serve as mediators of improved hematopoietic recovery or as a tool for immune modulation. We have developed a serum-free culture medium for ex vivo expansion of $CD34^+$-enriched blood progenitor cells. When supplemented with hematopoietic growth factors interleukin (IL)-3, stem cell factor (SCF), and flt3-ligand, an approximately 100-fold hematopoietic cell expansion is reached without additional manipulation within 7 to 10 days. Colony-forming progenitors are amplified at levels comparable to total cell numbers. Long-term culture–initiating cells (LTC-IC) also expand within 7 days, although to a lesser degree (about threefold). Injection of cultured cells at different culture periods into immune-deficient mice with the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) phenotype revealed maintenance of repopulating cells for a culture period of up to 4 days, whereas most of the repopulating potential was lost by culture day 7. The potential use of ex vivo culture protocols to purge mammary tumor cells from autologous hematopoietic grafts was studied using freshly isolated or culture-enriched primary breast cancer cells. No influence of hematopoietic stimulatory cytokines was seen; however, addition of transforming growth factor (TGF)-β1 resulted in an effective depletion of both total and clonogenic breast cancer cells in serum-supplemented cultures.

Hematopoietic cells did not contribute to tumor cell survival in mixing experiments. In contrast, in serum-free culture medium, mammary carcinoma cells were rapidly and effectively depleted within the first few days in culture. A specific molecular approach to purge breast cancer cells was exploited by the use of recombinant immunotoxins directed against the epidermal growth factor (EGF) or the erbB2 receptors, which were expressed in the tumor cells but not expressed in hematopoietic cells. A rapid, concentration-dependent elimination of primary breast cancer cells was achieved in ex vivo culture. Our data indicate that short-term serum-free ex vivo culture may allow effective hematopoietic reconstitution as well as tumor cell purging.

## INTRODUCTION

The implementation of peripheral blood progenitor cell transplantation has been followed by the development of a variety of protocols that are applied in high-dose chemotherapy with autologous hematopoietic stem cell transplantation.[1] Moreover, the clinical establishment of $CD34^+$ selection from leukapheresis products made it possible to culture hematopoietic cells in adequate volumes of culture medium in the presence of hematopoietic cytokines.[2–4] Ex vivo expansion has been designed to generate amplified numbers of progenitor cell populations, such as colony-forming cells, to shorten the period of bone marrow aplasia posttransplantation[5]; to provide time for the retroviral infection of progenitor cells[6,7]; or for the purging of contaminating tumor cells that are present within autografts.[7–9]

In most studies, the efficiency of the progenitor expansion has been assessed using semisolid clonogenic assays, numbers of $CD34^+$ cells, or the frequency of LTC-ICs.[10–12] More recently, immunodeficient mice have been used to measure the hematopoietic repopulation potential after ex vivo expansion.[13,14]

In patients undergoing high-dose chemotherapy with autologous hematopoietic transplantation, contamination of the graft with tumor cells has been observed and has been shown to be of clinical significance in retroviral marking studies in leukemia or neuroblastoma.[15–17] In breast cancer, contaminating tumor cells have also been shown to be capable of in vitro growth.[18] Therefore, purging procedures have been developed. Autologous grafts have been subjected to antibody-mediated selection of hematopoietic progenitor cells, i.e., enrichment for $CD34^+$ progenitors. With the currently available selection technology, however, it has been difficult to achieve $CD34^+$ cell purities of >90%—equivalent to an about two log expected depletion of tumor cells[19]—and contaminating breast cancer cells have been detected in $CD34^+$-purified fractions from both bone marrow[19] and peripheral blood.[20] We therefore investigated the influence of culture conditions used for hematopoietic cell expansion on the proliferation or survival of mammary carcinoma cells.

## MATERIALS AND METHODS

$CD34^+$ cells were isolated from patients with a diagnosis of solid tumors or lymphoma, after informed consent, by leukaphereses and subsequent $CD34^+$ purification (CellPro) as previously described.[21] $CD34^+$ cells were inoculated at a density of 3 to $5\times10^4$/mL into serum-free medium (CellGro; CellGenix GmbH, Freiburg, Germany) in the presence of recombinant hematopoietic growth factors in standard tissue culture flasks at 37°C and 5% $CO_2$.[21] Colony-forming assays and determination of LTC-IC was performed as previously described.[11] NOD/SCID mice were maintained and transplanted according to a previously described protocol for SCID mice.[22,23] Human engraftment was assessed by flow cytometry using human-specific antibodies against CD45 and HLA class I surface antigens.

Tumor cells were isolated from ascites or pleural effusions of patients with metastatic breast cancer and cultured in RPMI, 10% fetal calf serum (FCS) at a starting cell density of $5\times10^4$/mL.[9,24] Tumor cells were measured by immunostaining with anticytokeratin antibodies as described.[9,24] Recombinant immunotoxins recognizing the erbB2 and EGF receptor antigens have also been described.[9]

## RESULTS

Ex vivo expansion of total cell numbers followed a rapid time course with a more than 10-fold amplification within 1 week of culture (Fig. 1). Also, numbers of colony-forming cells increased exponentially in the presence of the cytokine combination, flt3 ligand, SCF, and IL-3. In contrast, numbers of LTC-IC increased to a relatively lower degree, by a factor of up to three within this time period (Fig. 1). When equal aliquots of approximately $0.5\times10^5$ $CD34^+$ cells were cultured for various time periods under the same conditions and injected into preirradiated (300 cGy) NOD-SCID mice, their ability to reconstitute human hematopoiesis in vivo was maintained for a period of only 4 days (Table 1). In contrast, on day 5 or day 7 of ex vivo expansion culture, no or nearly no human engraftment could be recorded in the mice (Table 1). These data show that primitive cell populations as assayed in vitro follow kinetics during serum-free ex vivo expansion of $CD34^+$ progenitor cells that are different from those of the in vivo repopulating cells.

To study the behavior of solid tumor cells during ex vivo expansion, primary breast carcinoma cells were used. These cells were isolated from malignant effusions, as numbers of contaminating tumor cells are usually very low within bone marrow. The tumor cells were identified using anticytokeratin immunostains. These stains were complemented by further immunohistochemical analyses to distinguish tumor cells from mesothelial cells, which might also grow from the effusion. The tumor cell origin of the analyzed cells could be verified by a positive BerEP4 stain and negative staining for calretinin. When primary tumor cells were

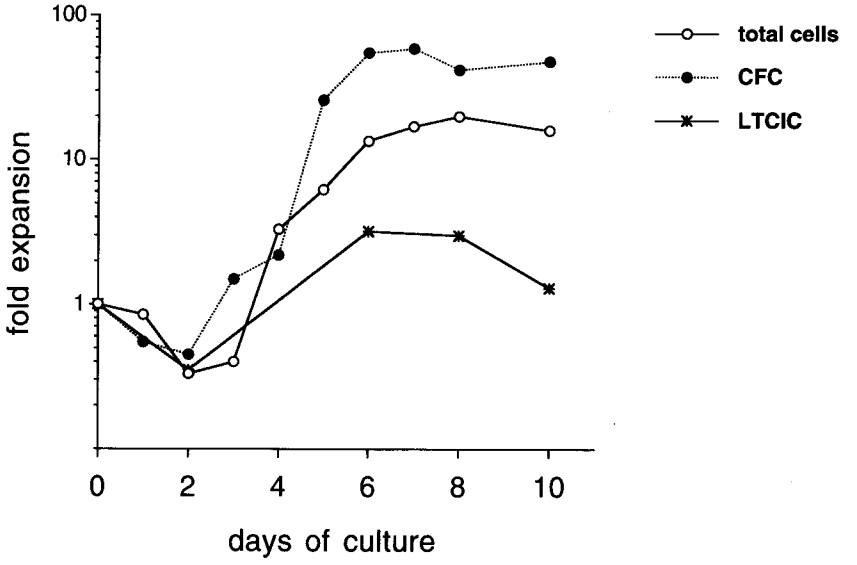


**Figure 1.** *Development of numbers of total nucleated cells, CFCs, and LTC-ICs during ex vivo expansion of CD34⁺ blood progenitor cells in serum-free medium in the presence of Flt3 ligand (300 ng/mL), SCF (100 ng/mL), IL-3 (100 ng/mL). The symbols give the mean values (total cell numbers, CFC) or the LTC-IC content determined via incidence analysis through limiting dilution culture of aliquots taken at the indicated intervals from a representative 100-mL clinical scale culture.*

grown in the presence of FCS, they started to proliferate several weeks after isolation and displayed purities of >95% cytokeratin-positive cells. When cytokines (in this case a combination of SCF, IL-1, -3, and -6, and erythropoietin, according to a clinically established ex vivo expansion protocol of plasma/serum supported CD34⁺ cell expansion) were added, no change was seen in their growth behavior (Table 2). In contrast, addition of TGF-β1 resulted in a fivefold reduction of tumor cell numbers, corresponding to a complete cessation of tumor cell growth, and induction of cell death. This result was observed both in the presence or absence of the hematopoietic cytokines, and regardless of whether total tumor cell numbers or clonogenic tumor cells were analyzed (Table 2).

The presence of CD34⁺ cells did not detectably influence the survival of mammary carcinoma cells in culture, as assessed after PKH labeling of the tumor cells and a 3-day observation period (Table 2). Coincubation of CD34⁺ cells and tumor cells at a ratio of about 1:100 also did not show altered tumor cell proliferation as observed when tumor cells were cultured alone (data not shown).

In contrast, when FCS-supported medium was exchanged for serum-free medium, a strong (10- to 100-fold) reduction in tumor cell numbers occurred

**Table 1.** Engraftment of human CD34+ blood progenitor cellsin NOD/SCID mice after various periods of suspension culture in Flt3 ligand, SCF, and IL-3

| *Mice transplanted (n)* | *Expansion culture (days)* | *% human CD45+ cells (mean ± SE)* | *Positive mice/total* |
|---|---|---|---|
| 10 | 0 | 10.9 ± 10.1 | 8/10 |
| 4 | 2 | 4.6 ± 3.5 | 3/4 |
| 4 | 3 | 3.1 ± 1.5 | 3/4 |
| 6 | 4 | 2.5 ± 2.8 | 4/6 |
| 3 | 5 | 0.3 ± 0.4 | 1/3 |
| 7 | 7 | 0.2 ± 0.5 | 1/7 |

*Mice received 300 cGy of γ-radiation and were transplanted with the full progeny of 3–5×10⁵ CD34+ blood progenitor cells cultured for various time periods. Human hematopoiesis was determined by flow cytometry using human-specific anti-CD45 antibody 5–8 weeks posttransplantation. Data from Goan et al.*[34]

(Table 2). This reduction was not further influenced by the presence or absence of CD34+ hematopoietic cells. In mixing experiments with CD34+ cells and tumor cells in serum-free medium, it became evident that the tumor cells were eliminated rapidly within the first 1 to 3 days (not shown).

Finally, single-chain recombinant immunotoxins directed against erbB2 or EGF receptors, which are present on mammary carcinoma cells, were used. These molecules also carried a modified *Pseudomonas* exotoxin as effector component.[9] In three of five samples expressing erbB2 and all six samples expressing the EGF receptor, efficient elimination of tumor cells was seen in serum-supplemented medium in a time-dependent manner (Table 2). This treatment also spared the CD34+ expanded hematopoietic progenitor cells.[9]

Altogether, we have seen that, among the components of ex vivo expansion protocols, use of serum-free medium or of recombinant immunotoxins is an efficient way to eliminate mammary carcinoma cells during suspension culture of CD34+ cells. The survival of hematopoietic repopulating stem cells is limited to a relatively short interval of 3 to 4 days. Yet, clinical protocols using ex vivo expansion may be a promising additive to reach an increased efficiency of tumor cell purging in patients with breast carcinoma receiving autotransplants.

## DISCUSSION

A number of previous studies have investigated the fate of primitive cell populations during ex vivo expansion of CD34+ progenitor cells.[3,10–12,21] Generally, these studies have used in vitro endpoints such as colony-forming cells (CFCs) or LTC-IC assays to measure the transplantation potential of expanded cells. Also,

**Table 2.** Influence of components of ex vivo expansion systems on proliferation or survival of mammary carcinoma cells

| *Conditions* | | *Duration* | *Tumor cell output/ input in* | *Change in total tumor cell numbers* | *Change in numbers of clonogenic* |
|---|---|---|---|---|---|
| *Variable* | *Control* | *(days)* | *control (n)* | *vs. controls* | *tumor cells* |
| S 136 E | FCS/RPMI | 7 | 4.5 | NC | NC |
| TGF-1 | FCS/RPMI | 7 | 4.5 | Fivefold reduction | 13–17-fold reduction |
| S136 + TGF-1 | FCS/RPMI | 7 | 4.5 | Fivefold reduction | >12-fold reduction |
| $CD34^+$ | FCS/RPMI S136 E | 3 | 1 | NC | Not done |
| Serum-free medium* | FCS/RPMI | 7–30 | 7.5 | 10–100-fold reduction | ND |
| Serum-free medium* | FCS/RPMI + S136 E | 7–30 | 6.5 | 10–100-fold reduction | ND |
| $CD34^+$ | Serum free* | 3 | 0.1 | NC | Not done |
| Anti-erbB2 immunotoxin | FCS/RPMI | 7 | >1 | 66–99% depletion in 3/5 cases | Not done |
| Anti-EGFR immunotoxin | FCS/RPMI | 7 | >1 | 73–99% depletion in 6/6 cases | Not done |

**Freshly isolated, nonprecultured tumor cells were used. NC, no change; ND, not detected (below levels of detection). Data adapted from Spyridonidis et al.[25]*

kinetic analyses at relatively short intervals have not been systematically performed. We found the kinetics of $CD34^+$ cell expansion and CFC expansion to be about two times faster than in our previous protocol using autologous patient plasma or FCS.[10,11] The cytokine combination used here has previously been found to confer an optimal LTC-IC expansion when starting with bone marrow $CD34^+$ $CD38^-$ cells.[12] Our LTC-IC results with a slight expansion should be in line with the (stronger) LTC-IC expansion observed by Petzer et al.,[12] when assuming that the additional CD38 depletion resulted in the enrichment of a more primitive and better expanding subpopulation of LTC-IC in the latter study. Wang et al.[14] observed analogous results to those of this study, when they incubated human hematopoietic cells on pre-established human bone marrow stroma feeders and found a short-term (4-day) maintenance of bone marrow repopulating cells, compared to a net expansion of LTC-IC and CFCs at later time points, when the in vivo repopulating potential had already declined. Bhatia et al.[25] confirmed these findings for suspension culture of human cord blood–derived $CD34^+$ $CD38^-$ cells with loss of repopulating cells after 8 days of suspension culture and, in this case,

an increase of repopulation potential after 4 days in cytokine-supported culture. The importance of flt-3 ligand for the maintenance of repopulating cells has been demonstrated previously by Dao et al.[26] They showed that flt-3 ligand was needed to maintain the repopulation potential for a period longer than 24 hours, up to 72 hours. In agreement with these observations, Larochelle et al.[27] found that the transplantation potential of $CD34^+$ $CD38^-$ cord blood cells was lost when they were incubated in the presence of other cytokine combinations.

The main components with an influence on the survival of breast cancer cells during ex vivo expansion were found to be serum-free medium and the use of recombinant immunotoxins. To a limited degree, TGF-β1 was also able to decrease numbers of tumor cells. Whereas some previous studies have shown an influence of hematopoietic cytokines such as IL-1 and -6 on tumor cell growth ex vivo using established breast cancer cell lines,[28,29] our results with primary breast cancer cells are in line with the findings of Emerman et al.[30] who, also using primary cells, found no effect of cytokines on tumor cell growth. The results with this serum-free medium preparation show that stem/progenitor cells do not have a major impact on tumor cell survival. The result from the short term (3-day) experiments using PKH-labeled tumor cells in the presence or absence of $CD34^+$ cells showed that in serum-free medium, an efficient tumor cell depletion is observed very early during the culture time.

Taking together our results, we propose that a relatively short incubation of $CD34^+$ blood progenitor cells is required for the maintenance of bone marrow–repopulating stem cells, offering the possibility to efficiently eliminate tumor cells from autologous transplants. This possibility is underscored by kinetic analyses of tumor cell elimination by immunotoxins, which also showed that these molecules may act within hours, up to a 3- to 4-day short time period.[9] Given the (already) low incidences of solid tumor cells within autologous transplants in patients with breast carcinoma, a combination of the purging mechanisms that may be achieved during ex vivo expansion could, in addition to immunoselection of transplants, lead to practically tumor-free transplants for clinical application.

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# CD34 Selection and Autografting in Patients With Low-Grade Non-Hodgkin's Lymphoma

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## ABSTRACT

The efficacy of an immunomagnetic purging method for selecting $CD34^+$ cells from leukapheresis products (LP) was assessed in 31 patients with follicular non-Hodgkin's lymphoma. Peripheral blood stem cells (PBSC) were collected after granulocyte colony-stimulating factor (G-CSF)-supported cytotoxic chemotherapy, and $CD34^+$ cells were selected using the Isolex 300 devices (Baxter Immunotherapy, Irvine, CA). The mean purity (± standard error [SE]) of the $CD34^+$ cell population was 96.3 ± 1.2%, and the mean recovery was 62.7 ± 5.7%. A reduction in the content of $CD19^+$ B cells was achieved ($1.7 \pm 1.4 \times 10^6$/kg vs. $0.05 \pm 0.01 \times 10^6$/kg, $P$=0.13). In 11 patients, the leukapheresis product contained t(14;18)-positive cells by polymerase chain reaction (PCR), whereas the selected $CD34^+$ cells of six patients became negative. Twelve patients received high-dose therapy consisting of total body irradiation (TBI) (14.4 Gy, hyperfractionated) and cyclophosphamide (200 mg/kg body weight), and one patient received BEAM (BCNU, etoposide, cytosine arabinoside, melphalan) followed by the reinfusion of a mean number of $7.4 \pm 1.2 \times 10^6$ $CD34^+$ cells/kg. No significant differences were observed with regard to the time needed for neutrophil and platelet reconstitution when a comparison was made with 104 patients who had received high-dose therapy supported by unmanipulated LP (13.6 ± 0.7 days vs. 13.9 ± 0.4, $P$=0.8, and 14.5 ± 1.3 vs. 13 ± 0.8 days, $P$=0.6, respectively). In conclusion, CD34-immunomagnetic selection provides cells of high purity, reduced tumor cell content, and conserved engraftment capability. Further selection of $CD34^+/CD19^-$ cells is envisaged to improve the purging efficacy of this method.

## INTRODUCTION

Peripheral blood hematopoietic stem and progenitor cells are increasingly used for the support of high-dose therapy. More recently, it has been demonstrated that

hematopoietic stem cells expressing the CD34 antigen can be successfully isolated from blood or bone marrow and provide fast hematopoietic reconstitution after high-dose therapy.[1–7] The selection of $CD34^+$ cells on a large scale has been used for purging purposes.[1,8,9] Essential prerequisites for any CD34 selection is that the tumor cells do not express the CD34 antigen and that the engraftment capability of the progenitor and stem cells is preserved.

Follicular lymphomas (FL) are characterized by the t(14;18) translocation, which brings the expression of the anti-apoptotic protein bcl-2 under the control of regulatory elements of the immunoglobulin heavy chain gene on chromosome 14.[10]Several groups including ours have previously demonstrated that $CD34^+$ hematopoietic stem cells and their $CD34^+/CD19^+$ B lymphoid progeny do not express the t(14;18) translocation, making the $CD34^+$ selection a suitable purging method for patients with FL.[11–13] In the study presented here, $CD34^+$ cells were enriched from 31 patients with FL using the Isolex 300 devices (Baxter Immunotherapy, Irvine, CA). The immunophenotype of the selected cells and the presence of residual tumor cells were evaluated. The engraftment capability of $CD34^+$ cells after high-dose therapy in 13 patients was compared to that of 104 unmanipulated leukapheresis products.

## PATIENTS AND METHODS

### Patients

Between November 1995 and June 1998, 31 patients were included in this study. Fourteen patients were women and 17 were men, with a median age of 48.5 years (range 32–59). Patient characteristics at the time of PBSC mobilization are given in Table 1. Informed consent was obtained from each patient before therapy. The study was conducted according to the guidelines of the Joint Ethical Committee of the University of Heidelberg.

PBSCs were collected using large-volume leukaphereses during marrow recovery enhanced with 300 μg/d subcutaneous G-CSF (Neupogen, Amgen, Thousands Oaks, CA) after cytotoxic chemotherapy. HAM (2 $g/m^2$ cytosine arabinoside every 12 h on days 1 and 2 and 10 $mg/m^2$ mitoxantrone, days 2 and 3) was administered to 30 patients. One patient received dexaBEAM (24 mg/d dexamethasone, days 1–7; 30 $mg/m^2$ melphalan, day 2; 60 $mg/m^2$ carmustine, day 3; 75 $mg/m^2$ etoposide and 200 $mg/m^2$ cytosine arabinoside, days 4–7).

Selected $CD34^+$ cells were used for the support of high-dose therapy in 13 patients (Table 2). The conditioning regimens consisted of TBI (14.4 Gy, hyperfractionated) followed by cyclophosphamide (200 mg/kg). One patient received BEAM (300 $mg/m^2$ carmustine, 1.2 $g/m^2$ etoposide, 800 $mg/m^2$ cytosine arabinoside, 140 $mg/m^2$ melphalan).

**Table 1.** Patient characteristics, $n$=31

| | |
|---|---|
| Age (median, range) | 48.5 (32–59) |
| Sex (M/F) | 17/14 |
| Histology | |
| Follicular lymphoma | 28 |
| Mantle cell lymphoma | 3 |
| Disease status at mobilization | |
| Complete remission | 9 |
| Partial remission | 22 |
| Previous radiotherapy | 3 |
| Bone marrow involvement | |
| Yes | 7 |
| No | 24 |

Hematologic short-term reconstitution was defined as the time to reach a white blood cell (WBC) count of $1\times10^9$/L, an absolute neutrophil count (ANC) of $0.5\times10^9$/L, and an unmaintained platelet count $>20\times10^9$/L.

A group of 104 patients who underwent high-dose therapy with PBSC support using unmanipulated leukapheresis products served as a control.

## CD34$^+$ cell preparation

CD34$^+$ cells were selected on the occasion of 32 procedures from large-volume (20 liters) leukapheresis products using the Baxter Isolex 300 SA (31 procedures)

**Table 2.** CD34 selections, $n$=32

| | |
|---|---|
| CD34% | |
| Before | 3.3 ± 0.4 |
| After | 96.3 ± 1.2 |
| CD34$^+$ cell recovery | 62.7 ± 5.7 |
| CD34$^+$ cells ($\times10^6$/kg) | 12.8 ± 1.4 |
| CD19$^+$ cells ($\times10^6$/kg) | |
| Before | 1.7 ± 1.4 |
| After | 0.05 ± 0.01 |
| CFU-GM ($\times10^3$/kg) | |
| Before | 1.4 ± 0.4 |
| After | 0.7 ± 0.2 |
| Minimal residual disease | |
| PCR-positive | |
| Before | 11 |
| After | 5 |

or 300i (one procedure) Magnetic Cell Separation Systems (Baxter Immunotherapy). Products containing more than 0.5% $CD34^+$ cells and more than $5\times10^6$ $CD34^+$ cells/kg were chosen preferentially for selection. The enrichment was performed on the day of leukapheresis, as in our hands overnight storage resulted in a substantial loss of viable $CD34^+$ cells. If possible, unmanipulated leukapheresis products were cryopreserved as back-up.

After a platelet wash of 10 minutes at 250*g*, the cells were resuspended in 100 mL phosphate-buffered saline (PBS, calcium- and magnesium-free) containing 1% human serum albumin (HSA) (Immuno, Heidelberg, Germany), 0.2% sodium citrate (Braun, Melsungen, Germany), and 0.5% immunoglobulin (Venimmun, Behring, Marburg, Germany). The cells were incubated with an anti-CD34 monoclonal antibody (Baxter) for 30 minutes at 4°C while rotating. After one wash, $4\times10^9$ freshly prepared sheep anti-mouse IgG1 (Fc) paramagnetic beads (Dynabeads M450; Dynal, Oslo, Norway) were added to the cells in a final volume of 200 mL. The suspension of cells and beads was incubated for 30 minutes at room temperature while rotating. The complexes of beads and cells were retained by the primary magnet of the Isolex 300SA, whereas unbound cells were removed by three washes. Rosetted cells were released from the immunomagnetic beads after incubation with chymopapain for 15 minutes at room temperature (two procedures) (8000 pKAT, ChymoCell-T; Baxter) or using a competitor peptide (PR34; Baxter) (30 procedures). After release, the beads were captured by repeated passages over the magnet. After washing, $CD34^+$ cells were resuspended in freezing medium containing minimal essential medium (MEM) (Seromed, Biochrom KG, Berlin, Germany), 7.5% dimethylsulfoxide (DMSO) (Merck, Darmstadt, Germany), and 4% HSA in a final volume between 5 and 15 mL. The cell suspension was frozen in 5 mL Nalgene tubes (Nalgene, Rochester, NY) using a computer-controlled freezing device (Cryo10-16 MR2; Messer-Griesheim, Germany). The tubes were stored at −196°C in the liquid phase of nitrogen.

### Immunofluorescence staining and flow cytometry

For immunofluorescence analysis, $10^6$ cells were incubated for 30 minutes at 4°C in the presence of an anti-CD34 fluorescein isothiocyanate (FITC)-conjugated HPCA-2 and an anti-CD19 phycoerythrin (PE)-conjugated monoclonal antibody (all from Becton Dickinson, Heidelberg, Germany). Isotype identical antibodies (Becton Dickinson) served as controls. Cells were washed twice with PBS, and red blood cells were removed using a FACS lysis solution (Becton Dickinson). Immunofluorescence analysis was performed using a five-parameter FACScan (Becton Dickinson) equipped with an argon-ion laser tuned at 488 nm and 0.3 W. Emission from FITC and PE was measured using filters of 530 nm and 585 nm, respectively.

The side scatter (SSC) characteristics vs. CD45 fluorescence dot plot were used to discriminate between the smallest hematopoietic cell population and erythrocytes or debris. The $CD34^+$ cells were analyzed in a fluorescence vs. SSC plot. Only cells with a lymphoid or lymphomonocytoid appearance were counted as $CD34^+$ cells, and their proportion was calculated in relation to that of $CD45^+$ cells. The percentage of false-positive events determined using isotype-specific control antibodies was <0.05% and was subtracted from the proportion of $CD34^+$ cells. The viability of the cells on the day of transplantation was studied using propidium iodine (PI) exclusion measured in a $CD34^+$-gated population.

### Minimal residual disease

The presence of tumor cells in the leukapheresis products and in the enriched $CD34^+$ cell population was studied using a sensitive nested-PCR method for the t(14;18) translocation in patients with follicular lymphoma. The samples were amplified for the bcl-2/IgH rearrangement using nested oligonucleotide primers.[12,14,15] The sensitivity of this PCR technique permits the detection of one t(14;18)-positive KARPAS-K422 cell in $10^5$ to $10^6$ normal mononuclear cells (MNC).[12] A patient-specific positive control was included in every PCR to compare the bcl-2/IgH fragment lengths and to exclude the presence of contaminating DNA.

### Statistical analysis

Clinical and laboratory data were evaluated according to standard statistical methods using commercially available computer programs (Statworks; Cricket, Philadelphia, PA). Statistical significance was examined using the Mann-Whitney *U* test and the Student *t* test. Correlation was studied by simple regression analysis. A significance level of $P<0.05$ was chosen. Unless otherwise indicated, all results are expressed as mean ± SE.

## RESULTS

### Immunomagnetic selection of $CD34^+$ cells

$CD34^+$ cells were enriched from 32 LP obtained from 31 patients with follicular lymphoma using the Isolex 300 devices for immunomagnetic cell selection. The number of $CD34^+$ cells harvested per kilogram body weight varied between 2.2 and $97.9\times10^6$ (median $24.8\times10^6$).

After enrichment, the mean purity of the $CD34^+$ cell population was 96.3 ± 1.2% with a mean $CD34^+$ cell recovery of 62.7 ± 5.7% (Table 2). Purity and

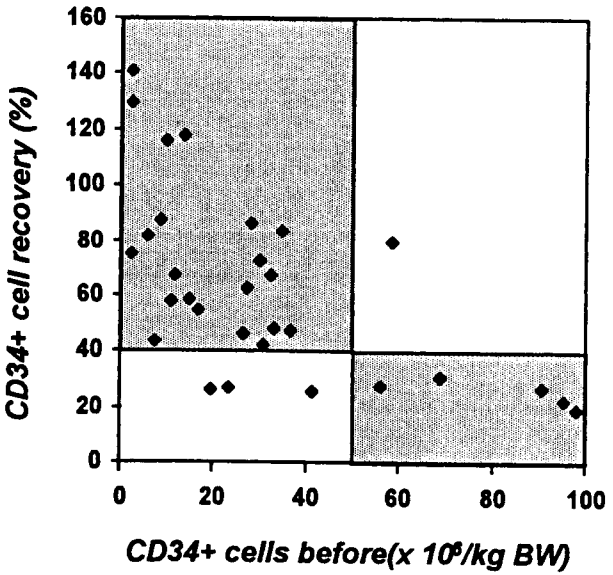


**Figure 1.** *The recovery of CD34⁺ cells after immunomagnetic selection inversely correlates with the number of CD34⁺ cells in the leukapheresis products ($P \leq 0.001$, $r = 0.64$).*

recovery of $CD34^+$ cells were not related to each other. The recovery of $CD34^+$ cells was inversely related to the number of $CD34^+$ cells contained in the LP ($r = -0.64$) (Fig. 1). In Table 3, the immunophenotype of the $CD34^+$ cells before and after enrichment is shown. A significant reduction in the proportion of $CD34^+$/Thy-$1^+$ and $CD34^+$/Leu-$8^+$ cells was observed, while no changes were found in the proportion of $CD34^+$/HLA-$DR^-$ and $CD34^+$/$CD38^-$ cells. The viability of the $CD34^+$ cells after selection was 98.7 ± 0.14%.

## Minimal residual disease

The number of $CD19^+$ cells after $CD34^+$ cell selection decreased from 1.7 ± $1.4 \times 10^6$/kg to 0.05 ± $0.01 \times 10^6$/kg ($P$=0.04) (Table 2). Using a sensitive nested PCR for the assessment of the t(14;18) translocation, the presence of tumor cells in the population of selected $CD34^+$ cells was examined. Eleven of 32 leukapheresis products contained PCR-positive cells. The median purity of $CD34^+$ cells was 98.1% (range 94.3–99.5%). After selection, the $CD34^+$ cell population became PCR-negative in six patients. The $CD34^+$ cells of these patients contained a mean number of $CD19^+$ cells of 0.08 ± $0.02 \times 10^6$/kg and was not significantly different from the five PCR-positive samples, which had a mean number of 0.04 ± $0.03 \times 10^6$/kg.

**Table 3.** Immunophenotype of CD34+ cells

| | *Before* | *After* | *P* |
|---|---|---|---|
| CD34+/HLA-DR− | 0.78 ± 0.2 | 0.63 ± 0.1 | 0.2 |
| CD34+/CD38− | 0.41 ± 0.2 | 0.54 ± 0.4 | 0.2 |
| CD34+/Thy-1+ | 50.5 ± 4.5 | 38.2 ± 4.4 | 0.002 |
| CD34+/Leu8+ | 83.7 ± 4.9 | 70 ± 7.5 | 0.014 |
| Viability | 99.2 ± 0.2 | 98.7 ± 0.1 | 0.03 |

## Hematologic reconstitution

Selected CD34+ cells were used for the support of 13 high-dose therapy cycles (Table 3). The remaining 18 patients had not been transplanted at the time of this report. On average, a mean number of 7.4 ± $1.2\times10^6$ CD34+ cells/kg were autografted. As assessed by PI exclusion, the mean viability of the CD34+ cells after thawing was 93 ± 1.87%. The patients engrafted with a mean time to leukocyte ($1.0\times10^9$/L), neutrophil ($0.5\times10^9$/L), and platelet ($20.0\times10^9$/L) recovery of 12.8 ± 0.7, 13.6 ± 0.7, and 14.5 ± 1.3 days, respectively (Fig. 2).The patients remained in hospital for a median of 15 days (range 12–21) after autografting and had a median of 3 days of fever (range 1–10). They required a median of six platelet and four erythrocyte transfusions (range 2–10 and 2–8, respectively).

The engraftment ability of the CD34+ autografts was compared with that of unmanipulated PBSC autografts containing 9 ± $0.7\times10^6$ CD34+ cells/kg which were used for the support of high-dose therapy in 104 patients. The mean time

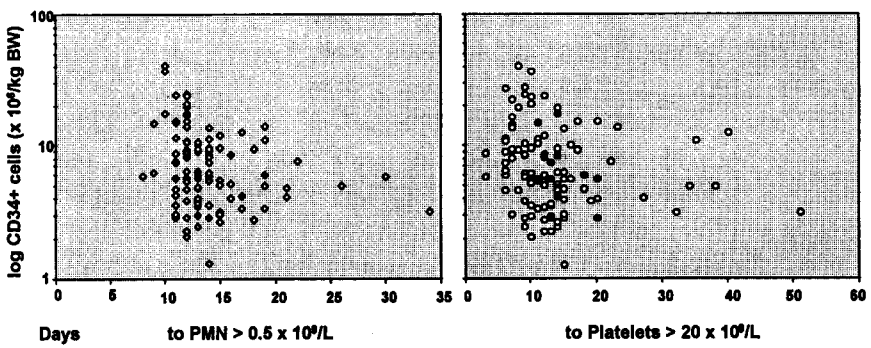


**Figure 2.** *The hematopoietic reconstitution ability of the selected CD34+ cells (closed symbols) is similar to that of CD34+ cells contained in unmanipulated autografts (open symbols). The time to recover leukocytes to $1\times10^9$/L, neutrophils to $0.5\times10^9$/L, and platelets to $20\times10^9$/L was 12.8 ± 0.7 vs. 12.6 ± 0.3 days (P=0.8), 13.6 ± 0.7 vs. 13.9 ± 0.4 days (P=0.8), and 14.5 ± 1.3 vs. 13 ± 0.8 days (P=0.6), respectively.*

needed for leukocyte, neutrophil, and platelet recovery was not different from that of selected $CD34^+$ cells (12.6 ± 0.3 [$P$=0.8], 13.9 ± 0.4 [$P$=0.8], and 13 ± 0.8 [$P$=0.6] days, respectively) (Fig. 2).

One patient with follicular lymphoma had graft failure. He had received $5.6\times10^6$ $CD34^+$ cells/kg, and no back-up cells were available for a second autografting. The purity of the $CD34^+$ cells was 96.4%, and the viability after thawing was 93.8%. He had stage IV disease with bone marrow involvement and entered PR following six cycles of previous cytotoxic chemotherapy. The latter was always associated with prolonged periods of cytopenia. The conditioning regimen consisted of TBI and cyclophosphamide. He developed severe pneumonia of unknown etiology and died of respiratory failure on day 88.

## DISCUSSION

Hematopoietic progenitor and stem cells collected from peripheral blood after G-CSF-supported cytotoxic chemotherapy contain a greater number of $CD34^+$ cells and are less likely to be contaminated by tumor cells than bone marrow.[16,17] In the study presented here, we examined the requirements and conditions for successful $CD34^+$ cell enrichment, aiming at a maximum of purity and recovery in patients with follicular lymphoma. In line with our previous results in patients with breast cancer,[6] the purity of the $CD34^+$ cell population was >90% and was associated with a recovery of >60% for most of the patients.

The great majority of our patients had prompt and stable hematologic reconstitution. However, one patient who received $5.6\times10^6$ $CD34^+$ cells/kg had graft failure and died of respiratory failure. The reason for graft failure in this patient is not clear. He had prolonged periods of pancytopenia after conventional cytotoxic chemotherapy, which might indicate impaired marrow function due to stromal defects. Graft failure has also been observed after autografting with whole bone marrow or unmanipulated leukapheresis products.

Confirming previous observations on the lack of malignant $t(14;18)^+$ cells in $CD34^+$ hematopoietic progenitor and stem cells from patients with follicular lymphoma,[7,11–13] the large-scale selection of $CD34^+$ cells resulted in the depletion of residual t(14;18)-positive tumor cells from six of 11 PCR-positive leukapheresis products. Persistence of PCR-positive cells in the fraction of $CD34^+$ cells was probably related to small amounts of contaminating mature $CD19^+$ cells. The significance of residual PCR-positive cells in the autografts of patients with low-grade non-Hodgkin's lymphoma is not clear.[15,18–20] Immunologic methods result in the purging of PCR-detectable lymphoma cells in about 50% of the cases.[15,19,21] Furthermore, patients receiving marrow autografts without molecular evidence of residual lymphoma cells have a better disease-free survival than those receiving PCR-positive autografts.[14,19] Gene-marking studies, like those performed for

neuroblastoma and acute myeloid leukemia, may help to resolve whether lymphoma cells contaminating the autograft can contribute to relapse after transplantation.[22,23] Certainly, the presence of chemo/radiotherapy-resistant tumor cells in vivo is also of great relevance. To eradicate minimal residual disease persisting after high-dose therapy, we are currently evaluating an adjuvant immunologic treatment using CD3×CD19 bispecific antibodies.

In conclusion, the selection of $CD34^+$ cells provides highly enriched hematopoietic progenitor and stem cells. More effective pretreatment schedules may reduce the content of potentially contaminating tumor cells. In some patients with FL, positive selection as the sole modality for purging is apparently not sufficient to obtain PCR-negative autografts. Double purging including CD34-positive and CD19-negative selection may therefore be envisaged.

## ACKNOWLEDGMENTS

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# Stem Cell Reinfusion Over Two Consecutive Days, Plus Delayed G-CSF, Hastens Engraftment

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## ABSTRACT

Outpatient reinfusion of mobilized peripheral stem cells over 2 consecutive days can minimize the side effects associated with larger volumes, increased dimethylsulfoxide, and elevated numbers of mature cells in apheresis products. Engraftment in the first nine patients receiving split reinfusions was compared to 20 previous single-reinfusion transplants. Both groups received the same number of $CD34^+ \times 10^6$ cells/kg, 4.5 ± 0.8 and 4.7 ± 2.7, and started granulocyte colony-stimulating factor (G-CSF) 24 hours before reinfusion began. Results are expressed as the mean ± standard deviation. *P* values <0.05, using a *t* test for unpaired samples, are statistically significant.

| | *Single reinfusion* | *Split reinfusion* | *P value* |
|---|---|---|---|
| Neutrophils | | | |
| Days <100 | 5 ± 2.5 | 3 ± 1.7 | 0.0299 |
| Days to >100 | 11 ± 2.5 | 9 ± 0.8 | 0.0322 |
| Days to >500 | 13 ± 2.6 | 11 ± 1.2 | 0.0168 |
| Platelets | | | |
| Days to >20,000 | 22 ± 7.9 | 17 ± 5.9 | 0.1077 |
| Days to >50,000 | 31 ± 14.6 | 21 ± 8.6 | 0.0713 |
| # of transfusions | 4.2 ± 1.6 | 3.0 ± 1.3 | 0.0678 |

Fractionating stem cell reinfusion significantly reduced neutropenic days and hastened myeloid engraftment. There was a trend toward faster platelet recovery. To further improve platelet recovery, G-CSF was delayed until 48 hours after completion of split reinfusion. $CD34^+ \times 10^6$ cells/kg, 4.5 ± 1.2, was not statistically different from the two previous groups of patients.

| | *Single reinfusion* | *Split reinfusion, delayed G-CSF* | *P value* |
|---|---|---|---|
| Neutrophils | | | |
| Days <100 | 5 ± 2.5 | 5 ± 1.2 | 0.4665 |
| Days to >100 | 11 ± 2.5 | 10 ± 0.8 | 0.3144 |
| Days to >500 | 13 ± 2.6 | 11 ± 0.7 | 0.0350 |
| Platelets | | | |
| Days to >20,000 | 22 ± 7.9 | 14 ± 1.8 | 0.0037 |
| Days to >50,000 | 31 ± 14.6 | 18 ± 2.5 | 0.0213 |
| # of transfusions | 4.2 ± 1.6 | 2.8 ± 0.8 | 0.0046 |

Compared with the single reinfusion group, the first 17 patients receiving split reinfusion/delayed G-CSF had significantly faster, more consistent platelet recoveries with fewer transfusions. Delayed G-CSF saved approximately $2000. Myeloid engraftment was postponed initially, but accelerated under the influence of G-CSF; days to >500 was still significantly faster. Delays in early myeloid recovery did not cause infectious complications; one of nine patients in the split reinfusion group and one of 17 in the split reinfusion/delayed G-CSF group required hospitalization.

## INTRODUCTION

Hematopoietic recovery after high-dose chemotherapy and autologous transplant depends on the source and quantity of $CD34^+$ progenitor cells reinfused. Mobilized peripheral cells (PBSC) have virtually replaced bone marrow as the source of progenitor cells because larger numbers of $CD34^+$ cells can be collected, which engraft faster and reduce the morbidity and cost of transplant.[1–4] Several groups, including our own, have demonstrated rapid, multilineage engraftment by reinfusing $4–5\times10^6$ $CD34^+$ cells/kg of body weight.[5–7] The side effects of progenitor infusion such as chills, bronchospasm, shortness of breath, and fluid overload can be worse with PBSC due to their larger volumes, more dimethylsulfoxide (DMSO), and increased number of granulocytes. Cells could be processed and volume reduced prior to transplant[8] or simply reinfused over 2 consecutive days to minimize the side effects. The first 26 patients to receive split reinfusions were compared with a historic group of 20 patients who had received their stem cells in a single reinfusion. Patients receiving split reinfusions started their G-CSF either 24 hours before the first infusion of cells or 48 hours after completion of stem cell reinfusion to determine the effects on multilineage engraftment.

## MATERIALS AND METHODS

### Patients

Both groups, due to small numbers of patients, were heterogeneous with respect to sex and diagnosis. The historic group contained five males and 15 females, with 16 solid tumor patients and four hematopoietic patients. The split reinfusion group contained five males and 21 females with 16 solid tumor patients and 10 hematopoietic patients.

### Mobilization and leukapheresis

All patients gave informed consent for peripheral stem cell (PSC) collection leukapheresis following standard induction chemotherapy, appropriate for their disease, followed by growth factor.

Breast cancer patients received one of the following regimens: FAC: 5-fluorouracil, 500 mg/m$^2$, adriamycin, 50 mg/m$^2$, cyclophosphamide, 500 mg/m$^2$; CAVe: cyclophosphamide, 500 mg/m$^2$, adriamycin, 50 mg/m$^2$, etoposide, 240 mg/m$^2$; CAT: cyclophosphamide, 500 mg/m$^2$, adriamycin, 50 mg/m$^2$, taxol, 175 mg/m$^2$.

Non-Hodgkin's lymphoma patients received one of the following regimens: CHOP: Cytoxan, 800 mg/m$^2$, adriamycin, 50 mg/m$^2$, vincristine, 2 mg; DHAP: Ara-C, 100 mg/m$^2$, cytoxan, 90 mg/m$^2$.

Myeloma patients received CVAD: Cytoxan, 1 gm/m$^2$, vincristine, 1.6 mg, adriamycin, 32 mg/m$^2$, dexamethasone, 160 mg.

Growth factor support consisted of 5 μg/kg G-CSF (Amgen) beginning 2 days after completion of therapy and continuing through leukapheresis. Daily apheresis began when the total circulating CD34$^+$ cell count exceeded $50\times10^6$ and continued until $4–5\times10^6$ CD34$^+$ cells/kg had been collected. High-speed (85–110 mL/min) large volume (18–20 liters) procedures were performed on a Cobe Spectra (Cobe BCT) or Baxter CS3000 (Baxter Fenwal). A single sample was taken from the final product bag for total nucleated cell count, CD34$^+$ analysis, and sterility testing.

### Progenitor cell processing and storage

PSC products were platelet and plasma depleted. Cells were historically frozen based on the total nucleated cell count, $500–700\times10^6$ nucleated cells/mL, and are currently frozen based on CD34$^+$ cell count with $2.0–2.5\times10^6$/kg in each bag. Bag volumes for both groups ranged between 40 and 70 mL and contained a final concentration of 10% DMSO and 10% cryoprecipitated autologous plasma. All cells were frozen using a Cryomed rate-controlled freezing unit (Forma Scientific).

## CD34 Labeling

Samples were counted using a Sysmex F800 (Baxter) or a Cell Dyne 3500 (Abbott) hematology instrument. Based on the white blood cell count, $0.5–1.0\times10^6$ nucleated cells from whole blood or leukapheresis products were incubated with 20 μL anti-HPCA-2-PE (Becton Dickinson) at room temperature in the dark for 20 minutes. Additional incubation for 10 minutes with 2 mL FACSlyse (Becton Dickinson) followed by vortexing and centrifugation for 2 minutes at 1000*g* effectively removed the red blood cells. Cells were washed 1× with phosphate buffered saline (PBS) and resuspended in 1 mL PBS + 0.2% paraformaldehyde. A minimum of $7.5\times10^4$ cells was acquired on a FACSort flow cytometer. Analysis was performed using Paint-a-Gate software by gating on a plot of CD34 fluorescence vs. side scatter.

## Consolidation therapy and reinfusion

All patients gave informed consent to receive high-dose therapy appropriate for their diagnosis, with PSC support following protocols approved by our Institutional Review Board.

Patients with breast cancer received MTB: mitoxantrone, 30 mg/m$^2$, thiotepa, 300 mg/m$^2$, BCNU, 300 mg/m$^2$.

Patient's with non-Hodgkin's lymphoma received BEAC: BCNU, 300 mg/m$^2$, VP-16, 800 mg/m$^2$, cytoxan, 3.2 gm/m$^2$.

Patient's with myeloma received VP/melphalan: VP-16, 600 mg/m$^2$, and melphalan, 120 mg/m$^2$.

Transplantation occurred 48 hours after completion of chemotherapy. Just before reinfusion, patients were premedicated with 250 mg hydrocortisone and 50 mg benadryl and connected to a continuous infusion of Ringer's lactate. PSC were thawed in a 40°C water bath to a semiliquid state, transferred to a 60-cc syringe, and pushed over 20 minutes through a 23-gauge needle into the side arm of the IV to dilute the cells and DMSO. This procedure, which was identical for all groups, was originally performed as a single maneuver and subsequently divided equally over 2 consecutive days.

Growth factor therapy with 5 μg/kg G-CSF initially began 24 hours before the first infusion of stem cells, but later was delayed until 48 hours after completion of transplant.

## RESULTS

There was no difference in the number of infused CD34$^+$ stem cells/kg ($\times10^6$) between the patients receiving a single vs. split reinfusion, 4.7 ± 2.2 vs. 4.6 ± 1.1,

**Table 1.** Neutrophil engraftment: single vs. split reinfusion

| | *Single reinfusion* | *Split reinfusion* | *P value* |
|---|---|---|---|
| Days ANC <100 | 5 ± 2.5 | 3 ± 1.7 | 0.0299 |
| Days ANC >100 | 11 ± 2.5 | 9 ± 0.8 | 0.0322 |
| Days ANC >500 | 13 ± 2.6 | 11 ± 1.2 | 0.0168 |

respectively ($P$=0.44). Administration of G-CSF 24 hours before transplant and stem cell infusion over 2 days positively affected neutrophil engraftment (Table 1). The number of neutropenic days was reduced, with significantly faster myeloid engraftment. Although not statistically relevant, there was a trend toward faster platelet recovery and fewer transfusions (Table 2).

In an effort to hasten platelet recovery, G-CSF administration was delayed to 48 hours after completion of the split transplant instead of 24 hours before reinfusion. Results are summarized in Table 3 for platelet recovery and in Table 4 for neutrophil recovery. There was a continued trend toward faster platelet recovery. Conversely, the number of neutropenic days increased from 3 to 5 and initial myeloid engraftment to >100 was significantly delayed.

The combination of split infusion and delayed G-CSF hastened platelet recovery (Table 5) compared with our original protocol, which initiated G-CSF 24 hours before a single infusion of stem cells. The improvement in platelet engraftment to 20,000 and 50,000 was both statistically and clinically relevant, as was the decrease in the number of platelet transfusions. Postponing the administration of G-CSF did not negatively impact myeloid recovery or the number of neutropenic days (Table 6) compared with the initial protocol of a single infusion preceded by G-CSF.

## DISCUSSION

The side effects associated with stem cell reinfusion can be more severe when the cells derive from large-volume, hypercellular leukapheresis that require more DMSO for adequate cryoprotection. Reinfusion of stem cells in two sessions on consecutive days not only minimized the anticipated side effects but also had an

**Table 2.** Platelet engraftment: single vs. split reinfusion

| | *Single reinfusion* | *Split reinfusion* | *P value* |
|---|---|---|---|
| Days platelets >20,000 | 22 ± 7.9 | 17 ± 5.9 | 0.1077 |
| Days platelets >50,000 | 31 ± 14.6 | 21 ± 8.6 | 0.0713 |
| No. transfusions | 4.2 ±1.6 | 3.0 ± 1.3 | 0.0678 |

**Table 3.** Platelet engraftment, split reinfusion: immediate vs. delayed G-CSF

| | *24 hours before* | *48 hours after* | *P value* |
|---|---|---|---|
| Days platelets >20,000 | 17 ± 5.9 | 14 ± 1.8 | 0.1426 |
| Days platelets >50,000 | 21 ± 8.6 | 18 ± 2.5 | 0.1824 |
| No. transfusions | 3.0 ± 1.3 | 2.8 ± 0.8 | 0.3734 |

unexpected positive impact on multilineage engraftment in our patients. In contrast, Abdel-Razeq et al.[9] reported a randomized study comparing multiple- vs. single-day infusions of PSC in 60 consecutive patients with both hematologic and solid tumors and found no difference in myeloid or platelet engraftment. The number of infused PSC, mobilized with growth factor alone, ranged between 2 and $3\times10^6$/kg. We infused larger numbers of PSC, which had been mobilized following chemotherapy and G-CSF. The combination of chemotherapy and G-CSF has been shown to mobilize $CD34^+$ cells with a higher clonogenic potential and more primitive subsets than $CD34^+$ cells mobilized with G-CSF alone.[10,11] These quantitative and qualitative differences in PSC may account for the different outcomes. It is not clear why multiple PSC infusions should affect hematopoietic recovery. Perhaps the first infusion of stem cells home, interact with the stroma to initiate recovery from cytotoxic therapy, and present a stimulated microenvironment to the stem cells in the second infusion.

Splitting the reinfusion had a greater impact on neutrophil rather than platelet recovery, which may be related to the use and scheduling of G-CSF. The utility of daily subcutaneous G-CSF at a dose of 5 μg/kg after PSC transplant has been clearly demonstrated;[12] however, the optimal timing of G-CSF administration is still unknown. There are studies to suggest that delaying G-CSF to day 5 or 6 after PSC transplant does not effect myeloid or platelet recovery and is cost-effective.[13,14] Conversely, Colby et al.[15] report that early initiation of G-CSF after PSC transplant, day 1 vs. day 4, hastens myeloid recovery and reduces hospital stay and antibiotic utilization thereby lowering the overall cost. Platelet recovery and transfusion dependence was not addressed in the study. When we delayed the start of G-CSF to 48 hours after the second reinfusion, initial myeloid engraftment to >100 and neutropenic days were negatively affected. Platelets continued to trend

**Table 4.** Neutrophil engraftment, split reinfusion: immediate vs. delayed G-CSF

| | *24 hours before* | *48 hours after* | *P value* |
|---|---|---|---|
| Days ANC <100 | 3 ± 1.7 | 5 ± 1.2 | 0.0096 |
| Day ANC >100 | 9 ± 0.8 | 10 ± 0.8 | 0.0083 |
| Day ANC >500 | 11 ± 1.2 | 11 ± 0.7 | 0.1687 |

**Table 5.** Platelet engraftment: single reinfusion vs. split/delayed G-CSF

| | *Single reinfusion* | *Split/delayed G-CSF* | *P value* |
|---|---|---|---|
| Days platelets >20,000 | 22 ± 7.9 | 14 ± 1.8 | 0.0037 |
| Days platelets >50,000 | 31 ± 14.6 | 18 ± 2.5 | 0.0213 |
| No. transfusions | 4.2 ± 1.6 | 2.8 ± 0.8 | 0.0046 |

toward faster recovery with fewer transfusions. Compared with a single infusion 24 hours after the first injection of G-CSF, platelet engraftment was significantly improved (Table 6). These observations suggest a competition for stem cell differentiation into myeloid and megakaryocyte lineages which can be shifted depending on the scheduling of growth factors such as G-CSF.

We will continue to transplant PSC over 2 consecutive days but will delay G-CSF only 24 hours after the last infusion in an effort to achieve a balance between neutrophil and platelet recovery. Availability of additional cytokines, such as stem cell factor, and modified forms, such as pegylated-erythropoietin, present the challenge of combined or sequential use with G-CSF to optimize multilineage engraftment after PSC transplant.

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**Table 6.** Summary of myeloid and platelet recovery

| | *Days* | | | | |
|---|---|---|---|---|---|
| | *ANC* | | | *Platelets* | |
| | *<100* | *>100* | *>500* | *>20,000* | *>50,000* |
| Single reinfusion, G-CSF 24 hours before | 5 | 11 | 13 | 22 | 31 |
| Split reinfusion, G-CSF 24 hours before | 3 | 9 | 11 | 17 | 21 |
| Split reinfusion, G-CSF 48 hours after | 5 | 10 | 11 | 14 | 18 |

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# Final Report of the First Prospective, Stratified, Randomized Trial Comparing G-CSF–Primed Bone Marrow Cells With G-CSF–Mobilized Peripheral Blood Cells for Pace of Hematopoietic Engraftment and Disease-Free Survival After High-Dose Therapy and Autotransplant

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## ABSTRACT

It has been widely reported that peripheral blood stem cell (PBSC) autotransplantation produces not only more rapid neutrophil recovery but also more rapid platelet recovery than bone marrow (BM) autotransplantation. These observations are based on uncontrolled trials in which recovery data from PBSC mobilized by chemotherapy and/or hematopoietic growth factors were compared with historical data from autotransplants with BM harvested under resting (unstimulated) conditions. To confirm (or deny) these observations, we conducted a prospective, stratified, randomized trial wherein all participating patients had both BM and PBSC harvested after 5 days of granulocyte colony-stimulating factor (G-CSF, 16 μg/kg/d) administration. Seventy patients consented to participate in this trial after conditioning with ifosfamide (20.1 g/m$^2$), carboplatin (1.8 g/m$^2$), and etoposide (3.0 g/m$^2$) or with mitoxantrone (75–90 mg/m$^2$) and thiotepa (0.9–1.2 g/m$^2$) with or without paclitaxel (360 mg/m$^2$). Patients were randomized to receive either BM ($n$=25) or PBSC ($n$=22). Patients ($n$=23) whose PBSC collection was inadequate according to preestablished criteria were removed from randomization and infused with both BM and

PBSC products. Hematologic recovery times for all patients were followed. We have observed a median platelet recovery (transfusion independent, >20,000/μL for 3 consecutive days) of 25 days for both BM-alone and PBSC-alone transplants ($P$=0.500). Patients who had inadequate PBSC mobilization and who were transplanted with both products had a median platelet recovery of 37 days, which, when compared with the groups receiving BM or PBSC alone, was significantly different ($P$=0.009). Median neutrophil recovery (>500/μL for 3 consecutive days) was 18 and 16 days, respectively, for BM alone and PBSC alone ($P$=0.446) with the time to recovery curves intertwining, while the median neutrophil recovery in the group of patients who had inadequate PBSC mobilization, when compared with the BM and PBSC groups, was significantly different at 22 days ($P$=0.008). We also examined the duration of disease-free survival after autotransplant as a function of which product was reinfused and have found that there is no differences for patients receiving BM, PBSC, or the combined products. These data indicate that there is little, if any, clinical advantage to the use of PBSC over BM as a source of stem cells for autologous transplant when G-CSF is given before harvesting hematopoietic stem cells.

## INTRODUCTION

Historically, BM has been used effectively as a source of hematopoietic stem cells to rescue patients from the myeloablative effects of escalated doses of radiochemotherapy in both the allogeneic and autologous settings.[1] Recently, however, cells collected from the blood, referred to as PBSC, have been employed for autologous stem cell transplant with increasing frequency.[2–5] Several investigators have reported that when PBSC are employed, the posttransplant period of neutropenia and thrombocytopenia are significantly reduced.[2,4–13]

It has been suggested that PBSC are a superior source of hematopoietic progenitors for autotransplant and should replace the use of BM for both autologous and allogeneic transplantation. This contention is based principally on a shortened period of posttransplant aplasia that has been associated with PBSC and on the associated reduced risk of transplant-related morbidity/mortality.[4] It has also been suggested that PBSC are superior to BM because of relative ease, safety, and low expense of collection and because of the possibility that PBSC are associated with reduced rates of disease relapse posttransplant.[14,15]

We have noted[16] that all of the reported observations of rapid PBSC associated engraftment are in the context of *mobilized* PBSC. In fact, no investigator performing PBSC transplants wherein mobilization was not employed has reported accelerated engraftment after unmobilized PBSC transplant.[17,18] In contrast, the BM transplant data, which have been used as the historical control in all of the reports of rapid engraftment for PBSC, have been derived from the use of *resting*

BM (i.e., BM harvested from individuals who are not receiving hematopoietic growth factor therapy and who generally have not received any myelosuppressive chemotherapy within the month or more before BM harvesting). From this observation, we hypothesized that the accelerated engraftment associated with PBSC is the result of the priming before stem cell harvesting and is not due to the anatomic derivation of the stem cells. Alternatively stated, our hypothesis was that BM harvested following a mobilizing treatment with cytokines should produce as rapid a hematologic recovery as PBSC harvested following the same treatment.

Recently, Beyer et al.[19] and Schmitz et al.[20] have presented prospective, randomized trials comparing *resting* BM with *mobilized* PBSC autologous transplants. These authors observed more rapid recovery of both circulating neutrophils (2 days and 1 day faster, respectively) and circulating platelets (7 days faster in both trials) with the mobilized PBSC. Because the conditioning of the patients before harvesting was different for BM collections (no conditioning) than for PBSC collections (chemotherapy followed by G-CSF[19] or G-CSF administered as a single agent for PBSC mobilization[20]), these results are not inconsistent with our hypothesis.

To test our hypothesis, we have conducted a randomized, stratified, prospective trial comparing blood- and marrow-derived stem cells harvested under identical growth factor–stimulating conditions. The following is a report of our data describing rates of posttransplant hematologic recovery and also the duration of posttransplant disease-free survival.

## MATERIALS AND METHODS

### Patients

Beginning in June 1993, patients were recruited from our referred patient population. To be eligible for this study, patients were required never to have had evidence of their malignant disease in their bone marrow by histologic criteria and could not have had a leukemic disorder. The majority of the patients in the trial had either breast cancer or lymphoma (Table 1). Patients were informed of the trial and gave written consent to participate, as approved by the Scientific Review Committee of the H. Lee Moffitt Cancer Center and as approved and annually reviewed by the Institutional Review Board of the University of South Florida.

### Mobilization/priming, harvesting, and cryopreservation

Patients received G-CSF (filgrastim), 16 μg/kg intravenously per day for 9 days. On day 6, BM was harvested, and on days 7 through 10, leukaphereses were

**Table 1.** Characteristics of patients in study: patient characteristics broken down by age, sex, underlying disease, and transplantation protocol employed

| | *BMSC* | *PBSC* | *Both* |
|---|---|---|---|
| *n* | 25 | 22 | 23 |
| Age (years) | | | |
| Mean ± SD | 44 ± 6 | 46 ± 9 | 45 ± 12 |
| Range | 33–60 | 27–63 | 23–61 |
| Sex (F/M) | 23/2 | 18/4 | 21/2 |
| Diagnosis | | | |
| Breast cancer | 22 | 15 | 14 |
| Stage II or III | 6 | 4 | 2 |
| Stage IV | 16 | 11 | 12 |
| Lymphoma | 1 | 5 | 5 |
| Other | 2 | 2 | 4 |
| Protocol | | | |
| ICE | 10 | 12 | 14 |
| MITT | 8 | 5 | 2 |
| TNT | 7 | 5 | 7 |

performed to harvest PBSC. The dose and timing of G-CSF administration were selected in 1993 based on published reports.[21,22]

BM was harvested under general anesthesia using conventional instruments and techniques. One to one-and-a-half liters of marrow were harvested and then processed to remove red cells and granulocytes before freezing. Red blood cells, removed from harvested BM in processing, were washed and returned to the patients as an auto transfusion.[23]

Leukaphereses were performed with an Haemonetics V-50 or MCS instrument (Haemonetics, Braintree, MA). Five to 10 liters of blood, depending on hematocrit, were leukapheresed daily. In the V-50, 16 cycles of separation in a pediatric size bowl (125 mL) were carried out, followed by a recirculation cycle to further reduce red blood cell load. In the MCS, 16 cycles of separation were carried out, interspersed after every fourth cycle by a recirculation cycle. Leukapheresis products were further processed to remove remaining red blood cells and granulocytes before freezing.

Freezing was done in either tissue culture medium 199 (Life Technologies, Rockville, MD) or Plasmalyte acetate buffered saline infusion solution (Baxter, Chicago, IL) supplemented with 10% autologous plasma and 10% dimethylsulfoxide (DMSO). A controlled-rate freezer was employed to assure temperature reduction at the rate of 1°C per minute. Stem cells were stored immersed in liquid nitrogen.

## High-dose chemotherapy

Patients were treated before autotransplant with one of three regimens. Patients who had failed prior anthracycline therapy were given induction therapy of ifosfamide, carboplatin, and etoposide (mini-ICE).[24] Patients responding to mini-ICE were conditioned for transplant with 20.1 g/m$^2$ ifosfamide, 1.8 g/m$^2$ carboplatin, and 3.0 g/m$^2$ etoposide (ICE).[25,26] Patients who had anthracycline-responsive disease were conditioned for transplant with 90 mg/m$^2$ mitoxantrone and 1.2 mg/m$^2$ thiotepa (MITT)[27] or with 360 mg/m$^2$ paclitaxel plus 75 mg/m$^2$ mitoxantrone and 0.9 g/m$^2$ thiotepa (TNT).[28]

## Randomization, stem cell infusion, and supportive care

Forty-eight hours after high-dose chemotherapy with ICE or 7 days after MITT or TNT, stem cells were reinfused. Before randomization, patients were stratified according to diagnosis (breast cancer vs. other) and transplant regimen (ICE, MITT, or TNT). A computer random number generator was employed to determine which stem cell product should be returned.

The assigned product was thawed in a 37–40°C sterile water bath and reinfused by gravity flow through an infusion set equipped with a mesh filter. All patients received G-CSF (10 μg/kg intravenously per day) beginning on the day following stem cell reinfusion and continuing until after neutrophil recovery (absolute neutrophil count [ANC] >1000/μL for 3 or more consecutive days). In the event that neutrophil recovery had not been achieved in 28 days after transplant, the study design called for reinfusion of the other harvested product. This only occurred in two patients, both of whom were conditioned with MITT. Because the median day of engraftment occurred more than a week before day 28 in all strata, second product infusions were not given any special treatment in our log-rank analysis.

Patients were nursed in HEPA-filtered laminar flow air rooms and provided with appropriate supportive care for all medical conditions arising in their transplant course as previously described in detail.[26,27]

## Hematopoietic assessment of collected cells

All cells collected were enumerated and their size profile recorded using a Sysmex K1000 blood cell analyzer (Baxter Diagnostic, Chicago, IL). Smears were made of all collected products, which were stained using Leukostat staining kits (Baxter Scientific, Chicago, IL). One hundred stained cells were evaluated to determine the mononuclear cell fraction. Granulocyte-macrophage colony-forming units (CFU-GM) and CD34$^+$ cell fractions were determined as described in detail previously.[29]

In brief, to measure the fraction of cells that were positive for the CD34 antigen, $5\times10^5$ cells to be assayed were suspended in 0.1 mL Dulbecco's phosphate buffered saline (DPBS) (Gibco, Grand Island, NY) and admixed with 20 μL phycoerythrin-tagged anti-CD34 (HPCA-2; Becton Dickinson, San Jose, CA). After 30 minutes of incubation at 4°C, the cells were thrice washed with cold DPBS and resuspended in 0.1 mL. The cell suspension was placed on a microscope slide, and CD34-positive cells were scored by eye using fluorescence microscopy.

To measure the presence of CFU-GM, $2\times10^5$ cells were plated in 35-mm petri dishes containing 1 mL Iscove's medium as well as 0.8% methylcellulose (Dow Chemical, Midland, MI) or, in later studies, 1.15% methylcellulose (Stem Cell Technologies, Vancouver, British Columbia) and supplemented with 20% fetal bovine serum and 1 ng granulocyte-macrophage colony-stimulating factor (GM-CSF) (Genzyme, Boston, MA). After 14 days of incubation, colonies of >25 cells were scored.

### Study design and statistical methods

To demonstrate that one stem cell product was superior to the other by producing a one-third reduction of neutrophil and platelet recovery times with 80% power at the $P<0.05$ level using a two-tailed analysis, we projected the need to accrue 50 patients to each trial arm. We performed periodic evaluations of the data, directed at early stopping of the trial. The criteria for early stopping were to have more than 40% of the projected accrual and $P>0.35$.

All laboratory and patient data for these studies were maintained in computer database files. In comparing days to engraftment, the log-rank test was employed. Graphic representation of engraftment was produced using the Kaplan-Meier method for computing probabilities of events. Comparisons of cell content returned to the patients were carried out using the Kruskal-Wallis nonparametric analysis of variance (ANOVA) method, as it was apparent that the numbers of cells collected were not normally distributed. All statistical tests were carried out using Statistica software (StatSoft, Tulsa, OK), and all values of $P$ were computed from two-tailed tests.

## RESULTS

### Cells collected and infused

Seventy patients gave informed consent and had both BM and PBSC collected after treatment with G-CSF. BM harvests were considered adequate if more than $2\times10^8$ mononuclear cells/kg were collected, and if the BM had a $CD34^+$ cell concentration of at least 0.5% (more than $10^6$/kg). PBSC harvests were considered adequate if, after four collections, at least $10^6$ $CD34^+$ cells/kg had been collected.

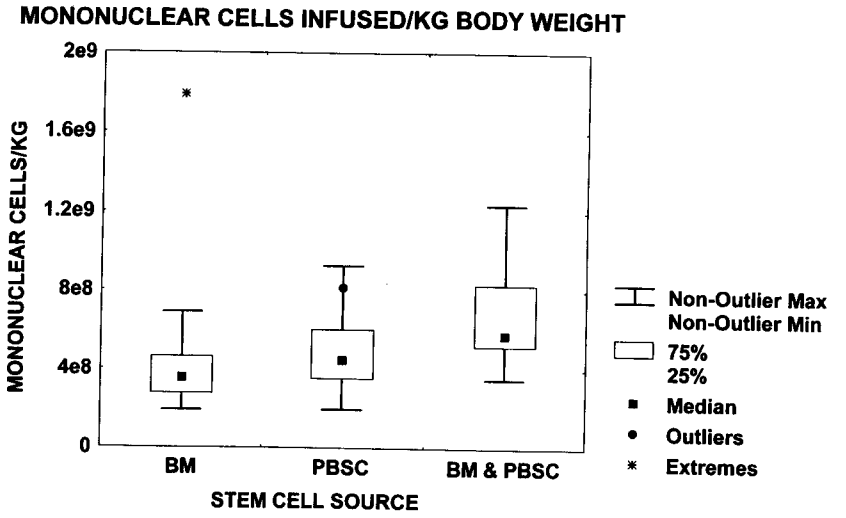


**Figure 1.** *Mononuclear cells infused per kilogram of patient body weight. Mononuclear cells per milliliter were determined using Sysmex cell analyzer, and were combined with product volume and patient body weight to determine cells/kg. Box and whisker representation with median, minimum, maximum, and inner quartiles gives sense of distribution.*

Twenty-three patients had adequate BM harvests but inadequate PBSC harvests by these criteria. These patients were not randomized and, instead, were reinfused with both products. There were no patients determined to have inadequate BM harvests.

The mean number of light-density nucleated cells per kilogram of patient body weight (Fig. 1) collected and infused (± standard deviation [SD]) was $4.7\times10^8$ ($\pm1.8\times10^8$) for patients who were randomized to receive PBSC ($n$=22) and $3.8\times10^8$ ($\pm1.5\times10^8$) for patients who were randomized to receive BM ($n$=25). The pairwise comparison yielded $P$=0.0531. By contrast, patients whose PBSC collection was determined inadequate for transplantation, and who therefore received both products ($n$=23), were infused with $7.2\times10^8$ ($\pm3.5\times10^8$) light-density nucleated cells per kilogram. Testing for significance of the differences reflected by the values for all three groups using the Kruskal-Wallis ANOVA yielded $P$=0.0001. Thus, while the total number of cells infused in the groups randomized to PBSC or BM was, essentially, not significantly different, the number infused in the group receiving both products was significantly greater than for either the patients receiving BM or PBSC alone.

The mean number of $CD34^+$ cells per kilogram of patient body weight (Fig. 2) collected and infused was $1.4\times10^6$ ($\pm1.0\times10^6$) for patients randomized to receive PBSC and was $2.3\times10^6$ ($\pm1.6\times10^6$) for patients randomized to receive BM (pairwise $P$=0.0654). For the patients whose PBSC collections were inadequate

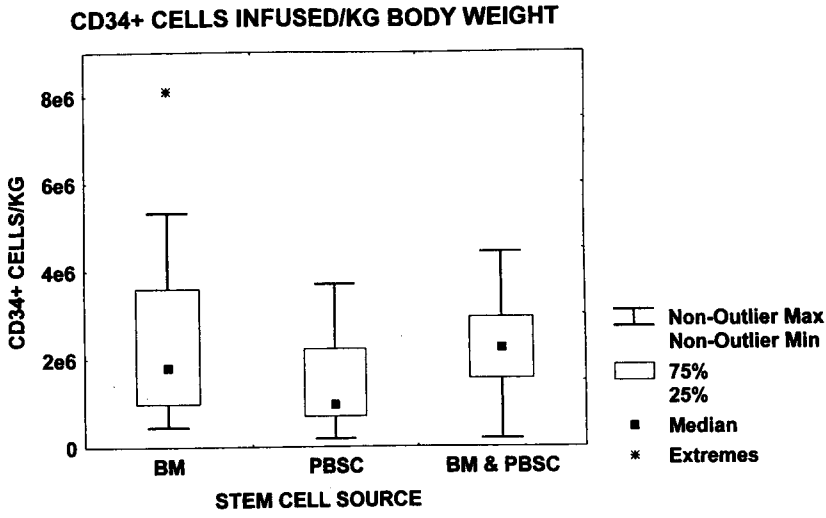


**Figure 2.** *$CD34^+$ cells infused per kilogram of patient body weight. $CD34^+$ fraction was determined as described in text, and multiplied by mononuclear cells/kg to yield $CD34^+$/kg.*

and who, thus, were infused with both products, $2.4\times10^6$ ($\pm1.7\times10^6$) $CD34^+$ cells were given. Testing for significance of the three-way difference by Kruskal-Wallis ANOVA yielded $P$=0.0639. The patients infused with both products received the same number of $CD34^+$ cells as did the patients receiving BM alone.

The mean number of CFU-GM per kilogram of patient body weight (Fig. 3) collected for patients who were randomized to receive PBSC was $1.3\times10^4$ ($\pm1.4\times10^4$), and the number of CFU-GM per kilogram collected for patients randomized to receive BM was $2.2\times10^4$ ($\pm1.9\times10^4$). By pairwise analysis this was not significant, with $P$=0.0842. The patients who were not randomized but who received both products due to inadequate PBSC collection were infused with $1.7\times10^4$ ($\pm1.6\times10^4$) colony-forming cells per kilogram. Testing for significance of the three-way difference by Kruskal-Wallis ANOVA also yielded no significance, with $P$=0.1645. The patients receiving both products received the same number of CFU-GM as did the patients receiving PBSC alone.

## Granulocyte and platelet recoveries

For patients conditioned with ICE, MITT, or TNT, the median time to ANC >500/μL was 16 days after infusion with PBSC ($n$=22) and 18 days after infusion with BM ($n$=25) (Fig. 4). Comparing the two groups by log-rank test produced $P$=0.44631. In contrast, the group of patients who received infusions of both products due to inadequate PBSC collection experienced a median of 22 days to

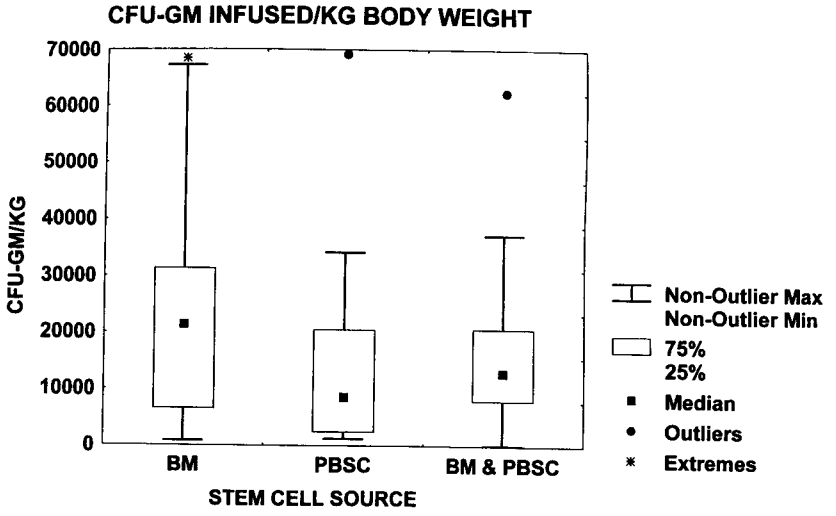


**Figure 3.** *CFU-GM colony forming cells infused per kilogram of patient body weight. $2 \times 10^5$ cells were plated in 1 mL Iscove's medium supplemented with 1.2% methylcellulose, 10% fetal bovine serum, and 1 ng/mL GM-CSF. Fourteen-day colonies were enumerated and combined with mononuclear cells/kg to yield CFU-GM/kg.*

achieve an ANC of ≥500/μL. When all three groups are compared by modified log-rank analysis, the resultant statistic was $P$=0.00846. Patients receiving both products recovered neutrophils more slowly.

For both the patients who received PBSC ($n$=22) and those who received BM ($n$=25), the median time to achieve self-sustained platelet counts of 20,000/μL for 3 consecutive days was 25 days. When the group of patients who received both products—for whom the median day to achieve a platelet count more than 20,000/μL was 37—was added into the analysis, the new statistic was $P$=0.00893 (Fig. 5). The patients who received both products had significantly later platelet recoveries.

## Disease-free survival

All patients in the study were followed for relapse of their disease and death due to any cause. Surviving patients in this study have been followed for a median of 16 months (range 6 to 41 months) after stem cell infusion. Evaluating all patients in the study for freedom from relapse, we found that there was no statistical difference in disease-free survival among the three patient groups ($P$=0.8458) (Fig. 6). Because the majority of patients in the study had stage IV breast cancer, we examined the outcomes for only the patients with that diagnosis and stage to eliminate any possibility that disease and stage could be confounding factors in the

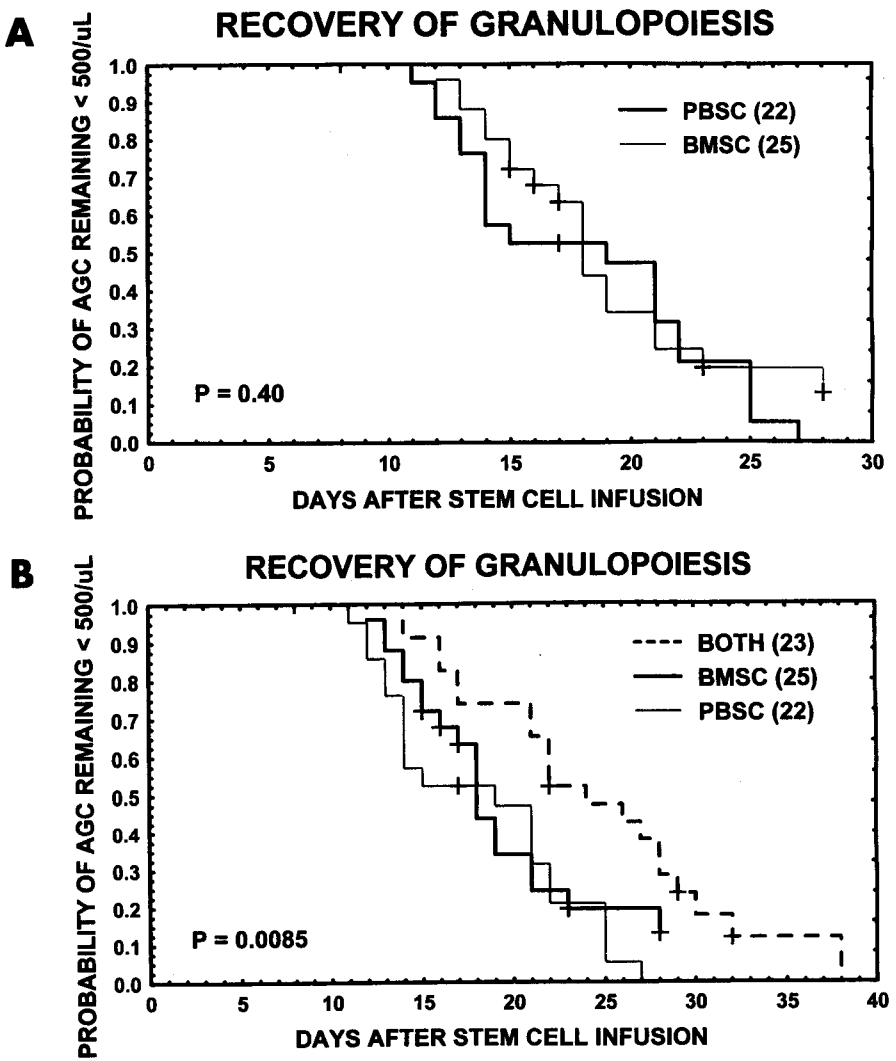


**Figure 4.** *Recovery of absolute granulocyte count to >500/μL: Kaplan-Meier representation of the probability of granulocyte count remaining <500/μL as a function of time after stem cell infusion. A: Two-way comparison with PBSC and BMSC transplanted groups. Note the crossover of the lines. B: Three-way comparison with PBSC and BMSC transplanted groups, and also with group transplanted with both PBSC and BMSC due to inadequate PBSC collections.*

analysis. In this more homogeneous subset analysis, we found that there remained no statistical difference in relapse-free survival, irrespective of which stem cell product was transplanted ($P$=0.9070) (Fig. 7).

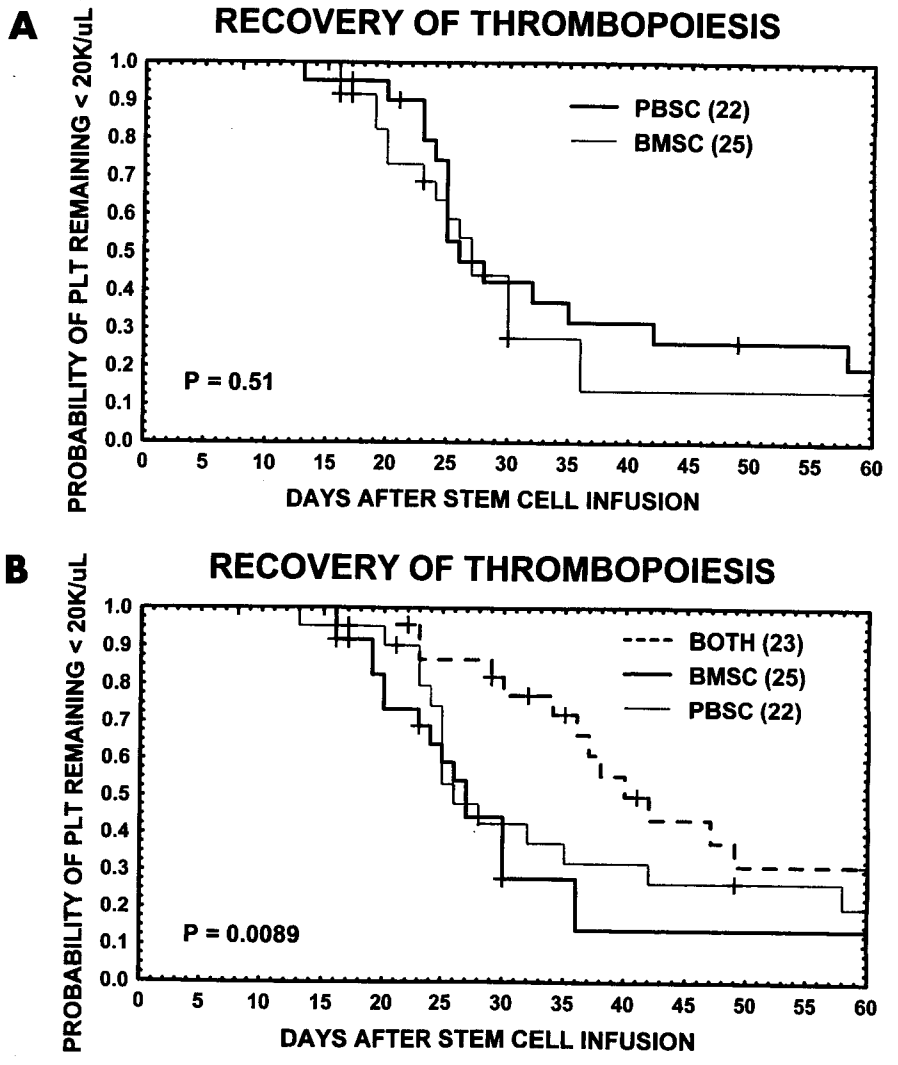


**Figure 5.** *Recovery of platelet count to >20,000/μL: Kaplan-Meier representations of the probability of platelet count remaining <20,000/μL and remaining transfusion dependent as a function of time after stem cell infusion. A: Two-way comparison with PBSC and BMSC transplanted groups. Note intertwined lines. B: Three-way comparison with PBSC and BMSC transplanted groups, and also with group transplanted with both products due to insufficient PBSC collections.*

## DISCUSSION

The use of hematopoietic progenitors derived from circulating blood for autologous hematopoietic reconstitution of an ablated host is accepted as an

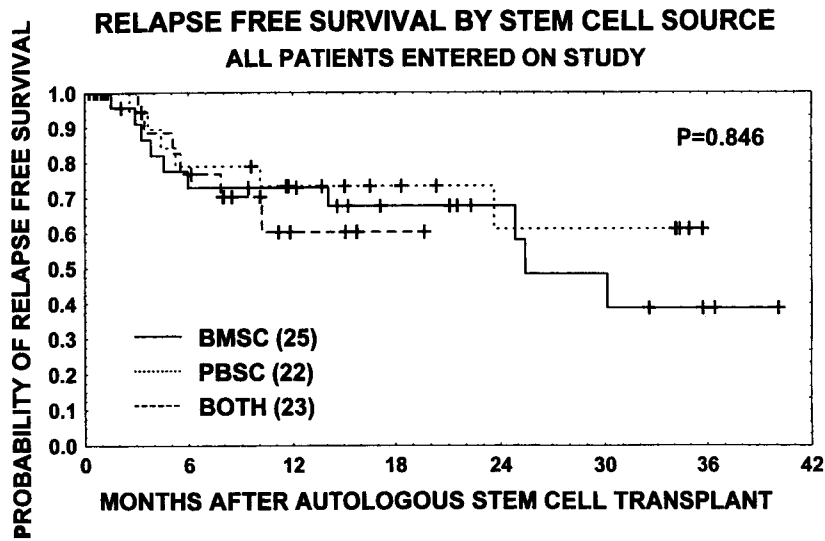


**Figure 6.** *Relapse-free survival, all patients: Kaplan-Meier representation of the probability of relapse as a function of time after stem cell transplant. In this analysis, toxic deaths are treated as censored data at the lefthand side of the plots.*

alternative to the use of bone marrow. In fact, it is widely contended that PBSC autotransplant is superior to BM autotransplant.[4] This contention is based on the premises that *1*) PBSC collection is safer due to the lack of need for general anesthesia and *2*) PBSC transplants produce more rapid engraftment of neutrophils and platelets than do BM transplants.[7,9,10,19] As a principal result of this latter observation, it has also been suggested that the PBSC autotransplant is safer due to reduced risk of opportunistic infections and less expensive due primarily to fewer days of posttransplant hospitalization.[4]

We observed that all of the reports of accelerated engraftment after PBSC transplant were comparing *mobilized* PBSC with bone marrow that was resting (i.e., no growth factors were given and many weeks had transpired since last chemotherapy exposure) at the time of harvest. Further, we were unable to find any reference in which unmobilized PBSC were transplanted and in which the effect of accelerated engraftment was reported.[17,18] There were preliminary reports, however, in which it appeared that bone marrow harvested after growth factor stimulation could produce more rapid engraftment.[30–35]

To account for all of these observations, we hypothesized that the treatment that mobilizes PBSC and not the anatomic location from which they were obtained is responsible for the rapid engraftment observed following PBSC autotransplant. This hypothesis would predict that in a randomized comparison of mobilized PBSC with resting BM for autotransplant, PBSC transplanted patients would

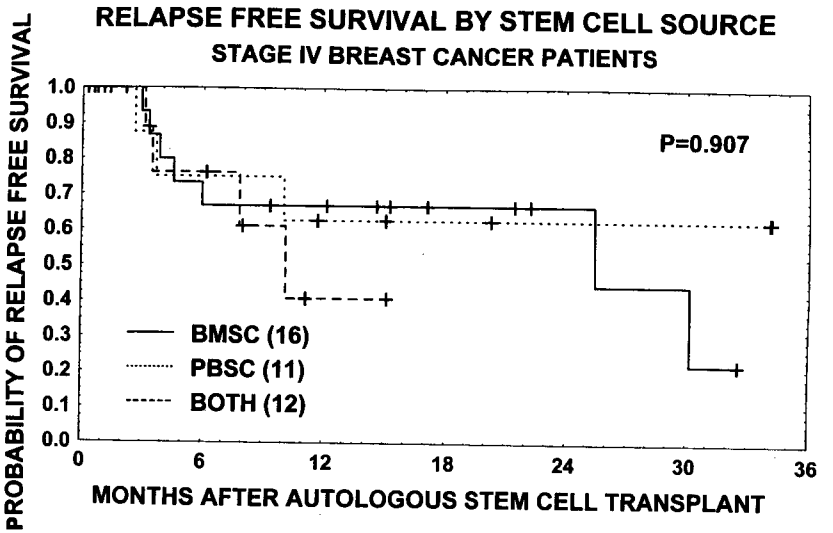


**Figure 7.** *Relapse-free survival, stage IV breast cancer patients: Kaplan-Meier representation of the probability of relapse as a function of time after stem cell transplant. In this analysis, toxic deaths are treated as censored data at the lefthand side of the plots.*

engraft more quickly. Such a study design has been recently reported by both Beyer et al.[19] and Schmitz et al.[20] These investigators reported, respectively, 2-day and 1-day shorter median times to neutrophil recovery for the patients randomized to receive PBSC. These differences are clinically very small. In both studies, the investigators observed 7-day shorter median times for platelet recovery for patients randomized to receive PBSC. This difference is a clinically meaningful one. These findings are not in contradiction with our hypothesis because, in both studies, BM was harvested in the resting state. Our hypothesis would predict that, in a randomized comparison of G-CSF-primed bone marrow with G-CSF-mobilized PBSC, engraftment rates for neutrophils and platelets would be indistinguishable. The data presented in this report reflect the veracity of our hypothesis.

An alternate explanation for the engraftment data presented in this report is that the hematologic recoveries after PBSC transplant were delayed by conditions of the study design. Specifically, it might be suggested that the collection of PBSC after BM harvesting might result in insufficient PBSC harvests. We collected PBSC on days 7–10 of G-CSF administration, when circulating $CD34^+$ cells are diminishing from peak values as recently shown.[36] Examination of the number of CFU-GM and $CD34^+$ cells infused per kilogram in this study reveals no difference between the patients randomized to receive PBSC and those randomized to receive BM. We cannot, however, completely exclude the possibility that circulating

$CD34^+$ cells and CFU-GM are not equivalent to their marrow-bound counterparts with respect to engraftment potential.

The median days to neutrophil and platelet recovery documented in this study are greater than other investigators have reported for G-CSF-mobilized PBSC,[7,9] an observation that might be taken to support the contention that our data are reflective of slower than expected PBSC engraftment. However, we have demonstrated in earlier studies[37,38] that the more dose-intense, cytotoxic transplant regimens we employed in this study are associated with slower posttransplant count recoveries. This observation, that transplant regimen dose intensity can delay engraftment, has been substantially corroborated in an animal model by Down et al.[39–41]

Perhaps the most revealing finding in this study is that patients who failed to mobilize adequate $CD34^+$ cell numbers after four PBSC collections and who were, as a result, transplanted with both BM and PBSC had a demonstrably longer period of posttransplant aplasia. These patients were transplanted with BM cell doses conventionally considered more than adequate, along with their PBSC collections. In the absence of inadequate PBSC collection data, these patients would have been indistinguishable from other autologous BM transplant patients, and the median times to hematologic recovery would have been increased for the entire population of patients receiving BM. In other words, at least some of the improved engraftment kinetics observed with PBSC transplants may be a function of transplanting only those patients who had adequate PBSC collections. The biologic implication of this consideration is that poor PBSC mobilization may be reflective of poor hematopoietic reserve and, in fact, may be a predictor of a high probability of delayed engraftment.

The finding that one-third of consenting patients could not be adequately mobilized for sufficient PBSC collection for transplant is of concern. This may be reflective of our patient population (predominantly breast cancer) and the associated types and amounts of previous cytotoxic therapy that the patients had received before referral. Prior therapy seems to produce more problems with PBSC collection than with BM collection.[42] Because of the stratification employed before randomization, this potential bias was minimized in the randomized portion of the trial. Interestingly, the third of patients who could not be randomized due to inadequate PBSC collection appear to resemble the other patients who were randomized (see Table 1). Thus, we cannot use disease and stage, surrogates for the type and amount of prior therapy, to explain why one-third of patients had inadequate PBSC collections.

We also examined disease-free survival to determine if PBSC transplant might impart a survival advantage, as has been reported for patients with non-Hodgkin's lymphoma.[14,15] As with engraftment, we found no statistical difference between the two products or with the transplant of the combined products. However, because of the relatively small size of this trial, which was designed for evaluating

hematopoietic reconstitution, we cannot exclude the possibility that small differences in disease-free survival may exist among the three groups of patients.

Within the past few years, autologous hematopoietic cell transplant using BM has rapidly declined, being replaced by PBSC transplant. The results of our study and, also, the results of two other trials in which unstimulated BM has been compared with mobilized PBSC[19,20] indicate that the advantages of PBSC over BM for autotransplant are not as great as had been previously suspected.[4] There may still be significant roles for BM as a stem cell product for autotransplantation; for instance, in small children, as a target for gene transfer therapy, and as a source of stem cells for ex vivo expansion.

The data from this study demonstrate the clinical equivalence of G-CSF-primed BM and G-CSF-mobilized PBSC when used for autotransplantation after three different high-dose chemotherapy regimens. This supports the hypothesis that the kinetics of stem cell engraftment is determined by the treatment given to the patient before collection of the stem cells rather than the anatomical compartment from which the stem cells are collected. However, these data should not be overinterpreted. Namely, it cannot be concluded that all PBSC mobilization methods will produce the same engraftment kinetics. Data from our program indicate that the PBSC mobilization regimen may profoundly affect the rate of hemátopoietic recovery,[43] a result that is supported by reports of differential cell recoveries in both animal[44] and human[45,46] studies.

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# CHAPTER 11
# MINITRANSPLANTS

# Canine Hematopoietic Stem Allografts Using Nonmyeloablative Therapy

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## ABSTRACT

A major limitation in the application of stem cell transplantation has been complications related to the conditioning regimens that have been intensified to the point where organ toxicities have been common, resulting in morbidity and mortality. Because of potentially severe complications, the use of transplantation has been restricted to relatively young patients at most transplant centers. Preliminary studies have shown that stable, allogeneic mixed donor-host chimerism can be accomplished in random-bred dogs by administering a sublethal dose of 200 cGy total-body irradiation (TBI) before, and a brief course of immunosuppression with mycophenolate mofetil (MMF) and cyclosporine (CSP) after, major histocompatibility complex (MHC)-identical marrow transplantation. MMF and CSP are capable of controlling both host-vs.-graft (HVG) and graft-vs.-host reactions and establishing a stable state of graft-host tolerance in this model as manifested by stable mixed donor-host hematopoietic chimerism. Based on additional preliminary data using lymphoid radiation instead of TBI, we hypothesize that the major role of TBI is to provide host immunosuppression. Current efforts are directed at replacing pretransplant radiation by anti–T cell reagents such as a monoclonal antibody (mAb) to the T cell receptor $\alpha\beta$ (TCR$\alpha\beta$). Initial data suggest that in vivo administration of an mAb against TCR$\alpha\beta$ enhanced allogeneic engraftment following an otherwise suboptimal dose of 450 cGy TBI similar to the degree of immunosuppression accomplished by an additional dose of 470 cGy TBI. In the MHC-haploidentical setting, cytokine-mobilized peripheral blood stem cells (PBSC) given after conditioning with 450 cGy TBI with postgrafting immunosuppression consisting of MMF and CSP resulted in sustained donor engraftment in three of six dogs. Current studies are underway evaluating a mAb directed at CD44, a cellular adhesion molecule expressed on hematopoietic as well as nonhematopoietic cells. The majority of dogs treated with the mAb in addition to MMF/CSP have engrafted. These approaches developed in the canine model have minimal toxicity, are non–marrow ablative and, thus, are safe enough

to be administered in the ambulatory care setting and may allow inclusion of older patients currently not eligible for treatment with stem cell allografting.

## INTRODUCTION

To have a successful hematopoietic stem cell (HSC) allograft, two immunologic barriers must be crossed: the first consists of host-vs.-graft (HVG) and the second is made up of graft-vs.-host (GVH) reactions. The high-dose conditioning regimens whose primary goal has been to eradicate the patient's underlying disease—for example, leukemia—also function to eliminate the HVG reaction by eradication of the host's immune response. The patients experience pancytopenia from which they are rescued by subsequent HSC allografts. The grafts also function to eliminate any residual leukemia via GVH reactions. Conditioning regimens have now been intensified to the point where organ toxicities are common, resulting in morbidity and mortality. Because of potentially severe complications, the use of transplantation has been restricted to relatively young patients at most transplant centers. Additionally, the optimal postgrafting immunosuppression necessary for control of GVHD often cannot be given to patients with organ toxicities such as those of the liver or kidney.[1]

To design effective conditioning regimens without excessive toxicity, four factors were taken into consideration. First, observations by Burchenal et al.,[2] who found that murine leukemia could only be eradicated by several thousand centigrays of TBI, indicated that some hematologic malignancies were incurable with even the most intense conditioning regimens alone. Second, HSC allografts not only rescue the patient from pancytopenia, but also provide a GVL effect which is in part responsible for many of the observed cures, as predicted from the murine studies of Barnes et al.[3] Weiden et al. published in 1979[4] and 1981[5] the landmark papers describing GVH/GVL effects in human allograft recipients. This led to the testing of donor buffy coat infusions to augment the GVL effect in marrow allografts[6] and to the use of donor lymphocyte infusions as therapy for patients who had relapsed after marrow allografting.[7–13] The third observation was that mixed hematopoietic chimerism sufficed in some cases to "cure" inherited disorders such as thalassemia major and sickle cell disease after conventional high-dose conditioning.[14,15] Last, HVG and GVH reactions are both mediated by T cells in the MHC-identical setting. Therefore, we have investigated the hypothesis that immunosuppression, primarily designed to prevent GVHD, can additionally be used to suppress HVG, thereby facilitating allogeneic engraftment.

We have done studies in a canine model to test the hypothesis that the currently used intensive cytoreductive conditioning therapy can be replaced by nonmyelotoxic immunosuppression. Immunosuppression is directed first at host cells before HSC transplant, and next at both donor and host cells after transplant.

The resultant stable mixed donor-host hematopoietic chimerism is a manifestation of mutual graft-host tolerance.

For assessment of mixed hematopoietic chimerism in the canine model, a polymerase chain reaction (PCR)-based assay of polymorphic mini- or microsatellite markers has been established.[16] This assay is informative in both sex-matched and sex-mismatched transplant settings and can be employed on different subsets of hematopoietic cells. Mixed chimerism is defined as the presence of 2.5 to 97.5% cells of donor or host origin, and these cut-offs are based on the sensitivity of the assay.

## MARROW TOXICITY OF TBI IN DOGS

In the absence of an HSC graft, a single dose of 400 cGy TBI delivered at 7 cGy/min is lethal despite intensive supportive care. Twenty-seven of 28 dogs died from problems related to prolonged pancytopenia.[17] At a dose of 300 cGy TBI, 14 of 21 dogs died, whereas at 200 cGy, only one of 19 animals died, with the remainder showing spontaneous hematopoietic recovery. When only 100 cGy is given, none of the animals (0 of 12) died. At 200 cGy, the granulocyte counts reached their nadirs at about 20 days after TBI, with a median value of 750/(L, while the platelet nadirs were reached between 15 and 24 days, with a median value of 7500/(L.

## MHC-MATCHED MARROW GRAFTS: IMMUNOSUPPRESSIVE TBI DOSE NEEDED FOR ENGRAFTMENT

The results of unmodified MHC-matched marrow allografts after TBI doses from 450 to 920 cGy are shown in Table 1. While a dose of 920 cGy was sufficiently immunosuppressive to result in 95% engraftment, a dose of 450 cGy led to rejection in the majority of dogs which either had autologous hematopoietic recovery (36%) or died of marrow aplasia (23%).[18,19]

## USE OF IMMUNOSUPPRESSIVE DRUG TREATMENT FOR INDUCTION OF MIXED CHIMERISM AFTER LOW-DOSE TBI IN RECIPIENTS OF MHC-MATCHED MARROW

At the myeloablative TBI dose of 450 cGy, two commonly used immunosuppressive GVHD prevention drugs, CSP and prednisone, were tested for their ability to promote engraftment. CSP was given at a dose of 15 mg/kg b.i.d. p.o. from day −1 through day 35, and prednisone was administered at 12.5 mg/kg orally b.i.d. on day −5 to 3 with subsequent taper through day 32.[20] The high dose of prednisone was based on a regimen initially used by Kernan et al. along with ATG[21] and later

**Table 1.** Marrow grafts from MHC-identical littermates after single-dose TBI (delivered at 7 cGy/min) without postgrafting immunosuppression[18,19]

| *Total body irradiation dose (cGy)* | *No. dogs transplanted* | *% of dogs with stable allografts* | *% of dogs surviving with autologous marrow recovery* |
|---|---|---|---|
| 450, myeloablative + supralethal | 39 | 41 | 36 |
| 600 | 23 | 52 | 17 |
| 700 | 5 | 60 | 0 |
| 800 | 5 | 80 | 0 |
| 920, immunosuppressive | 21 | 95 | 0 |

tested by Bjerke et al. with an anti-CD3 antibody for second transplants after rejection of the first graft.[22] Dogs given CSP had stable engraftment in all cases, which was significantly better than that seen in controls not given CSP ($P$=0.01). However, the high-dose prednisone was completely ineffective, and none of the dogs studied had sustained allogeneic engraftment. Next, the TBI dose was lowered to the sublethal range of 200 cGy and, in this setting, the use of posttransplant CSP alone failed to achieve engraftment (Table 2). By 4 weeks, all four dogs studied had rejected their allografts but all survived with autologous hematopoietic recovery.[23] To see if engraftment could be improved by using combinations of drugs, the antimetabolite methotrexate (MTX) was added to CSP, since previous data showed synergism of these two agents for prevention of GVHD in both dogs and human patients. Of six dogs studied, three became stable mixed chimeras and three rejected their grafts, although the rejections occurred much later than seen in dogs given CSP alone. A novel immunosuppressive drug, mycophenolate mofetil, was also tested in this model. MMF blocked the de novo purine

**Table 2.** Engraftment of MHC-identical littermate marrow using a sublethal dose of 200 cGy TBI (delivered at 7 cGy/min) before and immunosuppressive drugs for GVHD prevention after transplant[23] (and unpublished data)

| *Immunosuppression after transplant* | *No. dogs with stable grafts/ no. dogs studied* | *Duration of mixed chimerism (weeks)* |
|---|---|---|
| CSP* | 0/4 | 4, 4, 4, 4 |
| MTX†/CSP* | 3/6 | 2, 7, 11, >8§, >134, >134 |
| MMF‡/CSP* | 10/11 | 12, >49, >55, >56, (57, >62, >63, >73, >104, >130, >130 |

**Cyclosporine 15 mg/kg b.i.d. p.o. days –1 to 35; †methotrexate 0.4 mg/kg i.v. days 1, 3, 6, 11; ‡mycophenolate mofetil 10 mg/kg/ b.i.d. s.c. days 0–27; §euthanized because of papillomatosis.*

synthesis pathway by binding to inosine 5-monophosphate dehydrogenase, and this block interferes with lymphocyte replication. Previous data had indicated that MMF/CSP was superior to MTX/CSP for GVHD prophylaxis.[24] Consistent with those findings, only one of 11 MMF/CSP-treated dogs rejected its allograft at 12 weeks while the remaining 10 dogs have stable mixed chimeras between 49 and 130 weeks after transplant without any evidence of GVHD ($P$=0.06 compared with MTX/CSP and 0.01 compared with dogs given CSP alone). When the dose of TBI was lowered to 100 cGy, all six MMF/CSP-treated dogs rejected their marrow grafts within 3–12 weeks of transplant.

The role of peritransplant immunosuppression for induction of mixed chimerism has been demonstrated by studies using a mAb directed at the TCRαβ complex (unpublished data). In this study, mAb 15.9D5, given daily for 9 days beginning on day −1, was able to control HVG reactions in a manner that was comparable to that seen with CSP given for 35 days after transplant. Addition of this antibody to the MMF/CSP regimen may make it possible to lower the TBI dose needed to establish mixed chimerism to <200 cGy.

## TBI-INDUCED IMMUNOSUPPRESSION FOR ENGRAFTMENT OF MHC-MISMATCHED MARROW

With unrelated MHC-nonidentical marrow donors, engraftment was uniformly seen after conditioning with 1800 cGy TBI, was the exception after 920 cGy, and was never seen with lower TBI doses (Table 3).[25] Similar findings were made with marrow grafts in MHC-haploidentical littermates. In contrast, when cytokine-mobilized PBSC were substituted for marrow,[26] all nine MHC-haploidentical littermates had successful allografts after 920 cGy TBI (Table 4). The findings made here with cytokine-mobilized PBSC resemble those made previously in MHC-mismatched recipients given marrow supplemented by peripheral blood

**Table 3.** Results in dogs given single dose TBI at 7 cGy/min and marrow grafts from MHC-nonidentical donors; recipients were not given posttransplant immunosuppression[25]

| | *No. dogs* | | | |
|---|---|---|---|---|
| *TBI dose (cGy)* | *Studied* | *Graft rejection* | *Autologous recovery* | *Successful allograft* |
| 1800* | 10 | 0 | 0 | 10 |
| 920 | 57 | 47 | 0 | 10 |
| 450 | 5 | 5 | 0 | 0 |
| 380 | 5 | 5 | 0 | 0 |
| 300 | 5 | 5 | 2 | 0 |

**Three fractions of 600 cGy each, administered at 2.1 cGy/min.*

**Table 4.** Results in dogs given single dose 920 cGy TBI at 7 cGy/min and grafts from MHC-haploidentical littermates. Recipients were not given posttransplant immunosuppression.

| | *No. dogs* | | |
|---|---|---|---|
| *Stem cell source* | *Studied* | *Graft rejection* | *Successful allograft* |
| Marrow | 6 | 4 | 2* |
| PBSC† | 9 | 0 | 9 |

**P<0.01 vs. PBSC; †G-CSF- or G-CSF/SCF-mobilized.*[26]

buffy coat cells, which resulted in 95% engraftment (Table 5).[25] The virtually uniform engraftment seen in these dogs could be due to increased T-cell content of the graft, increased numbers of transplanted HSC, or other mechanisms yet to be determined in cytokine-mobilized PBSC.

## EFFECT OF MONOCLONAL ANTIBODIES TO CD44, CD18, OR MHC CLASS II (DR) ON ENGRAFTMENT

Several mAbs have been evaluated for their ability to promote engraftment of MHC-mismatched allogeneic marrow. CD44 and CD18 are both adhesion molecules expressed on hematopoietic cells and involved in heterotypic cellular

**Table 5.** Engraftment of MHC-nonidentical unrelated marrow grafts after 920 cGy TBI.

| | *Sustained allografts* | |
|---|---|---|
| *Additional therapy* | *% (no. of dogs with grafts/ no. of dogs studied)* | *P value†* |
| None[36] (and unpublished data)* | 18 (10/57) | — |
| Buffy coat cells[25] | 95 (19/20) | <0.001 |
| MTX posttransplant[36] | 60 (6/10) | 0.004 |
| MAb 6.4 (irrelevant)[36] * | 14 (1/7) | 0.86 |
| MAb S5 (αCD44)[37–39] * | 67 (14/21) | <0.001 |
| MAb 7.2 (αDR)[36] * | 43 (6/14) | 0.04 |
| MAb 7.2 + MTX[36] | 90 (9/10) | <0.001 |
| MAb 60.3 (αCD18) (unpublished data)* | 45 (5/11) | 0.04 |

**No posttransplant immunosuppression. mAbs S5 and 6.4 were given 0.2 mg/kg/day for six doses prior to TBI and marrow infusion. mAb 60.3 was given 1.0 mg/kg on day –1 followed by 0.3 mg/kg/day on days 0–14 where day 0 was the day of TBI and marrow infusion. mAb 7.2 was given 0.2 mg/kg/day on days –1 to 3 or days –5 to 0. †Monte Carlo simulation test[40] used for statistical comparisons to the group that received no additional therapy.*

interactions of immune function, whereas DR is an MHC class II molecule involved in alloantigen recognition, and mAbs against these molecules have proven useful in marrow allografting. Table 5 summarizes data in MHC-nonidentical unrelated recipients conditioned with 920 cGy TBI. Pretreatment with an irrelevant mAb 6.4 was ineffective in promoting engraftment, whereas significant increases in engraftment were seen with mAbs directed against CD44, DR, and CD18, although none of the mAbs was uniformly successful in achieving that goal. Nevertheless, the fact that recipient treatment with relatively small doses of mAb modified recipient immunity sufficiently for engraftment to occur in a majority of dogs was encouraging. The exact mechanisms of action by which mAbs improve engraftment have not yet been determined, although as far as mAb S5 (anti-CD44) is concerned, in vitro studies have shown that it interfered with the function of natural killer (NK) cells.[27,28] In turn, NK cells are known to mediate rejection of MHC-mismatched grafts in the canine model.[29,30] Not surprisingly, S5 failed to enhance grafts from MHC-matched littermates after 4.5 Gy TBI,[31] consistent with the notion that T cells and not NK cells reject MHC-matched grafts.[32]

## TOWARD NONMYELOABLATIVE REGIMENS FOR TRANSPLANTATION OF MHC-MISMATCHED MARROW

In recipients of PBSC grafts from MHC-haploidentical littermates, rejection uniformly occurred when the radiation dose was lowered from 920 to 450 cGy. When the recipients were given postgrafting immunosuppression with MMF/CSP, half of the dogs had sustained donor engraftment (unpublished data). When antibody S5 directed at CD44 was used in addition to MMF/CSP, virtually all dogs had sustained allogeneic engraftment (unpublished data).

## MIXED CHIMERISM IN THE TREATMENT OF HEMATOLOGIC DISEASES: SEVERE CANINE HEREDITARY HEMOLYTIC ANEMIA

Based on previous studies, it is known that severe canine hereditary hemolytic anemia due to pyruvate kinase deficiency can be corrected by conventional HSC transplants from healthy littermates following a conventional high-dose conditioning regimen.[33,34] Furthermore, the severe hepatic iron overload that existed before transplant regressed over time after transplant. The preclinical findings in this model encouraged the application of HSC transplants to the treatment of patients with beta-thalassemia [35].

Canine hereditary hemolytic anemia represents an ideal model to test the hypothesis that mixed chimerism can correct phenotypic manifestations of the genetic disease. Accordingly, a 5-year-old Basenji dog with pyruvate kinase deficiency was given 200 cGy TBI before, and MMF/CSP after, a marrow

transplant from a healthy MHC-identical littermate (unpublished data). Pretransplant liver and marrow biopsies showed extensive hemosiderosis and fibrosis and, in addition, there was extramedullary hepatic hematopoiesis. The dog's hematocrit was 24% with an uncorrected reticulocyte count of 30%. Twenty-one weeks after transplant, the hematocrit was 44% with a reticulocyte count of 1.2%, and the donor contribution among the dog's hematopoietic cells was approximately 80%. These very preliminary findings support the concept that mixed chimerism can be effective in correcting a nonmalignant hematologic disease.

## CONCLUSIONS

We believe that the current intensive cytoreductive and toxic conditioning regimens can be replaced by nonmyelotoxic immunosuppression. Allogeneic stem cell grafts create their own space in the host's marrow through a GVH reaction and do not require myeloablation through radiation or chemotherapy. Immunosuppression has a twofold effect: it is directed first at host cells before HSC transplant and next at both donor and host cells after transplant with the intent of establishing mutual graft-host tolerance. The resultant effect is to have stable mixed donor-host hematopoietic chimerism. Based on the results in the preclinical model in the MHC-identical setting, clinical studies have been initiated in the setting of both malignant and nonmalignant diseases. Early data in human patients with malignancies have shown the feasibility of establishing mixed chimerism using an outpatient transplant approach. Preliminary data from preclinical studies in the MHC-nonidentical setting will allow extension of this application to patients who do not have MHC-identical donors.

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# Allogeneic Progenitor Cell Transplantation After Nonmyeloablative Conditioning

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## NONMYELOABLATIVE CONDITIONING REGIMENS

Harnessing graft-vs.-leukemia without myeloablative therapy will depend on developing conditioning regimens that are sufficiently immunosuppressive to allow durable engraftment and yet have little nonhematologic toxicity. Likewise, in many cases, they will also need to be sufficiently active against the malignant disorder to provide time for the allogeneic cells to proliferate and thus exert their antitumor effect.

The purine analogs fludarabine and 2-chlorodeoxyadenosine (2-CDA) have been shown to be active against a variety of hematologic malignancies.[1–3] These compounds are also immunosuppressive, effectively inhibiting the mixed lymphocyte reaction in vitro.[4,5] Both fludarabine and 2-CDA have been shown to inhibit the mechanisms of DNA repair and may therefore potentiate the antitumor effect of some alkylating agents; however, they have little extramedullary toxicity and have been well tolerated in elderly and debilitated patients.[6–8] Therefore, purine analog–containing nonmyeloablative chemotherapy would seem an appropriate way of exploring induction of graft-vs.-leukemia without myeloablative therapy, in a procedure that has been named "minitransplant."

## MINITRANSPLANTS FOR ACUTE MYELOID LEUKEMIA AND CHRONIC MYELOGENOUS LEUKEMIA

At M.D. Anderson, we performed a pilot trial of purine analog–containing nonmyeloablative therapy for patients with AML or CML considered ineligible for myeloablative therapy—because of either age or medical condition—using fludarabine/idarubicin Ara-c or 2CDA/Ara-c in nonmyeloablative doses.[9,10] A total of 26 patients (AML 13, myelodysplastic syndrome [MDS] 4, CML 9) have been treated. Patient characteristics are summarized in Table 1.

Among the 17 patients with AML or MDS treated, three died before day 100 from infectious complications, but no other toxic deaths occurred. Three patients developed ≥grade 2 graft-vs.-host disease (GVHD), all of them responding to

**Table 1.** Characteristics of patients undergoing minitransplant for AML and CML at M.D. Anderson Cancer Center

| | |
|---|---|
| *n* | 26 |
| Age (years) | 60 (28–72) |
| Diagnosis and stage at BMT | |
| AML | |
| First relapse untreated | 2 |
| CR 2 | 2 |
| Refractory relapse or >CR 2 | 9 |
| MDS | |
| Untreated | 1 |
| First relapse untreated | |
| Refractory relapse or >CR 2 | |
| CML | |
| First chronic phase | 5 |
| Transformed | 4 |
| Median time to transplant (days) | 490 (77–3429) |
| Median no. of prior therapies | 2 (1–3) |
| Preparative regimen | |
| FlagIda | 17 |
| 2CDA/AraC | 9 |
| Donor type and cell source | |
| Sibling matched or 1 Ag mismatch | 20/3 |
| 6/6 matched unrelated | 2 |
| Syngeneic | 1 |
| PBSC/BM | 22/4 |
| GVHD prophylaxis (twin excluded) | |
| CSA or CSA/MP | 19 |
| FK/MTX | 6 |

therapy with steroids alone or in addition to antithymocyte globulin (ATG). Thirteen patients had neutrophil recovery a median of 10 days posttransplant (range 9–18) and 11 achieved platelet transfusion independence a median of 12 days posttransplant (range 8–78). Ten patients achieved complete remission (<5% bone marrow blast with neutrophil recovery and platelet transfusion independence). Chimerism analysis of the 10 patients achieving complete remission on day 30 revealed that seven of them had >80% donor cells either by cytogenetics or molecular techniques, one patient had autologous reconstitution, and two patients were inevaluable for chimerism either because of technical difficulties or lack of difference in enzyme restriction pattern between donor and recipient (syngeneic transplant). At 3 months posttransplant, six patients remained in remission, four of whom had >80% donor cells by similar studies, and by 1 year,

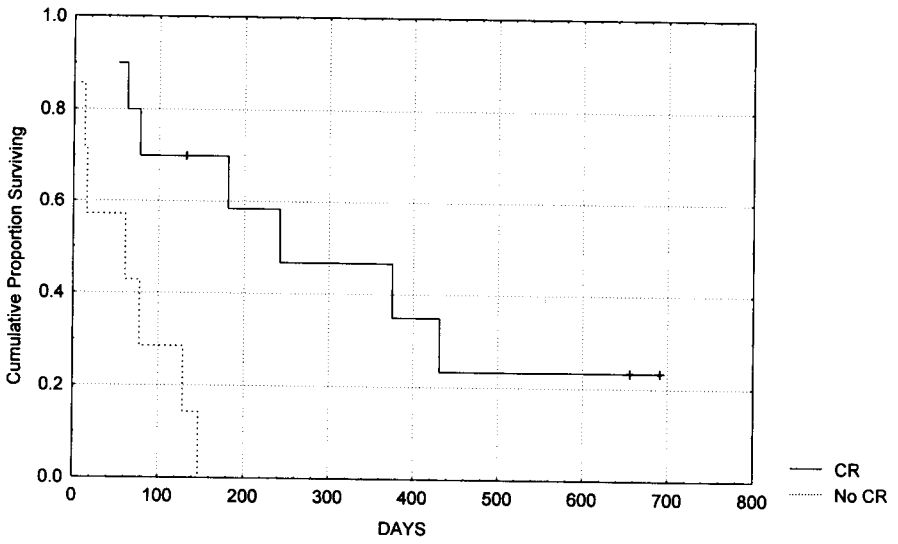


**Figure 1**

one of the two patients in remission remained 100% donor by molecular techniques and the other patient was inevaluable (syngeneic).

The median survival for patients achieving CR was 211 days, with three patients remaining alive at 5, 22, and 23 months after transplant. Nine patients have relapsed, of whom two were successfully reinduced, one with a second minitransplant and the other with a conventional syngeneic transplant using busulfan/cyclophosphamide conditioning. The median survival of the nonresponding patients was 61 days, with none of them responding to subsequent salvage maneuvers (Fig. 1).

This initial experience permitted us to conclude that donor cell engraftment and remissions can be achieved in patients with advanced AML using purine analog–containing nonmyeloablative chemotherapy followed by allogeneic peripheral blood stem cells. However, refractory patients relapse quickly, and this strategy should be further explored in patients in remission ineligible for conventional transplant techniques because of either age or medical condition.

Among the nine patients with CML, one patient died from acute GVHD on day 43, and no other treatment related deaths have occurred. All patients had hematologic recovery of both neutrophils and platelets a median of 13 days postinfusion, but two patients failed to have any evidence of donor cell engraftment (both were recipients of matched unrelated donor cells). Bone marrow on day 30 revealed complete cytogenetic remission in five patients and major cytogenetic response in another three, with one patient having insufficient metaphases. Five of

seven evaluable patients had cytogenetic progression during the first 3 months after transplant, one patient remains in complete cytogenetic remission 6 months after transplant, and the other relapsed 9 months posttransplant. All five of the progressing patients had immunosuppression withdrawn and received further infusion of donor lymphocyte with no responses reported to date.

These preliminary results suggest that the combination of fludarabine, idarubicin and Ara-c, although effective in achieving remissions, may not be optimal for CML. However, further study in more patients in nontransformed phases and longer follow-up is needed.

To try to improve on disease control, at M.D. Anderson Cancer Center we have explored the combination of the purine analogs fludarabine or 2-CDA with melphalan for patients with advanced hematologic malignancies who were considered poor candidates for a conventional allogeneic progenitor cell transplant.[11] From February 1996 to April 1998, a total of 63 patients with a variety of hematologic malignancies have been treated. Patient and treatment characteristics are summarized in Table 2. All patients received unmanipulated donor bone marrow or stem cells and FK506/methotrexate combinations for GVHD prophylaxis.

Fifty-six patients had neutrophil recovery at a median of 14 days (range 9–35), and 40 patients recovered platelet transfusion independence at a median of 22 days (range 9–118). All engrafting patients except one had documentation of >80% donor cell engraftment by day 30, with one instance of autologous reconstitution and one case of secondary graft failure. In this group of very poor prognosis patients ineligible for conventional transplant, the 100-day transplant-related mortality (TRM) was 50% (31 of 63), with four of eight patients in the 2-CDA/melphalan arm dying from multiorgan failure, leading us to close this treatment arm. The overall survival for patients in CR1 or untreated first relapse was 68% at 1 year, vs. 9% for patients with more advanced or refractory disease (Fig. 2). We concluded that fludarabine/melphalan combinations can allow engraftment of allogeneic progenitor cells including cells obtained from matched unrelated donors. Furthermore, this strategy can produce long-term disease control in patients with hematologic malignancies early in the course of their disease at an acceptable risk and toxicity in patients ineligible for conventional myeloablative transplant therapies. Treatment-related mortality and disease recurrence limits the usefulness of this approach in patients with refractory disease.

## MINITRANSPLANTS FOR LYMPHOID MALIGNANCIES

The use of allogeneic transplantation is limited in patients with lymphoid malignancies, such as chronic lymphocytic leukemia or lymphomas, because they typically are older. We have evaluated the induction of graft-vs.-leukemia as primary therapy for patients with lymphoid malignancies who are considered poor

**Table 2.** Patient and treatment characteristics for patients receiving melphalan and purine analog combinations

| | |
|---|---|
| *n* | 63 |
| Age (years) | 49 (22–71) |
| Diagnosis | |
| AML/MDS | 27/8 |
| CML | 16 |
| NHL/HD | 5/3 |
| ALL | 4 |
| Time to BMT, days (range) | 558 (69–6626) |
| Stage at BMT | |
| Acute leukemia | |
| CR1/CR2 | 1 / 2 |
| Untreated first relapse | 10 |
| Refractory or other | 35 |
| CML | |
| First chronic phase | 2 |
| Accelerated | 6 |
| Blast crisis/CP2 | 3/4 |
| Prior regimens | 2 (0–9) |
| Comorbid conditions | |
| Age >50 | 29 |
| Prior BMT | 17 |
| Poor organ function | 31 |
| PS 2 | 12 |
| Donor type | |
| 6/6 sibling | 31 |
| 5/6 sibling | 2 |
| 6/6 matched unrelated | 30 |
| Preparative regimen | |
| Fludarabine/melphalan | 55 |
| 2CDA/melphalan | 8 |

candidates for conventional transplant techniques.[12] Nine patients have been treated, of which eight were older than age 50. All patients with advanced CLL ($n$=5) or transformed lymphoma ($n$=4) were treated with one of two preparative regimens (fludarabine/cytoxan or fludarabine/Ara-c/platinum). Mixed chimerism was observed in six of the nine patients, with a percentage of donor cells ranging from 50 to 100% 1 month posttransplant. No regimen-related deaths were observed, and four patients achieved complete remission, one only after donor lymphocyte infusions.

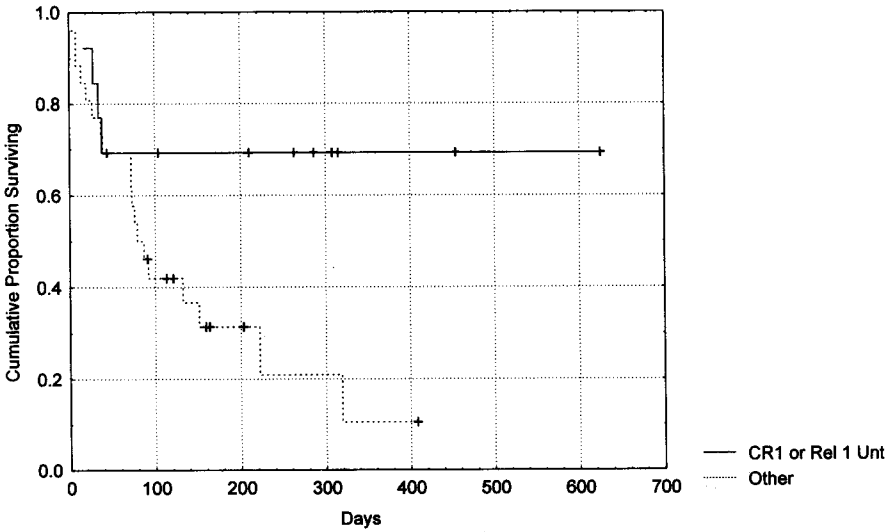


**Figure 2**

## SUMMARY AND CONCLUSIONS

The efficacy of graft-vs.-leukemia induction to treat relapses after allogeneic progenitor cell transplant in a variety of hematologic malignancies suggests that it may be possible to use the graft-vs.-leukemia effect as primary therapy for these malignancies without the need of myeloablative therapy. This type of strategy should be explored initially in patients considered ineligible for conventional myeloablative therapies because of either age or concurrent medical conditions.

We and others have demonstrated that nonablative chemotherapy using fludarabine combinations is sufficiently immunosuppressive to allow engraftment of allogeneic blood progenitor cells. Patients could then receive graded doses of donor lymphocytes, without rejection, to mediate graft-vs.-leukemia. Ideally, this therapy can be titrated to low levels of residual malignant cells using sensitive detection techniques. This novel approach to therapy would reduce the toxicity of the transplant procedure, allow it to be administered more safely to debilitated patients, and possibly extend the use of transplantation to older patients who are not presently eligible for bone marrow transplant procedures. Other possible indications include treatment of nonmalignant disorders and induction of tolerance for solid organ transplantation.

GVHD remains a major obstacle that needs to be overcome. Although a potentially lower level of inflammatory cytokines may be present after nonmyeloablative therapies, fatal GVHD still occurs. Methods to diminish GVHD after

allogeneic transplant such as selective T cell depletion, HSV-TK-transduced lymphocytes, and other nonablative combinations are currently being explored.[13–15]

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# CHAPTER 12
# GENE THERAPY

# Transduction of Primitive Hematopoietic Cells With Adenovirus-Polycation Gene Transfer Vehicles: New Strategies for Management of Hematologic Disease

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## ABSTRACT

Gene transfer into primitive hematopoietic cells has been proposed as a means to achieve a variety of therapeutic results. Unfortunately, many types of hematopoietic cells have proven to be largely refractory to standard methods of gene transfer. Consequently, we have investigated the use of new strategies to mediate high-efficiency transduction of human hematopoietic tissue. Previously, we have shown that under the appropriate conditions, adenoviral vectors can achieve limited gene transfer into primitive $CD34^+$ cells (Neering SJ, Hardy SF, Minamoto D, Spratt SK, Jordan CT: Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. *Blood* 88:1147–1155, 1996). To extend this approach, we have examined the use of polycationic compounds as a means to enhance the natural gene delivery capacity of adenoviral vectors. These studies have shown that a vehicle composed of adenoviral particles, coated with a polyamidoamine dendramer, is a highly efficient complex for the transduction of primitive human hematopoietic cells. To develop this method of gene transfer to be useful for immunotherapy strategies, we tested the virus-polycation complex (VPC) for transduction of primary myeloid leukemia cells. Using a VPC encoding a green fluorescent protein (GFP) reporter gene, our data show that 79% (± 13, $n$=7) of primary leukemic blast cells can be transduced using the VPC method. Moreover, approximately 70–80% of the gene transfer activity occurs within the first 2 hours of the infection period, and gene expression can be detected in as little as 3 hours. Also, cells transduced by this method continue to express the transgene for at least 6–7 days. Therefore, we suggest that this technique will be a highly effective strategy for the introductory of immune regulatory genes (such as CD80 and granulocyte-macrophage colony-stimulating factor [GM-CSF]) into primary tumor cells for the purpose of generating antileukemia vaccines.

## INTRODUCTION

A potentially interesting treatment modality that could be applied as an adjuvant therapy to acute leukemia is immunotherapy. Successful immunotherapy is based on the generation of a specific antitumor immune response.[1,2] Initial efforts in immunotherapy employed adoptive transfer of in vitro stimulated antitumor lymphocytes into melanoma patients. These studies showed that a clinical antitumor response could be achieved in approximately 30% of patients treated.[3] The logical extension of this work was to attempt in vivo lymphocyte stimulation via the administration of tumor-cell vaccines. This strategy has been tested for several malignancies but has shown only modest levels of success.[4] To further enhance the immunogenicity of tumor-cell vaccines, several investigators have employed gene transfer techniques as a means to introduce factors that may augment T cell costimulation and/or immunomodulation.[5,6] Evidence supporting the clinical utility of a gene transfer strategy for generation of leukemia vaccines has previously been shown in animal model systems. Several groups have shown that transduction of leukemic cells with genes such as CD80 and GM-CSF, followed by administration of the genetically modified tumor, can induce a significant and prolonged cytotoxic T lymphocyte (CTL)-specific antitumor response.[7,8] Given the lack of CD80 expression on human leukemic tumors (C.T.J., unpublished observation), it appears likely that such a strategy would also be worthy of clinical evaluation in leukemia.

While it has been feasible in laboratory studies to generate gene-modified tumor cells,[9] it has proven much more difficult to develop a sufficiently easy and practical means of transducing large numbers of primary human leukemic cells. Several investigators have successfully employed retroviral vectors to infect murine cells; however, to date retroviral transduction procedures have shown only low and inconsistent levels of gene transfer for primary human hematopoietic cells.[10–12] An alternative to retroviral gene transfer would be the use of adenoviral vectors.[13,14] Previously, we have shown that under the appropriate conditions, adenoviral vectors can achieve limited gene transfer into primitive $CD34^+$ cells.[15] In contrast to retroviral vehicles, adenoviruses can be easily generated in high concentrations ($>10^{12}$ viral particles/mL) and typically express their transgenes very effectively. Although adenoviral vectors do not integrate into the host cell genome, and are thus only transiently maintained, it is likely that transient expression will be sufficient for the purposes of inducing a vaccine response. While adenoviruses have been reported to infect some types of malignant hematopoietic cells,[16–18] the efficiency varies widely among differing cell types and patients. We believe that a more efficient technology will be required to mediate general use of this approach. Accordingly, we have developed a new type of vehicle that combines an adenoviral vector with a synthetic polyamidoamine dendramer.[19] Preliminary studies have

shown that such a hybrid vehicle can reproducibly infect primary acute myeloid leukemia (AML) cells with very high efficiency and speed. Thus, we propose to develop such a vehicle for use with the gene therapy based vaccination strategies already demonstrated to be efficacious in preclinical models. Our goal is to develop a simple, fast, and effective procedure for the generation of leukemia vaccines.

## MATERIALS AND METHODS

### Isolation of leukemic cells

Primary cells were isolated from leukemia patients under University of Kentucky protocol #98-BMT-91. Peripheral blood samples were obtained with informed consent and were subjected to erythrocyte depletion using standard $NH_4Cl$ lysis procedure. The resulting leukocyte population was then plated at $1.0\times10^6$ cells/mL in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and 10 U/mL penicillin and streptomycin. As indicated, cultures were further supplemented with interleukin (IL)-3 and stem cell factor (SCF) at 10 and 20 ng/mL, respectively (R&D Systems)

### Transductions

Virus was generated using 293 cells and twice plaque-purified by CsCl gradient preparation using standard procedures.[20] All infections were performed with an adenovirus vector encoding the GFP gene (C.T.J., unpublished data), and used a multiplicity of infection (MOI) of 250–500. VPC were generated by mixing adenovirus stocks with the polyamidoamine dendramer (Superfect; Qiagen) at a ratio of $5\times10^9$ viral particles per microgram polycation. The mixture was incubated at room temperature for 5–10 minutes then added directly to cell cultures. As indicated, cultures were washed to remove the virus or VPC after 2 hours and then replated in the same conditions.

### Flow cytometry

Except where indicated, all analysis of transductions was performed 48–72 hours postinfection. Before infection, each sample was phenotyped to determine the antigen profile of the leukemic population. After transduction, cells were stained with monoclonal antibodies specific to the leukemic cells and analyzed with respect to GFP fluorescence. Phycoerythrin-conjugated antibodies used to identify leukemic cells included reagents specific to CD33, CD34, HLA-DR, and CD15 (Becton Dickinson). All analysis was performed using a Becton Dickinson FACScan flow cytometer equipped with a 488 nm argon laser.

## RESULTS

### Transduction of primary AML cells

Leukocytes were isolated from peripheral blood of untreated AML patients and infected using either a conventional adenovirus vector encoding the GFP or the same vector complexed with a polyamidoamine dendramer compound (VPC, see Methods). Infections were done in both the presence and absence of exogenous cytokines, and cells were assayed for gene transfer efficiency 48–72 hours later. Shown in Fig. 1 is an example of one infection comparing the two methods of gene transfer. A shows the leukemic blast population (denoted by the box), and B, C, and D show uninfected, adenovirus only, or VPC infection, respectively. Table 1 summarizes our initial findings with seven patient samples. In the presence of exogenous growth factors, the VPC method yielded an average 79% gene transfer efficiency (± 13%), and the adenovirus alone yielded an average of 50% gene transfer (± 19%). In the absence of growth factors, the same gene transfer vehicles yielded efficiencies of 71% (± 11%) and 44% (± 10%), respectively.

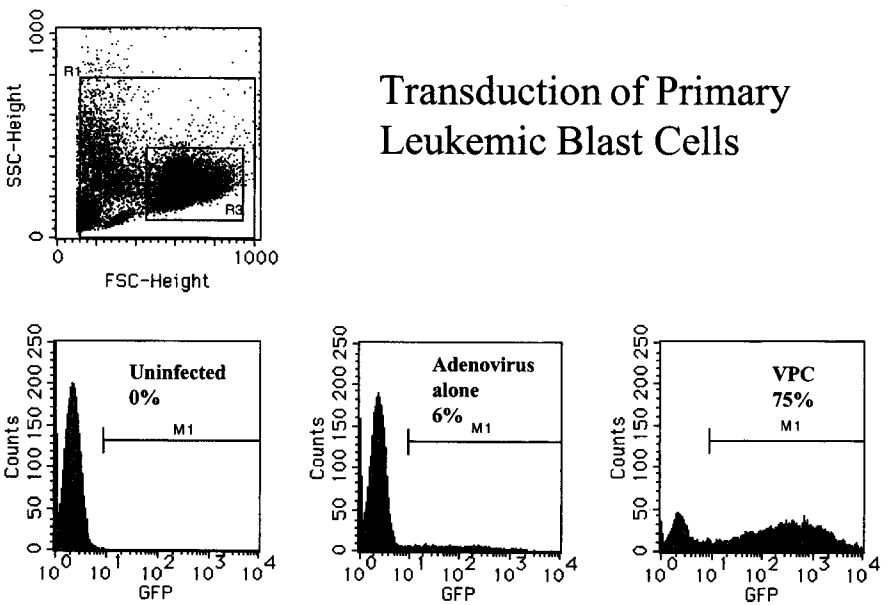


**Figure 1.** *Flow cytometric analysis of leukemic cells transduced with adenovirus or VPC. The upper left panel shows the forward vs. side angle light scatter of a leukemic leukocyte population. The box designated R3 indicates the blast population. Cells within the R3 box are shown for uninfected, adenovirus infected, or VPC infected samples (lower panels, left to right). Flow cytometric analysis was performed 24 hours postinfection.*

**Table 1.** Initial findings with seven patient samples

| | | % GFP positive plus growth factors | | %GFP positive minus growth factors | |
|---|---|---|---|---|---|
| *Experiment* | *FAB type* | *VPC* | *Adenovirus* | *VPC* | *Adenovirus* |
| 1. AML1229 | M5 | 89 | ND | ND | ND |
| 2. AML1230 | ND | 67 | 23 | ND | ND |
| 3. AML0119 | M7 | 96 | 66 | 83 (86%) | 45 (68%) |
| 4. AML0130 | M4 | 64 | 51 | 56 (87%) | 46 (90%) |
| 5. AML0206 | ND | 74 | 73 | 66 (89%) | 56 (77%) |
| 6. AML0211 | M4 | 73 | 35 | 68 (93%) | 28 (80%) |
| 7. AML0219 | M4/5 | 90 | 52 | 82 (91%) | 47 (90%) |
| Average | | 79 (±13) | 50 (±19) | 71 (±11) | 44 (±10) |

*Growth factors used were IL-3 (10 ng/mL) and SCF (20 ng/mL). ND, not done.*

### Kinetics of gene transfer

Previously, we have observed that transduction of normal $CD34^+$ cells occurs much more rapidly with the VPC complex than with conventional adenovirus vectors (C.T.J., unpublished data). The rate of gene transfer into AML cells was examined for two experiments in which VPC was compared to adenovirus alone (Table 2). For both experiments, cells were infected for 2 or 48 hours. The VPC-infected cells showed a high degree of infectivity after only 2 hours, whereas the adenovirus-infected cells were 80–90% reduced in comparison to the 48-hour infection. Thus, we conclude that like normal cells, infection of AML cells with VPC occurs with much faster kinetics.

## DISCUSSION

We have shown that primary AML cells can be readily transduced by a VPC and that such infection occurs with relatively fast kinetics. This suggests that the VPC may be an appropriate vehicle for the generation of gene-modified tumor cell vaccines for leukemia. Based on previous studies of other malignancies, we

**Table 2.** Rate of gene transfer for VPC and adenovirus alone

| | % GFP positive | | | |
|---|---|---|---|---|
| | 48-hour infection | | 2-hour infection | |
| *Experiment* | *VPC* | *Adenovirus* | *VPC* | *Adenovirus* |
| 6. AML021198 | 73 | 35 | 73 (100%) | 3 (9%) |
| 7. AML021998 | 90 | 52 | 64 (71%) | 9 (17%) |

envision a clinical plan as outlined in Fig. 2. Patients will possess the diagnosis of acute myeloid leukemia, established by peripheral blood and/or bone marrow morphologic and flow cytometric analysis, and be of poor prognosis as determined by adverse cytogenetics. Patients will be entered on study at the time of an initial presentation of a new hematologic malignancy or at the time of presentation with an untreated relapse. A volume of peripheral blood will be obtained, not exceeding 250 mL, which should allow for isolation of approximately $1–10\times10^9$ blast cells. Blood will be depleted of erythrocytes, and the mononuclear cell component, including the malignant population, will be isolated. Cells will be infected with a VPC comprising a replication incompetent adenoviral vector carrying the CD80 (B7.1) and/or GM-CSF genes. After infection, a sample of the product will be assayed to assess gene transfer efficiency, the ability to stimulate CTLs in vitro, and sterility. The resulting infected tumor cell product will be irradiated and cryopreserved in aliquots, in anticipation of later use as a leukemia vaccine. After standard induction and consolidation chemotherapy, patients whose infected tumor cells generated CTLs in vitro would then begin vaccine therapy. Cryopreserved cells will be thawed and administered using a dose escalation design. Part of the dosing strategy will be to explore the optimal time interval for administration of the vaccine. Objective response to the vaccine will be assessed by assays for cytolytic T cell populations specific for the tumor clone. Overall and disease-free survival will be calculated from the date of accrual. Major endpoints of this clinical trial include safety to patients, monitoring qualitative and quantitative immune function, inducing tumor-specific CTLs, and assessing time to relapse.

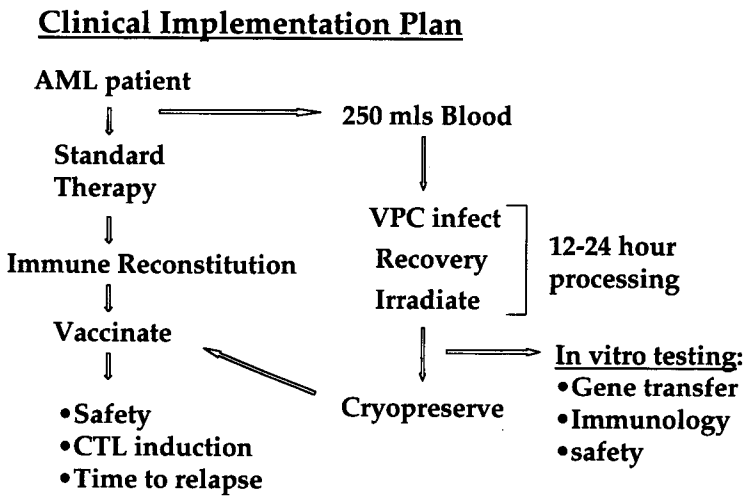


**Figure 2.** *Clinical implementation plan for the use of VPCs to generate gene-modified tumor cell vaccines.*

In preclinical studies, the only apparent shortcoming of this approach was that permanent cures were limited to instances in which tumor burden was relatively low. Thus, this strategy appears ideal in the context of an adjuvant setting, where tumor remission has already been induced by standard chemotherapy. In this scenario, the tumor-specific CTLs that are induced by the vaccine are only required to maintain a state of remission rather than attack an advanced malignancy. Furthermore, because tumor cells that do regrow in a relapsed leukemia patient are more likely to be drug resistant than cells that occur at presentation,[21] an induced CTL response may be particularly useful in this context. Finally, leukemia is also a potentially good general model for immunotherapy because, as a liquid tumor, it allows easy access to large numbers of tumor cells and lymphocytes. This feature allows for a variety of ex vivo manipulations.

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# Molecular Intervention in Myeloma Autografts

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In a clinical gene-marking trial, bone marrow harvested from 15 myeloma patients was maintained in long-term marrow culture (LTMC) for 3 weeks and exposed on days 8 and 15 to clinical-grade G1Na retrovirus supernatant. A mean of $8.23 \times 10^8$ gene-marked LTMC cells and $2.4 \times 10^4$ gene-marked CFU-GM were infused along with an unmanipulated PBSC graft into patients after myeloablative therapy. Gene transfer was observed in 71% of tested blood and bone marrow samples and 12% of bone marrow CFU-GM after transplant. The proportion of provirus-positive CFU was maximum at 3 months (mean 20%) and least at 24 months (3%) posttreatment. Semiquantitative PCR demonstrated that gene transfer was achieved in ~ 0.01% of total bone marrow and blood mononuclear cells. Gene transfer was also observed in circulating B lymphocytes, $CD34^+$ bone marrow progenitors, and CFU derived from these progenitors; however, gene-marked myeloma relapse has not been observed. These data suggest that long-lived hematopoietic stem cells may be successfully transduced in stromal-based culture systems, albeit below the level required for clinical utility at this time. While retroviral vectors may transduce rapidly cycling malignant plasma cells in culture, forming a basis for our gene-marking trials, most attempts at retroviral gene transfer into primary plasma cells demonstrate that transduction efficiency is low (1–4% of exposed cells). In contrast, we demonstrated that myeloma cell lines and, to a lesser degree, primary plasma cells could be infected with adenoviral vectors carrying marker genes. A phase I clinical trial of direct intratumoral injection of an adenoviral vector expressing IL-2 was initiated in patients with subcutaneous malignancy. Two patients with multiple subcutaneous plasmacytomas were injected. On tumor biopsy 14 days postinjection, vector-derived IL-2 was readily detected in tumor cells by PCR, and clinical inflammation was evident. On the basis of these studies, phase I vaccination trials using adenovirus engineered autologous plasma cells expressing IL-2 or the combination of IL-12 and B7-1 have been initiated in patients with residual disease posttransplant. Four patients injected to date have had no side effects attributable to the vaccine.

# A Vaccine of Melanoma Peptide Loaded Dendritic Cells: Preclinical and Clinical Data

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Dendritic cells (DC) are professional antigen presenting cells (APC) that can be used for an adoptive transfer to induce a specific T cell response in vivo against melanoma-associated antigens. We have recently demonstrated that Langerhans-type cells (LC) as well as mature interdigitating DC can be generated from $CD34^+$ hematopoietic progenitor cells (HPC) and from $CD14^+$ monocytes by the sequential use of hematopoietic growth factors including IL-4, GM-CSF, and TNF-(. HPC and monocyte-derived DCs were found to exhibit highly similar characteristics. For clinical use, HPC-derived DC were employed first and APC function was tested by IFN-( production of melan-A-specific CTL after stimulation with melan-A peptide–loaded DC. In a phase I clinical trial of adoptive immunotherapy, HLA-A1 or -A2 expressing melanoma patients received a total of four infusions of $5\times10^6$–$10^7$ DC loaded with MAGE-1, MAGE-3 (HLA-A1) or melan-A, gp100, tyrosinase (HLA-A2) peptides, respectively. Clinical side effects were mild and consisted of low-grade fever (WHO grade I–II) and some local inflammatory responses at the tumor site. To investigate the distribution and localization of infused DC, cells were labeled with $^{111}In$ or $^{99}Tc$. Results of in vivo tracing and immunologic responses will be presented (supported by SFB364).

# CHAPTER 13
# IMMUNOTHERAPY

# B43(anti-CD19)-Genistein Immunotherapy of B-Lineage ALL and NHL

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B43(anti-CD19)-genistein is a CD19 receptor directed protein tyrosine kinase inhibitor capable of inducing apoptosis in B-lineage leukemia and lymphoma cells. B43-genistein treatment resulted in 100% leukemia-free survival in SCID mice challenged with an otherwise invariably fatal and multidrug-resistant human leukemia (*Science* 267:886–891, 1995). B45-genistein was very well tolerated by cynomolgus monkeys at systemic exposure levels that were therapeutic against human leukemia and lymphoma in SCID mice (*Clin Cancer Res* 4:165–170, 1998). We are currently evaluating the safety of B43-genistein in patients with relapsed/refractory B-lineage acute lymphoblastic leukemia (ALL) or non-Hodgkin's lymphoma (NHL). Twenty-three patients have been treated with escalating doses of B43-genistein, with no dose-limiting toxicity. Objective responses have been seen in therapy-refractory ALL patients.

# Immunotoxins Containing Pokeweed Antiviral Protein for Immunotherapy in the Context of Bone Marrow Transplantation

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The CD19-directed immunotoxin B43-PAP is a promising new agent against B-lineage acute lymphoblastic leukemia (ALL). We have assessed the feasibility of using this agent in decreasing the pretransplant leukemic burden of pediatric patients undergoing allogeneic BMT. Seventeen high-risk remission ALL patients were treated with a 5-day B43-PAP pre-BMT window therapy (100 μg/kg/day × 5 consecutive days), before initiation of the pretransplant conditioning. This treatment was generally well tolerated with no dose-limiting toxicity. The probability of remaining in remission at 3 years was 74 ± 11%. Eleven of the 17 patients remain alive, free of leukemia at 1.8–4.5 years post-BMT, with a 3-year disease-free survival of 65 ± 12%.

# Targeting Immunotherapy for the Treatment of Leukemia and Solid Tumors

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## ABSTRACT

The prognosis for patients with leukemia and solid tumors remains poor in large part due to high relapse rates and significant toxicity from intensive combination chemotherapy. The development of more effective treatment strategies that do not depend entirely on the use of chemotherapeutic agents would be extremely useful. Recent advances in tumor immunology have provided the possibility of using antitumor immune responses in the treatment of patients. These advances are based on an increased understanding of T lymphocyte activation and tumor antigen expression. T lymphocytes, to become fully activated and functional, need at least two critical signals. The first is the recognition of foreign antigen through the T cell receptor, and the second is signaling through a costimulation receptor system. As many tumor cell types do not express costimulation receptors, it has been hypothesized that the lack of costimulation by the tumor cells may represent an important mechanism of escaping immune recognition and killing. To test this hypothesis for leukemia, murine models for acute myeloid leukemia (AML) have been used. Retroviral transfer and expression of the B7-1 (CD80) costimulation receptor has been shown to specifically stimulate the host immune system to develop effective antileukemic responses in these models. Further experiments have demonstrated that the antileukemic effect is primarily due to a $CD8^+$ cytolytic T cell response to one or more as-yet-unidentified MHC I restricted leukemia antigens. This response protects animals following subsequent exposure to B7 negative leukemia and is also able to cure animals with established leukemia in its early stages. Although promising, such viral-mediated approaches have several significant problems including safety, complicated implementation for the methodologies, and relatively low efficiencies of gene transfer and expression in cells derived from the human hematopoietic system such as leukemia. To this end, our laboratory has begun to develop alternative approaches designed to avoid these problems by targeting immunostimulatory protein conjugates to leukemia cells (as well as solid tumors) to generate specific antileukemic immune responses. This targeting approach uses the construction of chimeric proteins consisting of three different modular domains including *1*) a T cell costimulatory module, *2*) a linker or hinge module, and *3*) a

tumor or leukemia targeting module. Preliminary studies show that immunostimulatory conjugates can be produced, isolated, and transferred to both cell lines and primary leukemia and solid tumor cells. Functional studies in murine systems and using human leukemias are underway.

## INTRODUCTION

### Background

The acute leukemias, and specifically AML, are particularly good examples of the development of chemotherapeutic resistance and the consequences of increasing drug dose intensification.[1–4] Although induction chemotherapy is capable of effecting disease remission in 60–80% of patients with AML, long-term survival is achieved in only a minority.[5] There remains a great need for the development of alternative treatment approaches for patients with leukemia and other malignancies that are designed to *1*) circumvent drug resistance mechanisms, *2*) demonstrate specificity for cancer cells, and *3*) avoid toxic injury to normal tissues.

Several approaches have been designed to block or inhibit molecular pathways leading to chemotherapeutic resistance as demonstrated in the case of the P-glycoprotein drug transporter.[1–4,6–12] However, these studies have not proven to be less toxic and have shown that in most instances other drug resistance pathways are selected, thus reducing the regimen's ultimate effectiveness.[6–13] Other studies have used the ability of monoclonal antibodies (mAbs) or cytokines to target bacterial toxins or radiochemical conjugates to tumor cells.[14–18] Although such toxin conjugates have shown some encouraging results, they ultimately depend on the ability of the toxin to kill the malignant cells, which can develop toxin resistance or downregulate targeted surface molecules.[19–23] In addition, normal cells expressing the protein to which the conjugated toxin is targeted will also be exposed and potentially damaged by the toxin.[19–23] An alternative approach to eradicating tumor cells is to use the specificity of the immune system for unique immunostimulatory tumor antigens, thus providing a means to circumvent drug resistance mechanisms, avoid toxicity of either drugs or toxins from targeted conjugate molecules, eradicate minimal residual disease, and possibly maintain long term antitumor immunity.

### Enhancing antitumor immunity

Recent experiments have demonstrated that the modification of both murine and human tumor cells to express a wide variety of cytokines results in the rejection of genetically modified malignant cells as well as preexisting non–genetically

modified malignant cells.[24–28] The exact mechanism for the recruitment and expansion of these T cell subsets by different cytokines is still uncertain, although several critical observations concerning antigen recognition and T cell activation have provided some important clues.

The process by which T cells become activated by antigen presenting cells (APCs) depends on *1*) T cell receptor (TCR) recognition of antigen and signaling through the TCR (signal 1) and *2*) a costimulatory signal involving specific cell surface receptors on APCs and T cells (signal 2) followed by the induction of interleukin (IL)-2 and its receptor (IL-2R) by T cells, resulting in their proliferation and function.[29–31] A wide variety of surface molecules have been identified that are involved in the costimulatory second signal of the T cell response.[29–31] One particularly important costimulation pathway involves the B7 family of APC surface molecules including at least B711 (CD80) and B712 (CD86) and their T cell receptors, CD28 and CTLA-4.[29–32] Although CTLA-4 has a higher affinity for CD80 and CD86 than CD28, it is not a strictly redundant pathway, as it does not compensate for the lack of CD28-mediated costimulation in CD28 knockout/deficient mice.[33] Instead, engagement of the CTLA-4 receptor exerts a significant downregulatory effect on T cell activation both in vitro and in vivo.[34] Linsley et al. constructed an important reagent by fusing the extracellular domain of CTLA-4 and the human Ig Cγ1 chain.[35] This reagent has the ability to effectively and specifically block CD80/CD28 costimulatory signaling.[35]

Several recent studies have elegantly demonstrated the central importance of the B7 pathway in tumor-mediated costimulation.[30,34,36–41] Based on these observations, it has been hypothesized that tumor cells may escape immune recognition and killing by failing to satisfy the above requirements for T cell antigen recognition and activation.[29–32] For example, if antigen recognition occurs but tumor cells do not express a costimulatory molecule, then T cells may develop anergy or tolerance to the tumor cells. This situation has been experimentally observed in animal tumor models as well as in some human malignancies.[29–32] Thus, potential strategies for eliciting specific antitumor T cell responses have included attempts to express costimulatory surface receptors by gene transfer into tumors which otherwise do not express them, thereby supplying the critical costimulation signal for T cell activation. This second approach has been shown to work in several different animal tumor models, particularly ones involving melanomas, lymphomas, and murine sarcomas.[30,34,36–41]

Recent studies, including those from our own laboratory, have demonstrated a critical role for CD80-mediated costimulation in the generation of antileukemic immune responses.[42–44] This work has shown that retroviral transduction of B7-1 cDNA into unselected leukemia cells is able to induce a specific antileukemic response even against unmodified, preexisting leukemia. The antileukemic activity is primarily due to a $CD8^+$ cytolytic T lymphocyte (CTL) response to an as-yet-

unidentified MHC I–restricted leukemia antigen. Such studies have further demonstrated that the efficiency of this type of vaccine therapy is greatest when tumor or leukemic burden is lowest. In circumstances in which animals have very advanced leukemia, the costimulatory vaccine therapy rarely cures animals, although prolongation of life is commonly observed. Approaches that have been used and shown to improve the efficiency of this type of vaccine therapy include leukemic cytoreduction by a variety of treatments followed by vaccination or the combination of immunostimulatory cytokines with vaccination to augment antitumor immune responses.[24,45–47]

## IMMUNOSTIMULATORY TARGETING CONJUGATES

While the above studies have provided part of the experimental foundation underlying the importance of immune-mediated tumor cell recognition and eradication, there are several critical issues and problems that must be solved for such approaches to be rapidly and successfully extended to patients with leukemia. Several problems associated with the use of retroviruses for gene transfer include *1*) issues of safety, *2*) difficulty in preparation of virus producer cell lines with sufficiently high titers, *3*) decreased effectiveness at infecting hematopoietically derived human cells to produce significant levels of expression of a given gene product, and *4*) the lack of a straightforward way to target the expression to desired cell populations.[48–52] Our preliminary data using amphotropic packaging cell lines producing infectious retrovirus containing the human CD80 cDNA indicate that it may indeed be difficult to transduce sufficiently high numbers of human AML cells to produce significant levels of CD80 expression (unpublished data). This may in part be due to the relatively lower titers obtained for amphotropic compared with ecotropic producer cell lines, the decreased ability of human AML cells to proliferate in culture, which is a requirement for retroviral integration and expression, and the relatively low numbers of amphotropic virus receptors present on early hematopoietic stem cells.[48–53] The use of adenovirus, adeno-associated virus or other viral systems such as vaccinia or herpes viruses to transduce genes into hematopoietic derived cells suffer from some of these same disadvantages and, in addition, these viral systems often express immunogenic viral proteins that may obscure other specific responses being experimentally examined.[54–57] Mechanical methods have relatively low efficiency of gene transfer and still retain some of the issues of safety associated with transducing viral genomes.[58–64]

A possible solution to the problems associated with the transfer of genes using viruses as well as those noted above for toxin conjugates would be to develop nonviral, nontoxin targeting vehicles that would bind to leukemia cells but also provide important immunostimulatory signals to T lymphocytes. These costimulatory targeting conjugates would ideally be designed to take advantage of

leukemic cell surface molecules as well as providing T cell costimulatory signals to generate specific antileukemic responses. While the utility of such targeting molecules will likely be tested following ex vivo incorporation by tumor cells, it is possible that such conjugates could also be given directly to tumor-bearing animals or patients.

## MATERIALS AND METHODS

### Overview

Several nonviral approaches for the incorporation and expression of costimulatory receptors or other proteins into the membranes of tumor cells are possible (Fig. 1). While the ability to target such immunostimulatory molecules to tumor cells would appear to be the ultimate goal of such approaches, this may not always be necessary in that relatively pure populations of tumor cells are often available. In addition, in many cases, endogenous tumor antigens with unique specificity resulting from mutated genes or chimeric proteins arising from chromosomal translocations would be a prerequisite for the tumor cell being recognized by T

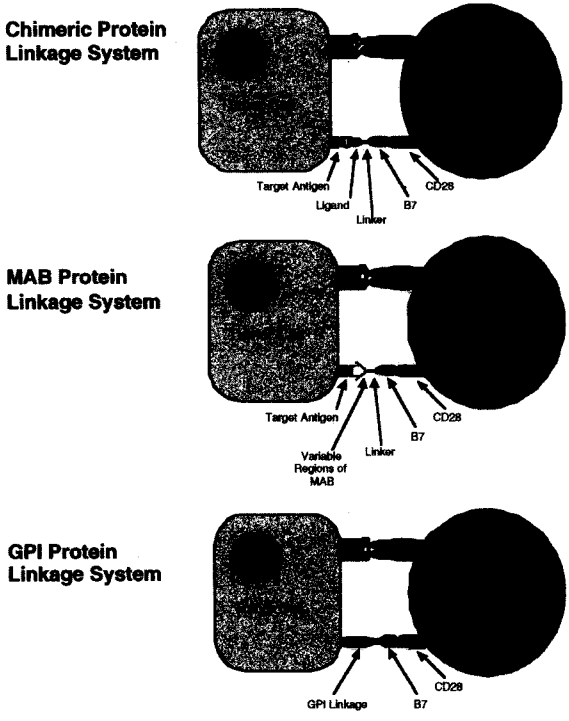



**Figure 1**

lymphocytes as foreign. Normal tissues or cells that did not express these abnormal antigens would not be expected to be recognized as targets just because they expressed a costimulatory receptor. Both approaches are currently being explored.

## Construction of target specific costimulatory conjugate cDNAs

The fundamental design of costimulatory targeting conjugates includes *1*) the T cell costimulatory counterreceptor, *2*) a peptide linker, and *3*) a protein sequence that will target conjugates to tumor cells. Much work has been done demonstrating that cDNA sequences encoding bacterial toxins can be effectively conjugated to a variety of cytokines.[65–69] These toxin/cytokine conjugates have also been shown to be able to bind and kill tumor cells expressing the receptors for the cytokines.[70,71]

Of particular relevance for AML is work using granulocyte-macrophage colony-stimulating factor (GM-CSF)/toxin conjugates to target leukemia cells.[70,71] These studies have shown that such conjugates are capable of targeting cells expressing the GM-CSF receptor, including AML cells. Because of the expression of GM-CSF receptors on nearly all forms of AML[72–75] as well as the successful production of chimeric GM-CSF/toxin molecules, we focused our initial studies on generating CD80/GM-CSF conjugates.

## Construction of costimulatory conjugates for generic use

The extracellular and transmembrane domains of the human CD80 cDNA were cloned by RT-PCR and linked to the cDNA sequence encoding the C-terminal third of the CD16 (Fc receptor) product which includes the GPI linkage signals.[76–79] Both the human CD80 cDNA and the CD16 region containing the Fc region and GPI signaling sequence were amplified from the Raji cell line.

Following the hybridization of the individual amplification products, the entire molecule was generated by PCR using oligonucleotide primers from the 5′ and 3′ ends. This product was then digested with *Hin*dIII and cloned into the retroviral vector LNCX. DH-5α bacteria were transformed, the chimeric retroviral vector was purified, and the insert was subsequently sequenced to ensure the absence of mutations.

## Expression systems

Recombinant viruses containing chimeric cDNAs encoding costimulatory receptors may be expressed in a variety of expression systems including bacterial, baculovirus, and mammalian cell lines. The first approach used was to transfect Cos cells (from green monkey kidney) to examine expression of chimeric molecules in transient cultures as well as in stable transfectants following selection

in G418. Thus, the LNCX-GPI-CD80 viral vector was transduced using lipofectin into Cos cells, which were subsequently selected for resistance to neomycin. Surviving colonies were cloned using sterile glass cylinders and expanded in culture. The expression of CD80 was determined by flow cytometric analysis using an anti-CD80 mAb (Becton Dickinson) or CTLA4Ig.[35]

### Expression and purification of costimulatory conjugates

Transfected Cos cells (Cos-hB71FC) were grown in 18× T-175 flasks. When confluent, the cells were removed by scraping and pelleted by centrifugation. The pelleted cells were lysed in 50 mM Tris-HCl, pH 8.0, 50 mM octyl-β glucoside, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin at 4°C, overnight. The lysate was clarified by ultracentrifuge at 93,000*g* at 4°C for 1 hour. The clarified lysate was passed through a protein G column (Pierce), crosslinked to anti-human B7 mAb using disuccinimidyl suberate (DSS). The column was washed with 40 mL of 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1% Triton X-100, then 20 mL 20 mM triethylamine, pH 10.5, plus 1% octyl-β-glucoside, followed by 10 mL of 50 mM Tris-HCl, pH 8.0, 1% octyl-β-glucoside. The protein was eluted using 50 mM glycine-HCl, pH 3.0, 200 mM NaCl, 1% octyl-β-glucoside as 1 mL fractions into tubes with 50 μL of 1 M Tris-HCl, pH 9.0. The appropriate fractions were pooled and then dialyzed against PBS for 24 hours with buffer changes every 8 hours.[80–83] The purified chimeric molecule was then tested directly.

### Testing incorporation of costimulatory conjugates into cell membranes

Either cell lines or de novo tumor cells were collected and washed three times with phosphate-buffered saline (PBS) and 1% fetal bovine serum (FBS). The cells were then resuspended in PBS and 1% FBS. Purified GPI-linked B71 and the target cells were incubated at 37°C on a rocking table at 5 rpm for 2–4 hours. The cells were then washed 3× with PBS and 1% FBS, then tested by flow cytometry for CD80 expression using an anti-human CD80 mAb (clone L307.4 from Becton Dickinson). This test was also repeated using cells that were irradiated with 32 cGy to determine how long the cells would continue to express CD80.

## RESULTS

### Target specific costimulatory conjugates

Based on published DNA sequence data and the work of Chan et al.,[70,71] we have generated murine CD80(mB7-1)/GM-CSF chimeric cDNAs. This was

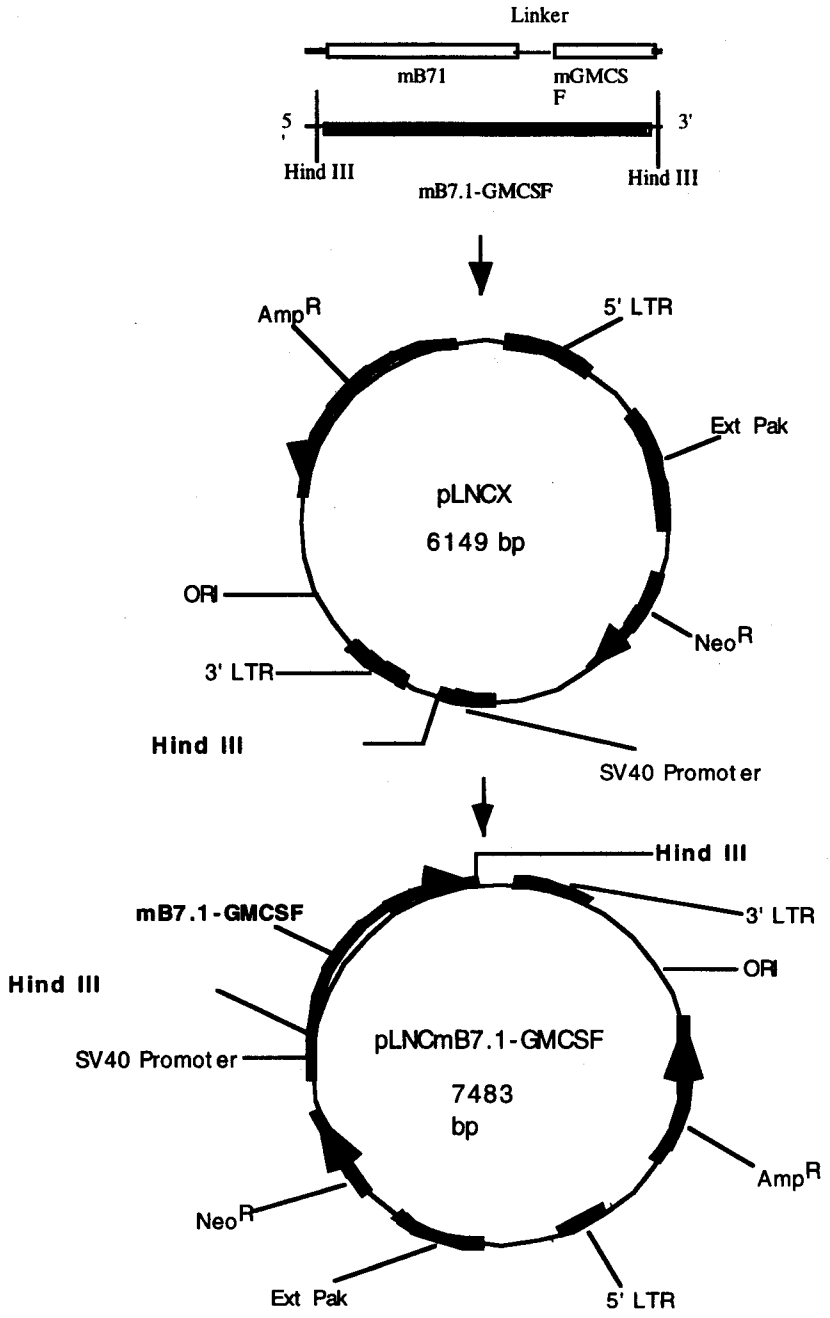
accomplished by first isolating murine CD80 cDNA from total RNA of lipopolysaccharide (LPS)-stimulated splenocytes by reverse transcription polymerase chain reaction (RT-PCR) amplification using oligonucleotide primers spanning either the entire open reading frame (ORF) of CD80 or the sequence just distal to the signal peptide (termed B7-1/MP for mature peptide). The 5′ primers were tailed with a *Hin*dIII restriction enzyme sequence while the 3′ primers were extended with an overhanging linker encoding the Gly4SerGly4SerGly4-SerGly4Ser amino acid sequence to serve as a hinge for the chimeric molecule. The murine GM-CSF cDNA was isolated from RNA of LPS-induced WEHI cells using a 5′ primer encoding both GM-CSF sequence and the extended nucleotide sequence coding for the overhanging linker noted above as well as a 3′ primer complementary to the 3′ end of the GM-CSF mRNA and extended with sequence encoding a *Hin*dIII restriction enzyme site.

The amplified CD80 and GM-CSF cDNAs with their overhanging linkers were gel-purified and allowed to anneal to form a single molecule followed by amplification using the murine CD80 5′ primers and the GM-CSF 3′ primers. The CD80/GM-CSF chimeric cDNA was then cloned into prokaryotic and eukaryotic expression vectors. A schematic representation of the strategy used to create this chimeric cDNA is shown in Fig. 2. Figure 3 shows the PCR amplicons for CD80 full-length and mature peptide, the secreted portion of GM-CSF, and the chimeric products after the reannealing PCR amplification of products.

After the cloning of this target specific costimulatory cDNA into the LNCX retrovirus, Cos cells were transfected and clones selected for neomycin resistance. While multiple clones were shown to express intact chimeric mRNA, it was not possible to demonstrate sufficient levels of chimeric protein either by using bioassays for GM-CSF activity or by Western blot analysis using antibodies directed to CD80. Subsequent experiments have now shown that this particular protein is unstable (possibly due to the linker used) and thus would not accumulate to levels allowing us to isolate sufficient amounts for biological studies. We therefore also began to explore an alternative approach.

## Costimulatory conjugates for generic use

Another approach for the transfer and expression of proteins on cell surfaces takes advantage of modifying proteins so that they can be directly incorporated into membranes. One such modification is the glycosylphosphatidylinositol (GPI) linkage, which anchors certain classes of proteins to cell membranes.[84,85] Proteins that are GPI-anchored are synthesized and then enzymatically processed such that a signal sequence (as yet not precisely defined) is recognized, resulting in the enzymatic addition of the GPI linkage to the protein. The GPI linkage then allows the protein to be inserted into the bilipid membrane of a cell and expressed on the


Linker
mB71
mGMCS F
5'
3'
Hind III
Hind III
mB7.1-GMCSF
Amp$^R$
5' LTR
Ext Pak
pLNCX
6149 bp
ORI
Neo$^R$
3' LTR
Hind III
SV40 Promoter
Hind III
mB7.1-GMCSF
3' LTR
ORI
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5' LTR

**Figure 2**

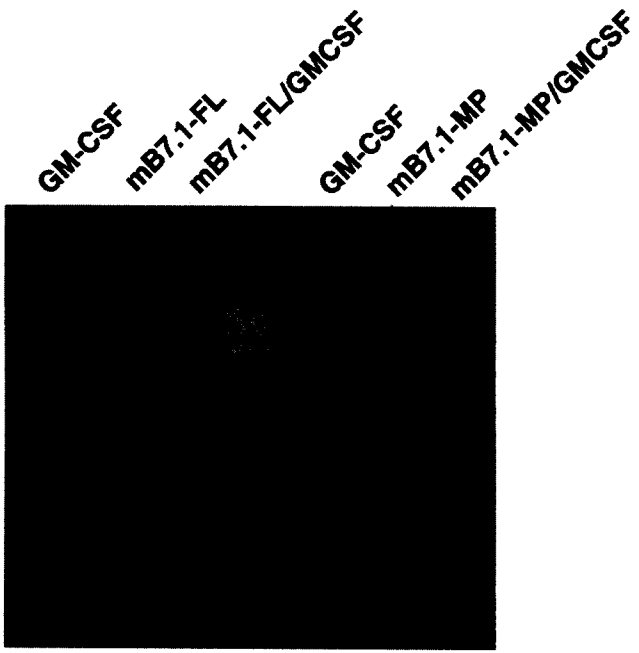


**Figure 3**

cell's surface. The possibility therefore exists for generating chimeric cDNAs composed of sequence encoding a desired protein joined to sequence encoding GPI linkage signals, thus providing the means to produce and purify GPI-linked receptors, ligands, or other signaling molecules.

To test this possibility, we cloned the cDNA sequence encoding the external domain of the human and murine CD80 receptor along with the cDNA sequence encoding the GPI linkage signals from the CD16 antigen as shown in Fig. 4.[77,79] The PCR products generated using this strategy are shown in Fig. 5. The chimeric cDNA was then cloned into the LNCX retrovirus and used to transduce Cos cells. The transduced Cos cells were selected for resistance to neomycin, and clones were isolated, expanded, and tested for surface expression of CD80. Several producer lines were isolated that expressed high levels of GPI-linked CD80 (Fig. 6).

Purification of the GPI-linked CD80 receptor was accomplished by affinity chromatography using either an anti-CD80 monoclonal antibody or CTLA4Ig directly coupled to Sepharose beads. Purified human GPI-CD80 was then tested for its ability to properly incorporate into membranes of various cell lines. Figure 7 shows the efficient incorporation of purified GPI-CD80 molecules into the membranes of a Ewing's sarcoma cell line our laboratory generated from a patient. Figure 8 shows the incorporation of human GPI-CD80 into the membranes of a human acute myelogenous leukemia cell line, MO7e. The incorporation of this

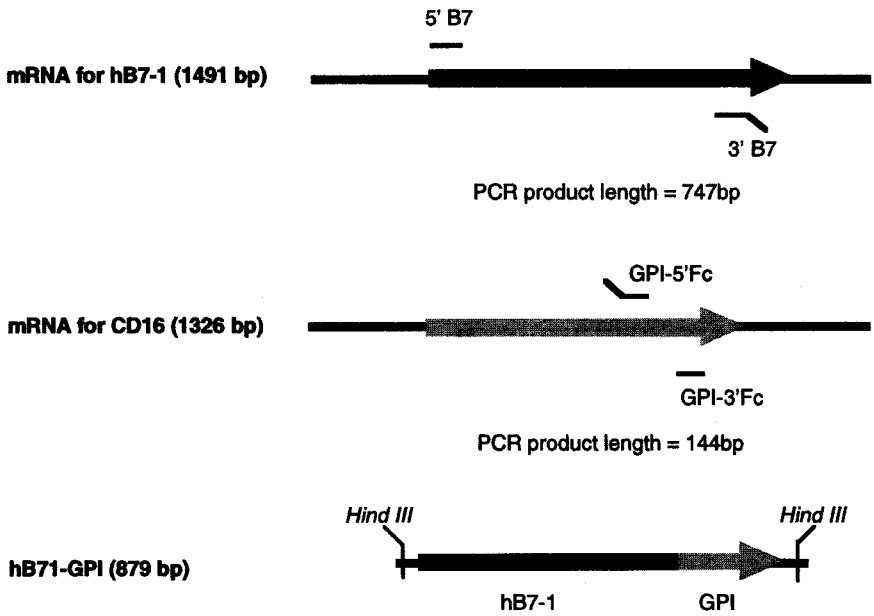


**Figure 4**

chimeric receptor molecule can also be incorporated into the membranes of de novo tumor and leukemia cells (Fig. 9). The functional costimulatory activity of GPI-linked CD80 has also been documented.[77,79]

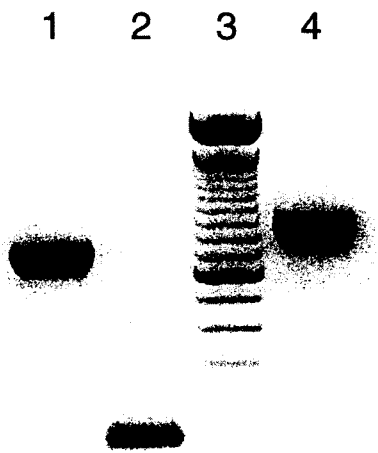


**Figure 5**

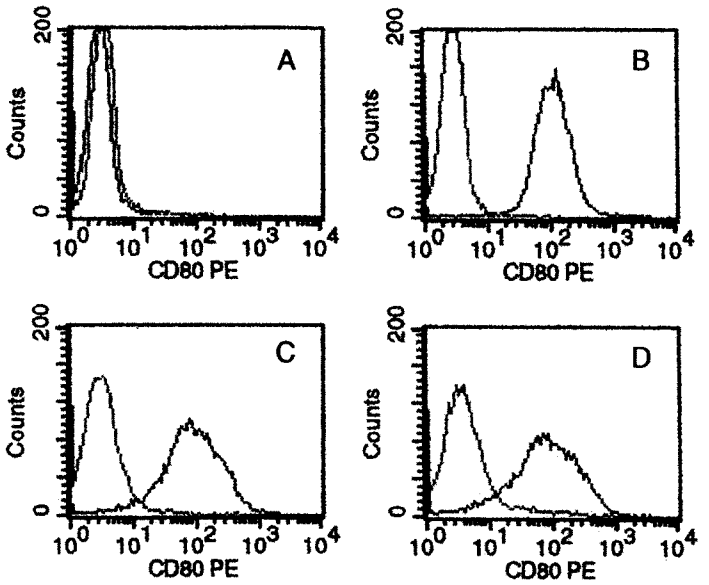


**Figure 6**

## DISCUSSION

While viral transduction of immunostimulatory molecules into tumor cells has demonstrated the utility of this approach in generating antitumor immune responses, viral approaches have several significant problems including safety, complicated methodologies, and relatively low efficiencies of gene transfer and expression in cells derived from the hematopoietic system such as leukemia. The approach of targeting immunostimulatory protein conjugates to tumor cells has several potential advantages over viral systems including *1*) the ability to target

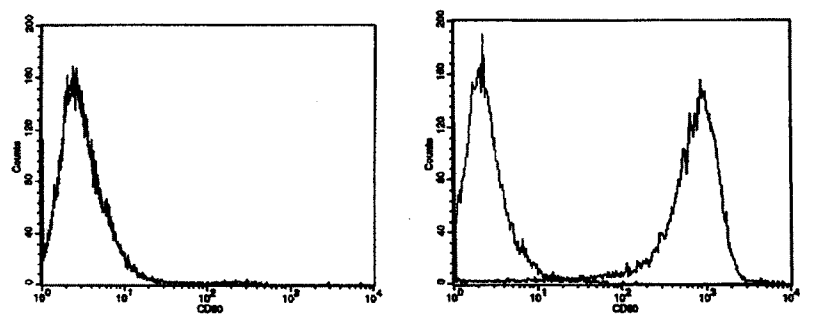

**Figure 7**

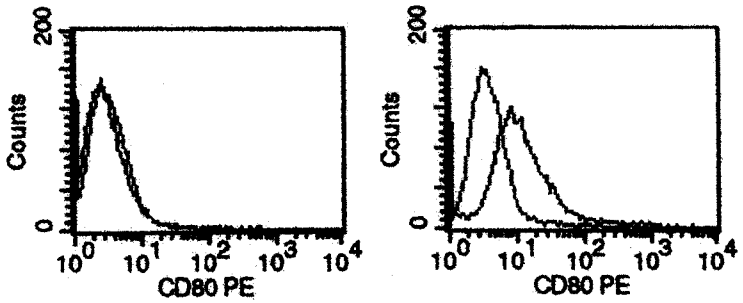


**Figure 8**

essentially any tumor surface molecule and lymphocyte receptor, *2*) the generation of synergistic antitumor immunostimulation effects from both the targeting moiety as well as the costimulatory component, *3*) the avoidance of the need to develop high-titer retroviral packaging cell lines, *4*) the ease of combining different immunostimulatory conjugates, and *5*) the absence of the requirement for viral integration and replication for expression of the desired product. Furthermore, tumor cell killing is not dependent on the chimeric targeting protein, such as in toxin-coupled conjugates. Instead, these costimulatory targeting conjugates are designed to activate tumor-specific T lymphocyte responses which, once activated, are able to effect collateral tumor cell killing as well as generate long-lasting antitumor immunity.

Several nonviral approaches for the incorporation and expression of costimulatory receptors or other proteins into the membranes of tumor cells are possible as described in this chapter. While the ability to target such immunostimulatory molecules to tumor cells would appear to be the ultimate goal of such approaches, this may not always be necessary in that relatively pure populations of tumor cells

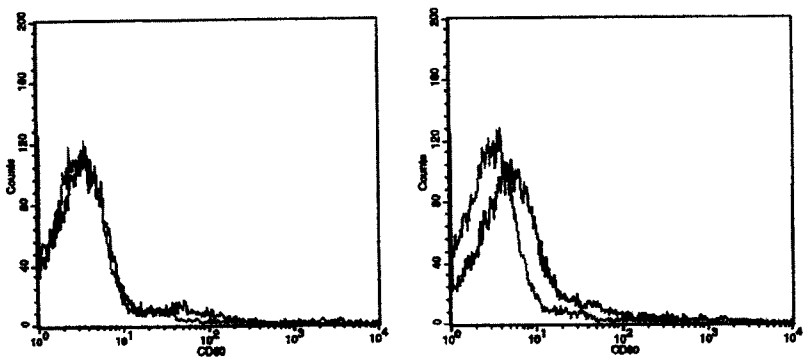

**Figure 9**

are often available. In addition, in many cases, endogenous tumor antigens, with unique specificity resulting from mutated genes or chimeric proteins arising from chromosomal translocations, would be a prerequisite for the tumor cell being recognized by T lymphocytes as foreign. Normal tissues or cells which did not express these abnormal antigens would not be expected to be recognized as targets just because they expressed a costimulatory receptor. As specific immunostimulatory tumor antigens are identified, the possibility of developing acellular, tumor vaccines by combining antigen preparations with targeted, immunostimulatory signals may prove possible.

Another critical aspect to the development of tumor vaccine approaches will be to determine when the most optimal time for vaccination should be in clinical settings. Clearly, the ability to mount a sufficient immune response is critical. This ability may be affected by the amount of tumor burden as well as the immunosuppressive effects of chemotherapeutic regimens, especially those involving conventional stem cell transplantation.

Alternative approaches to the circumvention of tumor cell resistance will likely include the enhancement of antitumor immunity through a variety of means as well as the exploitation of growth and apoptotic pathways. Although these latter approaches are in many cases still in the realm of the tissue culture dish or small animal models, they also represent part of the hope for more targeted and less toxic therapies for patients with cancer.

## ACKNOWLEDGMENTS

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# Adjuvant Immunotherapy in Conjunction With Autologous Stem Cell Transplantation

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## ABSTRACT

In the past few years, we have investigated the feasibility of inducing graft-vs.-leukemia (GVL)-like effects with cytokines given following autologous blood or marrow transplantation (autoBMT). AutoBMT for acute lymphoblastic leukemia (ALL) is associated with a high relapse rate. Our studies in an animal model of lymphoblastic leukemia/lymphoma (BCL1) suggest that immunomodulatory drugs such as alpha interferon (α-IFN) and recombinant interleukin 2 (rIL-2) may reduce the relapse rate when administered after syngeneic BMT during a state of minimal residual disease (MRD). A similar methodology was applied in recipients with acute lymphoblastic leukemia (ALL) and in patients with non-Hodgkin's lymphoma (NHL) and Hodgkin's disease (HD). In patients with NHL and HD, cytokine-mediated immunotherapy (CMI) consisted of concomitant administration of recombinant interleukin 2 (rIL-2) $6\times10^6$ IU/m$^2$/d $\times5$ days/week subcutaneously (SC) and α-IFN $3\times10^6$ U/day $\times5$ days/week SC with a total of 20 injections given over 3 months. A prospective randomized clinical trial is currently underway to confirm our encouraging pilot clinical trial in patients with NHL and HD with responding disease at risk to relapse following autoBMT. The trial has a potentially important change: the continued use of α-IFN for 6 months. In a parallel investigation, we are continuing to examine the use of α-IFN in recipients of autoBMT for ALL in an attempt to prevent disease recurrence. Follow up of high-risk patients with NHL, HD, and ALL, some treated more than 9 years ago, are most encouraging and suggest that relatively low-dose rIL-2 supplemented by α-IFN therapy, or even α-IFN alone in patients with ALL, may play an important role in maintaining remission in patients with minimal residual disease at high risk to relapse. The use of α-IFN as monoimmunotherapy in optimal doses should therefore be considered as an adjuvant treatment in high-risk ALL patients with minimal residual disease (rIL-2 may stimulate residual clonogenic tumor cells with IL-2 receptors and was therefore avoided). In NHL and HD, our long-term follow

up suggests, as published before, that the use of rIL-2 combined with α-IFN appears to be justified, although a formal study to compare the use of rIL-2 plus α-IFN with α-IFN alone is not yet available in humans. Taken together, immunotherapy with cytokines may represent a partial compensation mimicking GVL effects in conjunction with autoBMT.

## INTRODUCTION

AutoBMT is an acceptable treatment of choice for life-threatening disseminated malignancies that are chemoradiosensitive. The rate of relapse following autoBMT is >50% in patients with hematologic malignancies treated in first CR, but higher for more advanced stages of disease. High rates of relapse are also anticipated in patients with HD and NHL who have failed conventional front-line modalities or are refractory to primary treatment. AlloBMT provides a better probability of unmaintained long-term remission, frequently cure, in both acute as well as chronic leukemia and lymphoma and suggests the therapeutic efficacy of graft-vs.-leukemia/lymphoma (GVL) or graft-vs.-tumor (GVT) effects, which account for the advantage of alloBMT over autoBMT.[1–4] Since a genetically identical donor is not available for the majority of patients in need of high-dose chemoradiotherapy, autoBMT may represent not only the safest but most likely the only available approach for the majority of patients in need.

To reduce the incidence of relapse, our overall goal was to develop new approaches for induction of autoimmune-like GVL effects following autoBMT against minimal residual disease (MRD). The availability of an effective approach for controlling MRD with biotherapy rather than with more intensive myeloablative therapy is expected to reduce the life-threatening complications that would arise from further increases in the intensity of non-tumor-specific cytoreductive measures such as high-dose chemotherapy and radiation therapy included in an attempt to eradicate or control MRD. In view of the above, and based on successful studies in animal models of human ALL/NHL with the BCL1 murine B cell leukemia/lymphoma in cytokine-mediated immunotherapy (CMI) in murine leukemia,[5–7] we have introduced the use of CMI following autoBMT for reducing the rate of relapse.[8–11]

Based on these data and our current concepts on the treatment of disseminated malignancies, it seems reasonable to expect that the ultimate goal in the treatment of widespread disease will consist of maximal tumor eradication by conventional cytoreductive protocols, preferably autoBMT, toward accomplishing a state of MRD. Concomitantly, immunotherapeutic approaches will be used to contain MRD with the aim of controlling, rather than physically eradicating, the last tumor cell that may have escaped chemoradiotherapy.

## MATERIALS AND METHODS

### Conditioning for autoBMT

Conditioning regimen for patients with NHL and HD included thiotepa (40 mg/m$^2$ × 4 days), etoposide (200 mg/m$^2$ × 4 days), cytosar (200 mg/m$^2$ × 4 days), cyclophosphamide (60 mg/kg × 1 day), and melphalan (60 mg/m$^2$ × 2 days) (TECAM). Conditioning regimen for patients with ALL included cyclophosphamide and total-body irradiation or a combination of busulfan (without or with thiotepa) and cyclophosphamide.

### Details of the CMI protocol

Initially patients were treated as follows: rIL-2 $6\times10^6$ international units (IU)/m$^2$/d SC × 5 days/week combined with α-IFN $3\times10^6$ IU/d SC for 5 consecutive days. A total of 20 injections given over 4 weeks were followed by 4 weeks' rest. A second identical cycle was given over the following 4 weeks.[11]

More recently, we modified the protocol to prolong the period of α-IFN treatment. The current protocol consists of the following components that will be used for the prospective randomized trial: *1*) rIL-2 $6\times10^6$ IU/m$^2$/d SC × 5 days/week for a total of 5 injections followed by 2 weeks' rest; *2*) rIL-2 $6\times10^6$ IU/m$^2$/d SC combined with α-IFN $3\times10^6$ U/d × 5 days/week × 4 consecutive weeks (a total of 20 injections over 4 weeks) followed by 4 weeks' rest; *3*) α-IFN $3\times10^6$ U every other day, 3 injections/week SC for 6 months (a total of 72 injections).

### Onset of CMI after autoBMT

Patients with ML (Hodgkin's and NHL with responding disease) were eligible for the CMI protocol after autoBMT (regardless of the protocol used for conditioning) as soon as stabilization of peripheral blood counts was accomplished (while blood cells >$2.5\times10^9$/L and platelets >$75\times10^9$/L). Patients were treated with daily SC injections of rIL-2 (proleukin; Chiron) $6\times10^6$ IU/m$^2$/d, combined with α-IFN $3\times10^6$ IU (Roferon A; Hoffman LaRoche, Switzerland) $3\times10^6$ U/d, for 5 consecutive days each week for 4 weeks, followed by a month's break and then a second identical course.[11]

## RESULTS

The feasibility of inducting antitumor effects by CMI, consisting of a combination of rIL-2 and α-IFN, was already confirmed in a cohort of patients with malignant lymphoma at the stage of MRD following autoBMT.[11] Our first study

was a phase IIb clinical trial on 56 patients with MRD post autoBMT utilizing a combination of rIL-2 and α-IFN SC in an outpatient setting. Thirty-two patients had NHL and 24 patients had HD. Results were compared to 61 ML patients (NHL 36, HD 25, median age 35 years) who served as historical controls. Disease stage, sex, and age were statistically similar in the study group and the matched historical controls.[11] Fifty-six patients (38 men, 20 women), aged 10–53 years; 44 patients (79%) in the study group and 43 patients (70%) in the historical controls were stage III–IV at diagnosis, while 12 patients (21%) in the study group and 18 patients (30%) in the historical controls were stage I–II. Thirty-three patients (58%) in the study group and 37 patients (60%) in the historical control group had B symptoms. The frequency of NHL high-grade histology was 36% in the controls in comparison with 22% in the study group. Most of the patients in the study group and historical controls were transplanted in an advanced stage of disease, as 63% of the study group patients and 69% of the historical controls were transplanted after first relapse or more. There was no difference in the conditioning regimen between the immunotherapy-treated patients and the historical control group. The median time interval between autoBMT and CMI was fixed at 4 (2.5–10) months, in view of the need for adequate hematopoietic reconstitution before the initiation of cytokine administration.

The overall survival of patients with malignant lymphoma who received immunotherapy was significantly higher than that of patients who did not. Survival at 48 months was 90% (95% confidence interval [CI] 70–97) for the immunotherapy patients, and 46% (95% CI 30–60) for the historical controls ($P<0.01$). Similarly, the overall survival was significantly higher for the HD and NHL patients who received immunotherapy compared with the historical controls. The survival rates at 48 months were 100 and 80% (95% CI 43–95) vs. 57 (95% CI 312–75) and 42% (95% CI 24–58%), respectively ($P<0.02$). The disease-free survival (DFS) of all patients with NHL and HD who received immunotherapy was significantly higher than that of comparable patients in the historical control group who did not receive immunotherapy. The actuarial DFS at 48 months was 70 (95% CI 50–84) and 48% (95% CI 32–61), respectively ($P<0.01$). Similarly, the actuarial DFS was significantly higher for the NHL and HD patients after immunotherapy than for the historical controls. The actuarial DFS for NHL patients receiving immunotherapy at 48 months was 64 (95% CI 36–80) and 41% (95% CI 25–48) for patients who did not receive immunotherapy ($P<0.01$). The actuarial DFS for patients with HD receiving immunotherapy at 48 months was 88 (95% CI 50–96) and 60% (95% CI 34–78) for patients who did not receive immunotherapy ($P<0.042$).

The relapse rate was significantly lower for patients with malignant lymphoma who received CMI than for a similar cohort of patients belonging to the historical controls. Of the 56 patients who received immunotherapy, 11 (20%) relapsed (eight

NHL and three HD patients). Of the 61 patients who did not receive immunotherapy, 29 (46%) relapsed (21 NHL and eight HD patients) ($P<0.01$).

We are currently conducting a multicenter prospective randomized trial, investigating our newest rIL-2/α-IFN combination for intermediate and high-grade NHL and HD in an attempt to confirm the benefit of CMI in the setting of MRD. In this second ongoing study, we wanted to see whether modification of the immunotherapy schedule by reducing rIL-2 to 1 week ($3–6\times10^6$ IU/m$^2$/d), followed by combined rIL-2/α-IFN for 1 month and extending the α-IFN treatment period to 6 months ($3\times10^6$ U/d × 3/w) would improve efficacy of CMI. Thirty-eight patients (25 men, 13 women, median age 34, range 18–57 years) were enrolled in the new immunotherapy protocol. Twenty-four patients had NHL (nine high grade, nine intermediate, six low-grade) and 14 HD. Of the NHL patients, 13 were transplanted in remission (CR2 11, CR1 two), eight in partial remission and three with refractory disease. Of the HD patients, seven were transplanted in remission (CR2 six, CR1 one), five in partial remission, and two with refractory disease. The conditioning regimen was identical to the one described above (TECAM). The immunotherapy protocol was initiated as soon as stable engraftment was accomplished. Overall survival rate at 48 months was 83% (95% CI 62–93), while actuarial DFS was 65% (95% CI 42–76). For NHL patients, the figures were 76 and 61% for survival and DFS, respectively, while for HD patients the corresponding values were 91 and 71%, respectively. Toxicity was acceptable, and in no case was treatment interrupted because of toxicity.

As to the use of CMI in ALL, thus far we have treated only four patients with the combination of rIL-2 and α-IFN, of whom three are alive and disease-free from 2.5 to >8 years after autoBMT. To date, we have treated 11 patients with ALL with α-IFN alone post-autoBMT because of the concern that T cell receptor–positive leukemic cells may respond to rIL-2 administration. Eight of 11 patients are alive and disease-free 4 months to 6 years (median 3.5 years) after autoBMT.

## DISCUSSION

In clinical practice, as a rule, hematologic malignancies and solid tumors in humans are by and large nonimmunogenic; this explains why spontaneous antitumor responses cannot be anticipated and the high incidence of relapse from residual tumor cells escaping maximally tolerated doses of chemoradiotherapy. Recent clinical investigations based on most promising animal data suggest that immunotherapy mediated by cytokines may be successfully used after autoBMT.[6,7] Our previous clinical study suggested that similar results may be obtained in patients with both NHL and HD undergoing CMI following autoBMT.[11] As far as the use of CMI for patients with MRD, we have initiated a prospective randomized clinical trial after showing that the modified CMI protocol, as suggested by the data

presented, appears to be at least as good as the one used previously. In the present work, we have extended our observations to include patients with ALL treated mostly with α-IFN alone, but a few also with the full combination of rIL-2 and α-IFN. There are too few patients to determine which of the two protocols is better. Both are well tolerated and worthy of further study, preferably in a prospective randomized study. The potential advantages of cell therapy in conjunction with allogeneic blood or bone marrow transplantation for prevention of relapse by their GVL effects should be tested against the potential risk of procedure-related complications, including transplant-related toxicity and GVHD. Marrow aplasia or chronic GVHD may also represent a serious threat in the successful application of GVL.[12,13] For patients with no available matched donors, CMI may represent a reasonable replacement for GVL effects, whereas for patients with a matched sibling donor, the risk of GVHD needs to be considered against the better efficacy of tumor eradication by donor T cells. In contrast, there may be little risk to CMI after autoBMT, with the possible benefit of preventing relapse in up to 20% of the patients, if indeed the prospective study will confirm the published data.

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# Antitumor Immunotherapy in Autologous Transplantation

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In allogeneic bone marrow transplantation (alloBMT), malignant cell elimination is achieved not only through high-dose chemotherapy but also, of equal importance, by an allogeneic graft-vs.-tumor (GVT) effect. One way to decrease relapse rates after autologous BMT (autoBMT) would be to induce such immunologic GVT effects or at least components of it. An elegant way consists of the combination of an autoBMT performed first for disease reduction followed by a Mini or Immuno-Transplant from an HLA-matched sibling. Alternatively, only T cells from the donor may be infused after the autologous transplant. Such a strategy requires, however, an HLA-identical sibling donor to be available and will not be applicable for the majority of patients. Other strategies must be devised that take into consideration the likelihood that the patient's immune system has been dormant and tolerant of the cancer in the first place and has some "down time" until recovery after transplant. The immune defects listed in Table 1 are known to occur in cancer patients, and any strategy to induce GVT effects in autoBMT recipients will have to overcome those impediments. Immunotherapeutic strategies that are currently under study are summarized in Table 2.

Autologous graft-vs.-host disease (GVHD) can be induced with cyclosporine and augmented with interferon gamma and interleukin-2. Although about 50% of patients develop clinical GVHD, the tumor response, at least in breast cancer patients, has not been convincing.[1] On the other hand, some patients with advanced lymphoma may benefit from this treatment.[2] As with other immunologic treatments, randomized studies will have to show whether this particular therapy can prevent or delay relapse. This also applies to treatment with cytokine IL-2. Although occasional patients with advanced acute myeloid leukemia (AML) have responded to IL-2 treatment,[3] results in patients who have received IL-2 after autologous transplant are only borderline suggestive of a beneficial effect. Results from a randomized study from France in patients with AML and acute lymphocytic leukemia (ALL) seemed to confirm that there is no benefit of IL-2 postautograft.[4]

**Table 1.** Tumor cells escape recognition by T cells

| |
|---|
| Tumor cells: |
| • Deficient or ineffective HLA expression |
| • Lack of immunogenic peptides |
| • Defective antigen processing |
| • Lack of costimulatory molecules |
| • Production of inhibitory compounds |
| T cells: |
| • Lack of zeta-chain of T-cell receptor |
| • Defective signal transduction pathways |

However, the study is largely inconclusive as the protocol required the administration of a relatively high dose of IL-2 that caused premature discontinuation of the cytokine in the majority of patients.

Administration of interferon is problematic as it has side effects that can interfere with the recovery of the patient early after bone marrow transplant.[5] It may, however, have a clinical benefit particularly when combined with IL-2.[6]

Cellular treatments are promising but often cumbersome to prepare. Leukemia-specific T cells can be generated when antigen-presenting cells such as dendritic cells are used to generate specific T cells. Dendritic cells can be cultured from CD34 cells or monocytes from blood, bone marrow, or cord blood sources. These cells can be "spiked" with tumor antigens obtained by various methods (Table 3). These engineered dendritic cells can be reinjected into autologous recipients or used to expand T cells ex vivo. Clinical studies are underway using different techniques of spiking dendritic cells.

Since NK/LAK cells recognize tumor targets that lack or have mutated MHC antigens, they should theoretically be "broader" antitumor cells than T lymphocytes, which require intact MHC in addition to presentation of a specific antigen. The infusion of autologous LAK is tolerated early after transplant.[7,8] However, the process of obtaining and culturing LAK cells is cumbersome, and patients require IL-2 injections to maintain their activity in vivo.

**Table 2.** Antitumor immunotherapy in autologous transplantation

| |
|---|
| • Autologous GVHD |
| • Cytokines |
| • Ex vivo activation of autograft |
| • Monoclonal antibodies |
| • Immunization strategies |
| • Cellular therapies |

Table 3. Methods of providing dendritic cells with specific antigens

- Extracts from tumor cell lysates
  - acid elution of peptides
  - freeze/thaw purified tumor cell proteins
  - Heat shock protein (HSP)-peptide complexes
- Exposure to apoptotic cells
- Generation of dendritic cells from malignant CD34 cells
- Fusion or coculture of dendritic cells with tumor cells
- Transfection of dendritic cells with tumor cell RNA or cDNA of tumor associated antigens (TAA)

An LAK-like cell line has recently been cloned (NK-92).[9,10] It can readily be expanded ex vivo under serum-free conditions. On average, it is 20 times more cytolytic against tumor target cells than LAK cells, and it kills a broader range of tumor target cells due to the lack of KIR (kill cell inhibitory receptor), which inhibits the function of NK cells on contact with HLA on tumor targets. Studies in SCID mice performed at Memorial Sloan Kettering Cancer Center and in our laboratory have shown that mice inoculated with AML or melanoma have a significantly prolonged survival when treated with NK-92 cells.[11,12] Since the original NK-92 cells are IL-2-dependent, variants have been generated by transfecting the IL-2 gene. The IL-2-independent variants have been named NK-92ci and NK-92mi.[13]

Although some of the immunotherapies after autologous bone marrow transplant are promising, no single approach has yet demonstrated convincing benefit to generally recommend its integration into posttransplant management.

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# CHAPTER 14
# RADIOIMMUNOTHERAPY

# The Brambell Receptor (FcRB) and the Biological Half-Life of Clinical Antibodies

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The clinical utility and applications design of radioantibodies are influenced by an interplay between the physical half-life of the isotope and the biological half-life of the antibody. It has long been known that the catabolism of IgG is regulated by a protection receptor (FcRp) which makes it the longest-surviving of all plasma proteins. Recent discoveries have shown that FcRp is the same as the receptor involved in transport of IgG in the perinatal period from mother to young (FcRn). The FcRp and FcRn expressions of this molecule are generically termed the Brambell receptor (FcRB) in honor of the man who discovered and related both of these functions more than 30 years ago. The catabolic fates of IgG and its fragments are related to the expression of FcRB and the access of these components to this protection mechanism. This discussion is intended to foster a mechanistic approach to thinking about the metabolic fate and clinical potential of these molecules in their context as radiodiagnostics and radiotherapeutics.

During the 1950s and 1960s, Professor F.W. Rogers Brambell (Fig. 1) of the University of North Wales, Bangor, U.K., published an extensive series of articles that described a saturable transport receptor that mediates IgG transmission from mother to young. The receptor was shown to be expressed antenatally in yolk sac and placenta and neonatally in intestine for absorption from milk, depending on the species. He subsequently provided an analysis of IgG catabolism data which demonstrated an analogous saturable "protection" mechanism for catabolism in adult animals.[1,2]

Early studies on IgG showed a paradoxical finding: the fractional survival of IgG, in rodents and humans alike, was decreased when IgG concentrations were higher (Fig. 2); that is, for a specific IgG molecule, its likelihood of being catabolized was increased by the presence of other IgG molecules. A concentration-dependent catabolism mechanism (i.e., a catabolism receptor) would be expected to exhibit *less* fractional catabolism as it was saturated with higher concentrations of IgG, whereas there was no apparent upper bound on the amount of antibody that could be catabolized at maximal rates. This "maximal rate" corresponded to the catabolism of other non–renally filtered, nonprotected proteins, such as albumin,

**Figure 1.** *Francis William Rogers Brambell (1901–1970), Commander of the British Empire, Fellow of the Royal Society, Royal Medallist, Professor of Zoology, and Director of the Agricultural Research Council Unit of Embryology, University College of North Wales, Bangor, U.K. Photo courtesy of Dr. Michael Brambell.*

IgA, and IgM. Furthermore, it was shown that Fc of IgG, but no other classes, was both necessary and sufficient to mediate this activity. To resolve this paradox, Brambell et al. inferred that the only thing compatible with this finding was in fact a nonsaturable catabolic mechanism, and a saturable protection mechanism, in direct parallel to the antenatal and neonatal transport mechanism he and colleagues had previously established for IgG transmission. For protection from catabolism, he inferred a "protection receptor" on the walls of the vesicles of pinocytotically active cells that selectively rescued IgG from catabolism by lysosomes.[3]

The model we suggest to account for these findings is the isolation from the general pool in a special compartment or compartments of a part of the γ-globulin solution. The essential point is that, of the γ-globulin so isolated, only those molecules which attach to receptors are saved and returned to the circulation. It is suggested that isolation is effected by pinocytosis and that the receptors are intracellular in the walls of the vesicles.

As to the site of IgG catabolism, Brambell[4] averred "the catabolism of γ-globulin in the manner predicted could take place in any part of the body in which pinocytosis of plasma proteins occurs and that where this is not implicit in the hypothesis." Waldmann and Jones[5] later inferred that the vascular endothelium was most likely as the primary site of IgG catabolism, which is also the dominant tissue in the body for endocytosis. Furthermore, they discovered that Brambell's receptor mediates

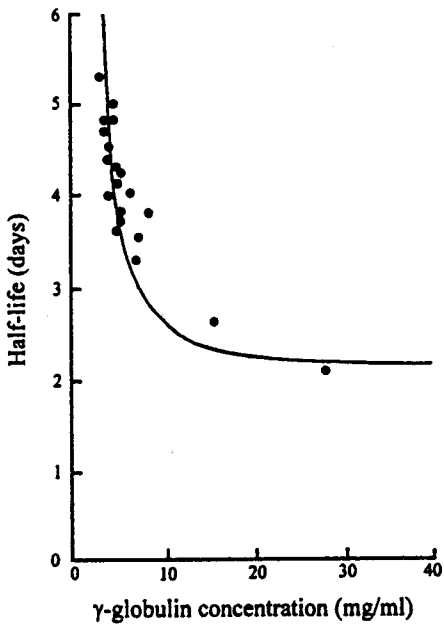


**Figure 2.** *Relation of half-life to serum concentration of γ-globulin in mice. Simulation (solid line) according to receptor model of Brambell with a=0.34, b=2.5. From Brambell et al.,[3] with permission.*

IgG binding by a pH-dependent mechanism: after nonspecific bulk endocytosis, IgG Fc binds to FcRB in the acidic environment (pH 6) of the endosome, the IgG attached to the receptor is directed away from the lysosomes via an alternate pathway, and then it is released in the physiologic neutrality (pH 7.4) of extracellular fluids after return to the cell surface. The overall process is summarized in Fig. 3, including the effect of IgG concentration on receptor saturation.

The receptor itself was purified first by Rodewald and Krahenbuhl[6] and later cloned by Simister and Mostov.[7] This cloned receptor was initially termed FcRn for neonatal rat intestine, the tissue from which cloning was first performed. It is notable that many animals, including humans, have little or no neonatal IgG transmission, with the major expression of this transport receptor occurring antenatally instead. Nevertheless, the term FcRn has been broadly applied to the transmission function of this receptor. It was subsequently confirmed that the same protein also mediates the IgG protection function (FcRp): animals knocked-out for the receptor showed accelerated IgG catabolism[8–10] (Fig. 4) as well as loss of perinatal transport.[11] In adult animals, the dominant expression is, in fact, as protection receptor (FcRp), making IgG the longest-surviving of all plasma proteins. Although an early report suggested that the FcRB might be involved in transport of IgG from blood to bile, its action in liver appears to be solely as FcRp, paralleling the basal endocytic

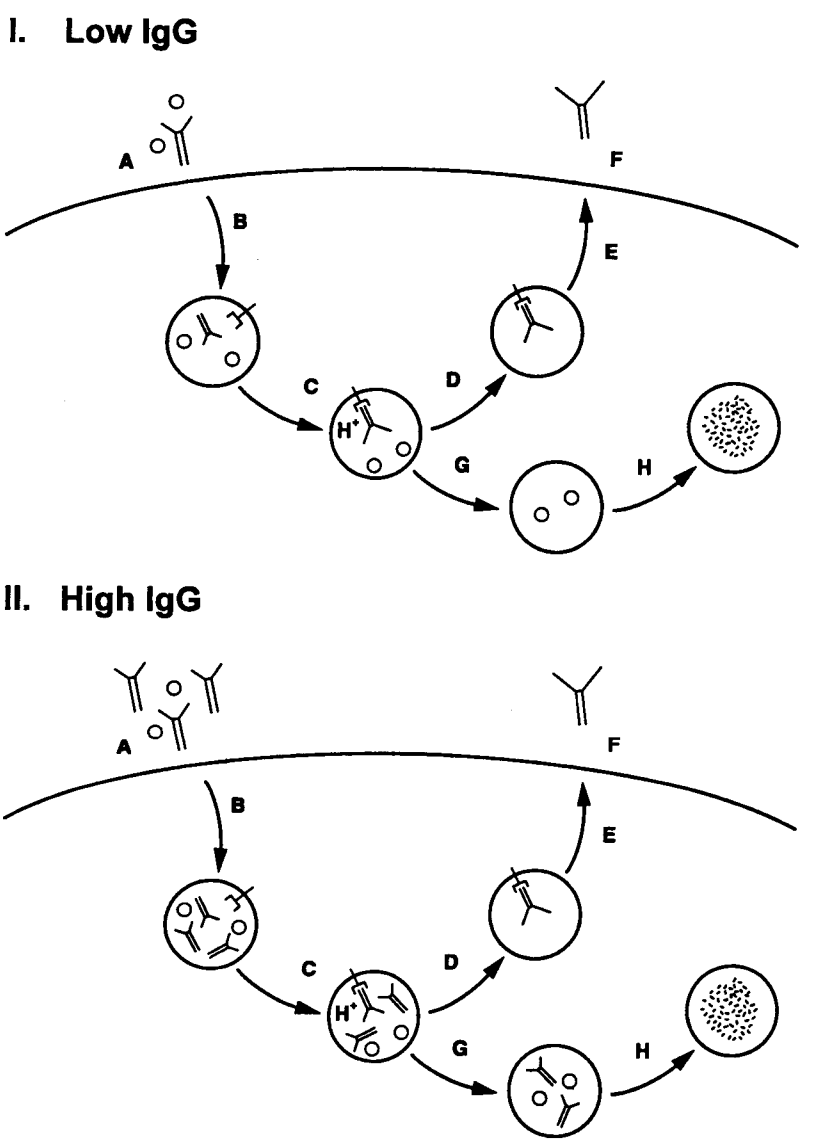


**Figure 3.** *Mechanism of γ-globulin protection from catabolism. IgG (Y) and plasma proteins (o) (A) are internalized into endosomes of endothelium (B), without prior binding. In the low pH ($H^+$) of the endosome (C), binding of IgG is promoted. D, E, F: IgG retained by receptor recycles to the cell surface and dissociates in the neutral pH of the extracellular fluid, returning to circulation. G, H: Unbound proteins are shunted to the lysosomes for degradation. With "Low IgG," receptor efficiently "rescues" IgG from catabolism. With "High IgG," receptor is saturated and excess IgG passes to catabolism for a net acceleration of IgG catabolism. All features excepting the role of pH in binding and endothelial localization were known to Brambell and inherent to his model.*[3]

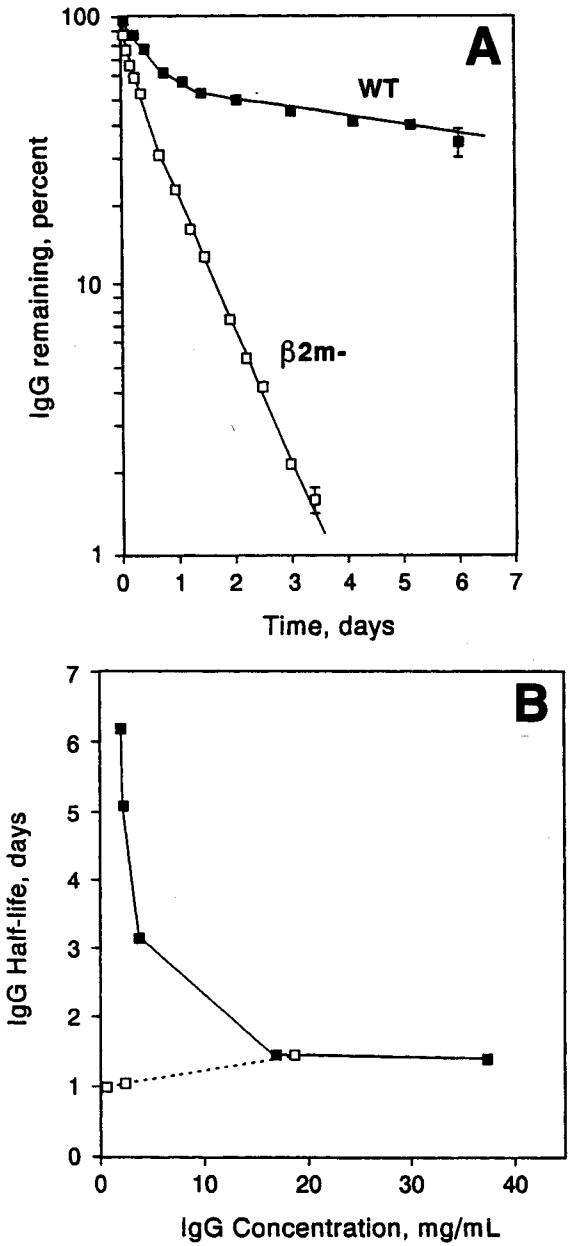


**Figure 4.** *The Brambell receptor protects IgG from catabolism. A: Mice knocked out for FcRB light chain β2m$^{-/-}$ show accelerated clearance of IgG, with the same resultant catabolic rate constant as albumin. B: Mice knocked out for FcRB lose the concentration-catabolism relationship inherent to FcRB function. Plasma IgG levels were manipulated by injection of exogenous IgG.* ■, *wildtype;* □, *mutant. From Junghans and Anderson,*[9] *with permission.*

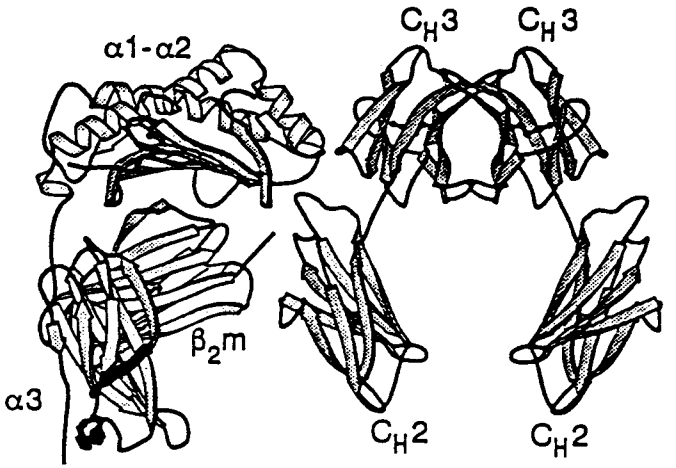


**Figure 5.** *X-ray solution of FcRB:Fc co-crystals. Lying-down configuration with 2:1 FcRB:Fc complexes are shown with a view looking parallel to the membrane. Contacts are observed between the α1-α2 domains and CH3-CH2 and between the β2m and CH2 of the first FcRB, with lesser contacts between the amino terminus of CH2 and the second FcRB in the receptor dimer. In this configuration, the complete IgG lies with Fab arms parallel to the plane of the membrane. The IgG interaction with monomer vs. dimer FcRB on or in cells is unresolved and remains a matter for investigation. Figure provided by Dr. P. Bjorkman.*

activity of that tissue (Telleman P, Junghans RP, manuscript submitted). To date, there is no evidence for an FcRn-like IgG transport activity in adult animals.

The Brambell receptor is composed of an MHC class I–related heavy chain and a $\beta_2$m light chain.[7] Crystal structures by Bjorkman and co-workers showed contacts of Fc with nongroove domains of the FcRB class I–related heavy chain and other contacts with the $\beta_2$m light chain (Fig. 5).[12] Fc histidines were involved in the contacts that explained the pH dependence of the binding, corroborated independently by Ward, Ghetie, and co-workers using molecular and pharmacokinetic techniques.[13] Extensive immuno-electron microscopy by Rodewald and co-workers showed that FcRB expression is predominantly in the endosomes and in the associated communication channels of the cell, with little or no surface expression, depending on the cell type.[1,14,15]

Confirming the earlier proposed localization of the protection receptor, the FcRB was shown to be most heavily expressed in the vascular endothelium by immunostaining and mRNA expression.[1,16,17] Given the enormous surface area of this "tissue" (1000 $m^2$), the ongoing pinocytotic activity sums to account for the major catabolism of plasma proteins that are not renally filtered. In mice from "clean" facilities with typically low IgG levels, the FcRp accounts for a near tenfold reduction in the catabolic rate of IgG relative to albumin, a nonprotected

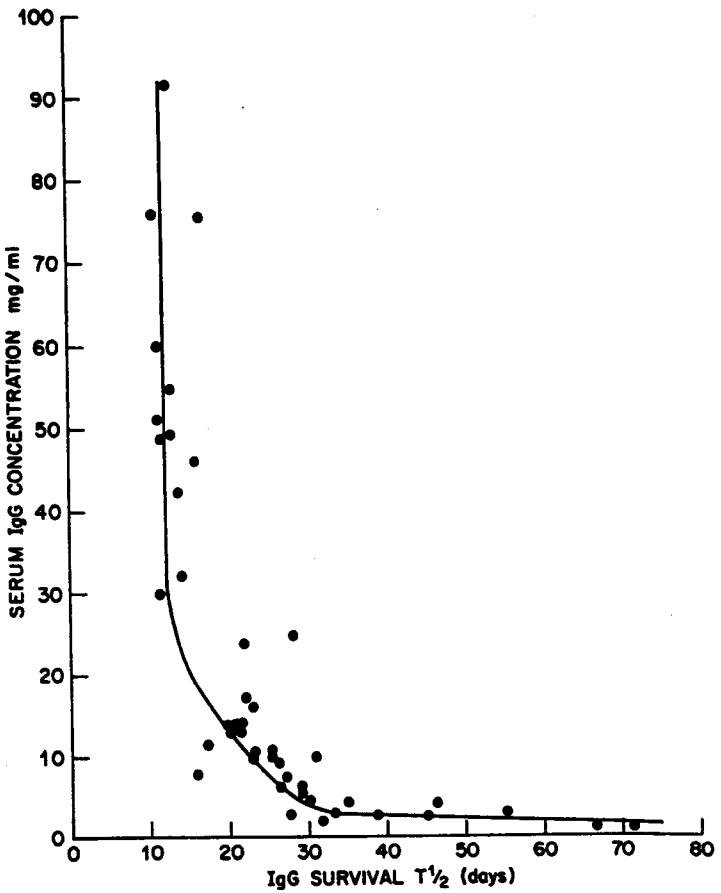


**Figure 6.** *The concentration-catabolism profile of IgG in humans. Similar to the profiles noted in mice (Fig. 2), the high IgG concentration correlates with high IgG catabolism and shortened survival in vivo. Note that the X- and Y-axes are switched relative to those in Fig. 2. From Waldmann and Strober,[20] with permission.*

protein, and correspondingly generates tenfold higher blood levels of IgG for the same number of IgG-producing plasma cells.[9] This has been interpreted to discount prior models of an "immunoregulatory feedback" mechanism to regulate IgG levels: all IgG synthesis is driven by the antigen stimulus and not affected by the blood levels of IgG per se.[18] Where IgG levels are higher, the impact of the FcRp on prolonging survival is progressively diminished until ultimately it is catabolized at the same rate as nonprotected proteins (Fig. 2).

Examining the profile of IgG catabolism in humans (Fig. 6), it is possible to infer a similarly important impact of FcRp on the long survival of IgG. At usual plasma IgG concentrations of 10 mg/mL, the average circulating half-life is 23

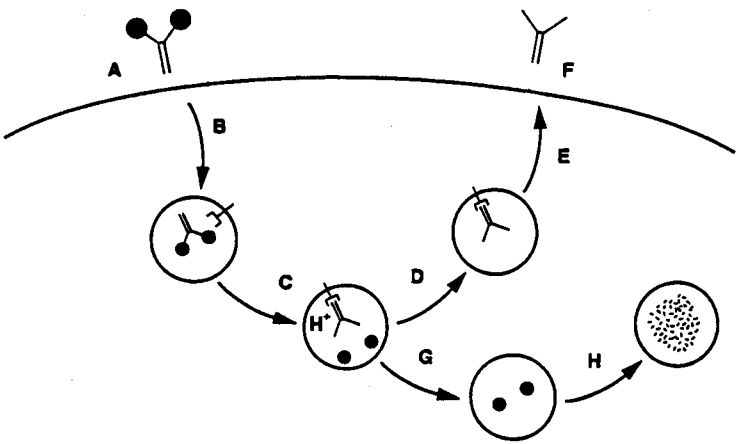


**Figure 7.** *The Brambell receptor is central to differential catabolism. Monomeric IgG plus antigen (A) internalizes into endosomes (B). Antigen dissociates from antibody in the low pH of the endosome (C), where binding of IgG to FcRp is promoted. D, E, F: Antibody retained by FcRp recycles to the surface, returning IgG to circulation, free of antigen. G, H: Released antigen is shunted to the lysosomes for degradation, yielding different net catabolic rates for antigen and antibody in the same complex.*

days.[19] This is near the elbow of the curve in Fig. 6. Accordingly, for patients with IgG deficiency, the $t_{1/2}$ would be expected to be substantially longer (>50 days); in patients with high IgG due to infection or IgG-producing myeloma, the $t_{1/2}$ would be expected to be much shorter, of the order of 10+ days.

Finally, a surprising feature was discovered in the metabolism of antigen-antibody complexes that directly pointed to the Brambell receptor and the peculiarities of its function. When a small, renally filtered protein antigen was bound to antibody in vivo, its survival was markedly prolonged, but the antigen-in-complex was nevertheless catabolized faster than the antibody in the same complex, termed "differential catabolism" (Fig. 6A).[1,9,20] This was interpreted as antigen dissociation in the acidic endosome, where Fc-FcRp binding is stabilized, with passage of antigen to lysosomal catabolism and return of antibody to circulation via the protection receptor. In this experiment, it was estimated that the IgG cycled through the endosomes eight times on average before undergoing catabolism and the antigen cycled through only three times before dissociating from the protected IgG in the acidic endosome environment and itself being catabolized. When the experiment was performed with mutant mice knocked-out for FcRp (Fig. 6B),[9] bound antigen was cleared at the same accelerated rate as (unprotected) antibody, demonstrating the central role of the FcRp in differential catabolism. In the absence of protection, all plasma proteins are catabolized following the first pass into the endosome.

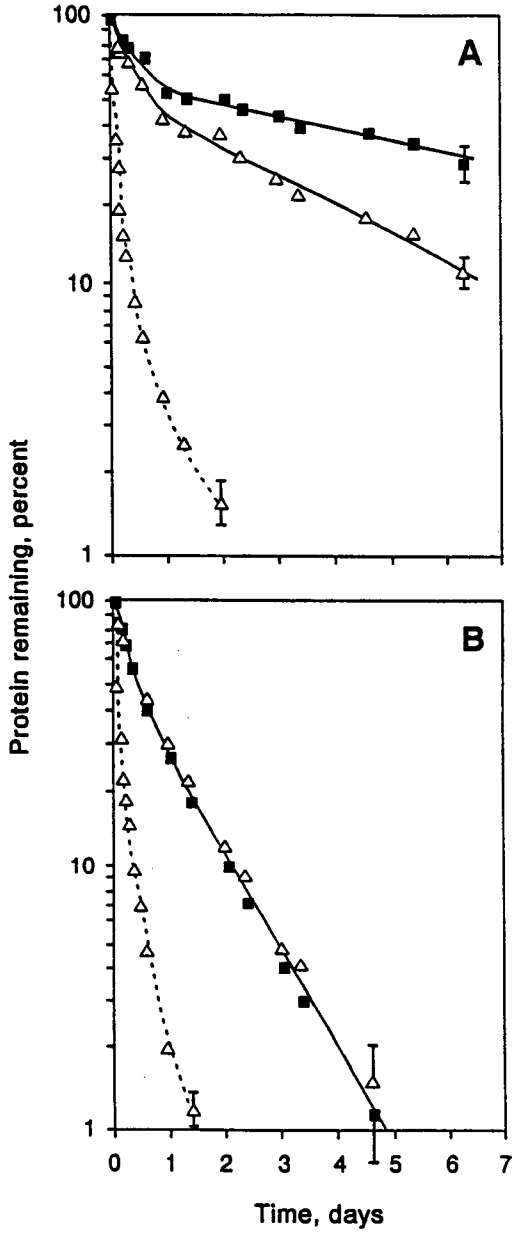


**Figure 8.** *Both panels show the survival in wildtype (A) or FcRB knock-out (B) mice of soluble Tac antigen in the presence of nonspecific (- -△- -) or specific (—△—) antibody. Also shown is the survival of specific antibody (anti-Tac, ■). When the receptor is deleted, the antigen and antibody pass together to lysosomal catabolism (upper). From Junghans and Anderson,[9] with permission.*

This differential catabolism mechanism suggests a means for "cleansing" antibody of bound antigen before return to circulation. This can be a factor in the setting of soluble antigen production in that the saturation of administered antibody by soluble antigen may be less than predicted if such cleansing were not a factor.[20,21]

With these facts at hand, several generalizations can be offered which are pertinent to radioimmunotherapies. To begin with, one considers whether the protein is small enough (e.g., ≤60 kDa) to be renally filtered. If so, this is the dominant means of catabolism. For example, in mice, Fab (50 kDa) is filtered with high efficiency, with a catabolic $t_{1/2}$ of about 1–2 hours and a survival $t_{1/2}$ of 4–6 hours.* Single-chain Fv molecules are still smaller and also rapidly filtered and catabolized. $Fab'_2$ molecules (100 kDa) have a somewhat slower catabolism, but it is not as slow as nonfiltered proteins, suggesting some disulfide reduction by serum thiols to yield two $Fab'_1$ molecules, that then will filter efficiently. For large molecules such as IgA and IgM, the catabolism is determined by the endocytic activity of the vascular endothelium, yielding half-lives of 1 day in mice and ~10–12 days in humans.[19] Complete IgG has the longest half-life, 5–7 days in mice and 23 days in humans, as mentioned above. It is noteworthy that the IgG is catabolized at the same site as other nonfiltered proteins, i.e., in the vascular endothelium, but its catabolism is delayed due to "protection" at those sites.

Curiously, the Fc fragment, although no larger than Fab, has a survival $t_{1/2}$ that is far longer than Fab in mice, and nearly as long (>50%) as that of complete IgG.[19] The Fc can interact with the FcRp protection mechanism while in plasma, but if this fragment is filtered as expected a priori based on size, this renal catabolism should dominate. Either there is some structural feature of the Fc that prevents renal filtration or the Fc is somehow rescued after filtration and returned to circulation. This paradox has never been resolved in the 30+ years since it was defined. Fc has been used as a carrier for many small proteins such as tumor necrosis factor (TNF), interleukin (IL)-2 and other growth factors to confer this advantage of long survivals. Apart from the still-undetermined issue of renal filtration, it is clear that the IgG Fc fragment itself as well as the Fc chimeric molecules in plasma are internalized by vascular endothelium and other tissues to endosomes and rescued by the FcRp while present in plasma.

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*The catabolic $t_{1/2}$ is based on the fraction of the antibody in blood plasma that is catabolized per day, whereas the so-called β or survival $t_{1/2}$ is delayed by the proportion of the antibody which is extravascular and thus not exposed to catabolism. The β rate constant is a complex composite of the catabolic rate constant and intercompartmental distribution rate constants without a fixed relation to the catabolic rate constant. For judging steady-state antibody levels of endogenously synthesized proteins or for judging mechanisms of catabolism, the catabolic $t_{1/2}$ is the more relevant quantity, whereas the β $t_{1/2}$ gives a better estimate of whole-body loss kinetics pertinent to exogenously administered proteins.[1,9,18–20]

The site on Fc for interaction with the FcRp is distinct from the sites for binding complement or hematopoietic FcR. Accordingly, it is possible to design Fc-containing molecules or to modify therapeutic IgG that will delete unwanted immune system interactions while preserving the long survival properties of IgG. For example, it has been hypothesized that such immune system interactions may have led to the excess deaths in one population of patients treated with TNF receptor-Fc chimeras, that such modifications might have helped to avoid.[22]

Aware of these features of IgG catabolism, one may consider finally the interplay between the biologic half-life, determined by the survival of antibody or antibody fragments, and the physical half-life, determined by the isotope decay, in estimating the efficacy and exposure to normal tissues. In general, when the $t_{1/2}$ of the antibody is less than that of the radioisotope (e.g., $^{90}$Y Fab), then the site of catabolism of the antibody will indicate the site of greatest potential exposure to the radioactivity. In general, the site of catabolism is either the kidney for filtered antibodies, or diffusely in the body (as vascular endothelium) for intact, nonfiltered antibody. In practice, there are few situations in which the therapeutic isotope has a half-life that exceeds that of complete immunoglobulins in humans, whether it be IgG, IgA, or IgM, which have minimum 5-day half-lives. On the other hand, where antibody catabolism is slow relative to isotope decay (e.g., $^{90}$Y IgG), the exposure to the kidney is less and whole-body toxicities, typically marrow, are what are dose-limiting. (The presence of murine glycosylation patterns on intact mouse IgG has been thought to divert these antibodies at least in part to the liver, but this is considered less of a factor in the evolving predominance of human antibodies.) The damage to normal tissue is mitigated where the isotope itself is excreted from the body after antibody catabolism with a time constant substantially more rapid than the isotope decay (e.g., $^{131}$I), and conversely the damage to the organ is enhanced where the isotope is efficiently retained in the catabolic tissue until decay (e.g., $^{212}$Pb). Some isotopes when liberated are chemically disposed to particular tissues for uptake, such as iodine to thyroid and yttrium to bone. However, strategies such as chemical blockade with KI and better chelators have greatly ameliorated these tendencies. In terms of targeting, it is important that the balance of biologic and physical half-lives are compatible with the objectives of tissue penetration, tumor concentration, and adequate dose delivery compatible with safety. While these kinetic features were previously recognized, the new understanding of the mechanisms of catabolism of IgG and its fragments will ultimately contribute to the rational design of antibody-based radioimmunotherapies.

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# Selection of Radioisotopes, Chelates and Immunoconjugates for Radioimmunotherapy

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Interest in radioimmunotherapy (RIT) has increased following positive clinical results in hematologic malignancies. This presents an opportunity to reexamine the optimal selection of radioisotopes, chelates, and immunoconjugates for clinical RIT.

*Radiometals.* The pure beta emitter, yttrium-90, is replacing iodine-131 for the treatment of measurable disease. Yttrium-90 can be administered on an outpatient basis and is readily available carrier-free at high specific activity. Lutetium-177 has less energetic beta emissions that could be exploited for the treatment of microscopic disease. Both radioisotopes can be attached to proteins through bifunctional chelates.

*Chelates.* Two types of chelates have been developed: backbone substituted DTPA derivatives and macrocyclics. The first rapidly complex trivalent radiometals, at high yield and with little interference from contaminating metal ions. In contrast, the macrocyclics complex radiometals more slowly, at reduced yields, and are sensitive to trace metal contamination. However, macrocyclic/radiometal complexes have higher in vivo stability. Chelates can be immunogenic, but their immunogenicity is dependent on that of the administered antibody.

*Immunoconjugates.* The biologic half-life of an administered antibody reflects the half-life of the antibody class in the species of origin. In general, the half-life increases with species size. Smaller-sized radioimmunoconjugates: scFv, dimeric Fv, Fab′ and $F(ab')_2$ target tumor faster and more homogeneously than IgG but also have lower tumor uptake due to their shorter blood half-lives. Smaller radioimmunoconjugates will only be valuable for therapy if their tumor uptake and retention can be increased. Tumor retention is positively correlated to the number of antigen binding sites. Dimeric Fv and $F(ab')_2$ demonstrate longer tumor retention than scFv and Fab′. The intracompartmental (i.c.) and intralesional (i.l.) administration of radioimmunoconjugates bypasses the delivery problem posed by the endothelial barrier. Both i.c. and i.l. administration can provide higher tumor uptake (>80% injected dose per gram of tumor). Tumor retention of i.c. and i.l. administered radioimmunoconjugates is based on size and the number of antigen binding sites, with IgM having higher retention than IgG.

*Conclusion.* Higher therapeutic ratios for clinical RIT will be possible through judicious selection and preclinical testing of radioimmunoconjugate components.

# Approaches to the Radiation Dosimetry of Red Marrow From Internal Radionuclides

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## ABSTRACT

Red marrow is the target tissue for certain radionuclide treatments (such as ablation therapy), and marrow is often the dose-limiting normal tissue in radioimmunotherapy of cancer. Radiation dosimetry is an important part of correlating the biologic response of red marrow to energy imparted by ionizing radiation. Dosimetry is also an essential part of treatment planning. Methods for determining the radiation absorbed dose to body organs from internally deposited radionuclides, such as those recommended by the Medical Internal Radiation Dose (MIRD) Committee of the Society of Nuclear Medicine, are well established. However, special problems are encountered in the application of MIRD techniques to the dosimetry of red marrow, and therefore marrow dose estimates can be highly uncertain. Dose calculations rely on physical models and activity measurements. Marrow dosimetry is difficult because *1*) the geometry of red marrow structure with respect to bony spicules and to blood supply is difficult to describe and model and *2*) the amount of radionuclide in marrow is difficult to measure and quantify. The complexity of marrow dose determinations has increased with the advent of treatment techniques that influence activity distributions at the cellular level. Although various investigators have proposed different approaches for characterizing marrow dose, many questions remain and standardized techniques have not been implemented in clinical trials. This chapter reviews many of the models, parameters, assumptions, and limitations of red marrow dosimetry. The relevance of absorbed dose to marrow toxicity is discussed, and opportunities for improving marrow dosimetry are described.

## INTRODUCTION

The radiation absorbed dose is defined as the energy imparted by ionizing radiation per unit mass in which it is absorbed. Absorbed dose is a useful quantity for prospective treatment planning in radiation therapy and for retrospective assessment of the biological effectiveness of radiation in both cancerous and normal tissues. Radiation doses to internal organs are estimated by calculation

using data from direct measurements. Direct measurements and calculations are subject to many uncertainties. Even so, a best estimate of absorbed dose to internal organs is of value to procedures that use radiopharmaceuticals for diagnosis and treatment of disease.

Several different methods are used to calculate the marrow absorbed, and the results of a dose assessment may vary considerably among investigators. This chapter reviews the most common approaches to marrow dosimetry, as well as many of the uncertainties involved.

## Why is red marrow dosimetry important?

Dosimetry is useful for treatment planning and evaluating dose-response correlations after treatment. From a radiobiologic perspective, marrow is one of the most sensitive tissues of the body to the effects of radiation. A large fraction of mitotically active stem cells may be killed by radiation doses of 300 to 700 cGy (rads), and the subsequent production of mature white blood cells, platelets, and red blood cells may be greatly reduced if their precursor cells are destroyed. The fraction of stem cells damaged by radiation is proportional to the marrow absorbed dose, and thus marrow is usually the dose-limiting normal tissue in therapies such as radioimmunotherapy of cancer. Marrow may also be the target tissue for certain radionuclide treatments, such as ablative therapy before transplantation,[1–2] and for therapy of acute and chronic leukemia.[3] Thus, a best estimate of marrow dose helps the physician to understand how much activity should be administered to achieve a desired therapeutic outcome without excessive toxicity.

## Why is red marrow dosimetry difficult?

Marrow is the most difficult tissue in the human body for dose assessment. Radiation physics calculations require well-defined geometric information, but the geometry of red marrow structure with respect to bony spicules in trabecular bone is difficult to describe and model. The mixture of soft tissue, blood, and bone varies from one bone type to another and with a given bone, depending on the ratio of trabecular to cortical bone. Radiation physics calculations also require information about the amount of activity present at any point in time, which may be difficult to measure. The cumulative absorbed dose from high levels of administered activity may alter the cellularity of marrow, and thus may alter the relative composition of cell types in marrow spaces. Each of these factors complicates the assessment of marrow dose.

Marrow is irradiated by activity in circulating blood, activity that resides in marrow itself, and activity in neighboring organs and tissues. Radioactivity administered to a patient is usually given intravenously, and thus the early contri-

bution to marrow dose is mostly from activity in circulating blood. The dose rate to marrow is greatest at early times postinjection, and the dose rate decreases as activity clears from the blood. Specific uptake and retention of activity in marrow tissue may increase and prolong this irradiation. The amount of activity on bone surfaces and in other nearby organs, as well as the total-body activity over time, must also be determined so that the total contribution to dose from penetrating gamma radiation may be assessed. The dose assessment method usually depends on the amount and type of information that can be obtained to describe the time-activity curves for marrow and other dose-contributing organs.

## METHODS

Three common methods are used to calculate marrow dose. The method of choice will depend on the purpose of the clinical study, the ability to obtain reliable measurement data, the relative need for data from highly invasive measurements, and the level of activity administered to patients.

### Dosimetry parameters

The radiation dose to internal organs is a function of

- the total activity administered to a patient,
- the fraction of the administered activity taken up by each major source organ or tissue,
- the retention of activity in organs over time, and the total number of radioactive decays,
- the energies, emissions, and physical half-life of the radionuclide (and decay products, if any),
- the spatial distribution of activity within tissues,
- the size and weight of the patient,
- the geometry and density of tissues absorbing radiation, and
- the cross-organ irradiation component for penetrating gamma radiation.

Each of these elements enters into a dose calculation. Simplified methods and computer software have been developed to facilitate the calculation.

### MIRD schema

A formal system for internal dosimetry was developed by the Medical Internal Radiation Dose (MIRD) Committee of the Society of Nuclear Medicine.[4–5] According to this schema, the absorbed dose to an organ or tissue such as red marrow is the sum of contributions from several different components. Radiations from internally deposited activity were classified as either *penetrating* (gamma

rays with energies ≥10 keV) or *nonpenetrating* (beta particles, Auger electrons, and gamma rays <10 keV in energy). The amount of energy deposited by penetrating radiation originating from activity in one organ that irradiates a neighboring organ or tissue was calculated by Monte Carlo photon transport codes using standard anthropomorphic models of the human body. The MIRD formalism assumes that all energy from nonpenetrating radiation is absorbed in the source organ and that there is no energy imparted by cross-organ irradiation by nonpenetrating radiation. Thus, the major contributions to marrow dose are from *1*) the nonpenetrating beta radiation from activity resident in the marrow itself, *2*) the fraction of the penetrating gamma radiation from activity resident in the marrow that is absorbed in the marrow, and *3*) the fraction of penetrating gamma radiation from activity resident in surrounding tissues that irradiates the marrow. Radioactivity must therefore be measured, at various times, in the marrow itself, in the major source organs surrounding the marrow (usually the liver, kidneys, lungs, spleen, gastrointestinal tract), and in the body as a whole to determine marrow dose. All radioactivity administered to the patient must be accounted for.

The amounts of activity present in marrow or in neighboring source organs are determined by direct photon counting of the gamma activity levels present or beta counting of tissue or fluid specimens. The amount of gamma radiation emitted by radionuclides is highly variable. Since yttrium-90 is a pure beta emitter, a gamma-emitting label (such as indium-111) must be added to measure and track the biodistribution of yttrium-90. In contrast, the amount of gamma radiation emitted by iodine-131 is high, and count rates from therapy levels of iodine-131 are usually too high for patient imaging and activity measurement. Therefore, organ uptake and retention data are usually determined from a test administration of antibody labeled with low-level amounts of radionuclide. Trace-labeled antibody is administered to the patient, and quantitative planar imaging is performed at selected times postinjection to obtain biodistribution data and time-activity curves for major organs and red marrow. The activity in marrow may also be inferred by measuring the activity in circulating blood or by counting the activity measured directly in a part of the total marrow, such as the sacrum or biopsy specimens.

Radiation doses to internal organs are usually expressed in terms of absorbed dose per unit administered activity. According to the MIRD schema,[4] the mean absorbed dose, $D$, to an organ or tissue is the product of the cumulated activity (the integral of a time-activity curve in a "source organ") and the $S$ value (a physics constant for a given radionuclide and source-target organ pair):

$$D_{(k \leftarrow h)} = \tilde{A}\, S_{(k \leftarrow h)}, \tag{1}$$

where $D_{(k \leftarrow h)}$ is the average organ dose (Gy or rads), $\tilde{A}$ is the cumulated activity (in units of Bq/second, or μCi/hour), and $S_{(k \leftarrow h)}$ is the mean absorbed dose to target

organ $k$ per unit cumulated activity in source organ $h$ (in units of Gy/Bq/second, or rads/μCi/hour). The cumulated activity, $\tilde{A}$, is obtained by measuring radionuclide uptake and retention in regions of interest at various time points. The $S$ value is dependent on the types and energies of all radiations emitted by the radionuclide, the geometry (size and shape) of the source and target regions (and the distance between them), and the elemental composition and density of body tissues. Values of $S_{(k \leftarrow h)}$ have been tabulated by the MIRD Committee.[5] Thus, it may only be necessary to determine the cumulated activity, $\tilde{A}_h$, for each source organ of importance in an individual patient.

Equation 1 is the fundamental equation in medical internal dosimetry. The source organ is any organ or tissue for which one is able to obtain quantitative information from direct or indirect methods on the amount of activity present over time, and the target organ is any organ or tissue for which an estimate of the absorbed dose is needed. The administered activity is represented by the symbol $A_0$ (with units of Bq or μCi). The total dose to a target organ is the sum of all the individual contributions from radioactivity in each source organ and in all remaining tissues.

Another quantity of interest is the source-organ *residence time*, $\tau$, which is the integral of the time-activity curve in a source organ in terms of fraction of administered activity. Thus, $\tau = \tilde{A}/A_0$. The mean dose to a target tissue per unit administered activity is therefore given by $D/A_0 = \tau S$. The *residence time* should not be confused with the concept of retention half-time for a radionuclide in an organ.

## Marrow dosimetry methods

Three different approaches have been proposed[6] for estimating radiation absorbed dose to marrow: *1*) the remainder-body method, *2*) the blood serum-activity method, and *3*) the direct-assessment method.

*Remainder-body method.* The assumption behind this method is that the radioactivity in marrow is not known or cannot be determined by direct measurements. Measurements are, however, made of activity in other major organs and in the whole body. The activity in red marrow is therefore assumed to be equal, per unit mass, to that of all other remainder tissues, such as muscle, fat, skin, and skeleton. The remainder activity is determined by subtracting the activity of the major organs from the whole-body activity. The dose to red marrow consists of the beta contribution from activity in the marrow plus the penetrating gamma contribution from activity in the measured source organs.

*Serum-activity method.* The assumption behind this method is that radioactivity is present in marrow, but that this activity does not specifically target marrow. The

activity in marrow is inferred by measuring the activity in circulating blood at various times after administering a radiolabeled compound and correcting for red cell hematocrit. Siegel et al.[6] recommended *1*) that the marrow specific activity is 0.2 to 0.4 times that of the blood, *2*) that the clearance of activity from circulating blood is equal to the clearance of activity from red marrow, and *3*) that the distribution of activity in marrow is uniform. A time-activity curve is constructed and a residence time for marrow is determined from the integral of the area under the time-activity curve. Marrow becomes a separate tissue from the remainder body tissue. The dose to marrow is then calculated using the conventional MIRD approach.

*Direct-assessment method.* The assumption behind this method is that activity in marrow can be measured and quantified. This method is preferred because it provides the most accurate dose assessment. Marrow activity may be determined by direct counting of activity in marrow of a vertebral body or the sacrum,[7] or by counting a marrow biopsy specimen.[8] These samples are assumed to contain a known fraction of total-body red marrow. Figure 1 shows a time-activity curve for red marrow obtained by direct measurements of activity in the right or left

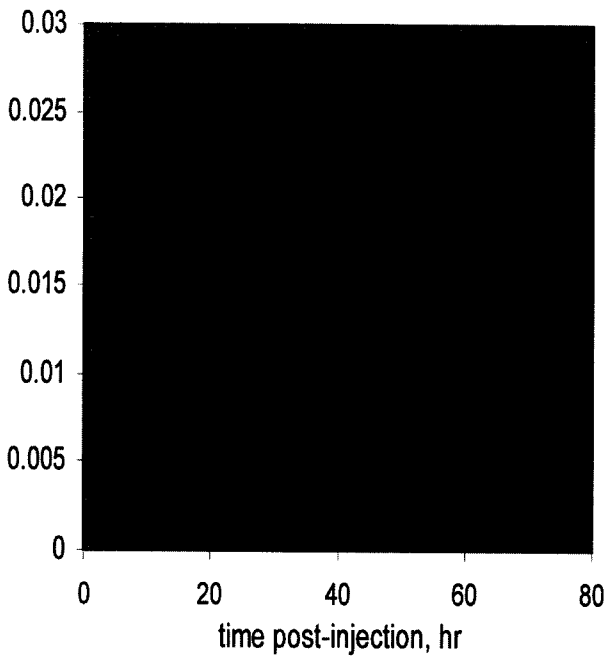


**Figure 1.** *Time-activity curve for a radionuclide in marrow of a patient, determined by direct measurement (planar imaging). The amount of marrow in the patient is not known, but the curve may be normalized by measuring the activity per gram of marrow in a biopsy specimen from the same patient.*

acetabulum. The curve is normalized *to percent administered activity per gram* (%ID/g) with measurement of a marrow biopsy specimen from the same patient. The area under the curve provides the residence time for a marrow dose calculation.

## RESULTS

Marrow absorbed doses should be estimated individually for each patient who receives an internal administration of a therapeutic radiopharmaceutical. The variation in patient-specific parameters dictates that each dose assessment be customized or tailored to each patient. Results will depend on the method, model, and assumptions used in the dose calculation.

## DISCUSSION

Many factors influence the calculated absorbed dose to red marrow from an internally administered radionuclide. The three approaches described above apply to different sets of starting assumptions. Additionally, the models on which the calculations are based may be subject to change from time to time, leading to differences in calculated results. Direct measurements of marrow radioactivity in vivo are also subject to normal statistical counting errors and measurement uncertainties. And finally, the dose-related response of patients to high marrow doses from protracted, low-dose-rate irradiation is highly variable.

### Choice of appropriate *S* value

The mean dose per unit cumulated activity (or *S* value) for each source organ and target organ pair has been calculated for more than 120 different radionuclides and for more than 10 different anthropomorphic models of the human representing different ages, sizes, and sexes. Since the *S* value accounts for transport of radiation as well as the physical structure of marrow tissue, the derivation of *S* values is complicated. As scientists revise their model representations of bone marrow structure and composition and refine their physics calculations for a broad spectrum of radiation emissions and energies, they obtain new sets of tabulated *S* values to choose from. Three major sets of marrow *S* values are in common use, and each will result in a different marrow dose result:

*MIRD-11/MIRDOSE1 (1975).* The first comprehensive tabulation of *S* values was completed by the MIRD committee[9] in 1975. The basis for the marrow *S* values was prior work by Spiers,[10–12] who measured chord-length distribution in skeletal trabeculae and marrow cavities and calculated the energy deposited by

individual radiation tracks. These early *S* values were used in a BASIC computer program called MIRDOSE, which was developed at Oak Ridge Associated Universities (Oak Ridge, TN) in about 1984 to implement the MIRD schema.

*ICRP-30/MIRDOSE2 (1989).* Revised *S* values for beta-particle energy deposition in total marrow space were calculated in 1976 by Whitwell and Spiers.[13] These *S* values were used as the basis for international radiation protection standards and calculations of radiation doses to workers after accidental intakes of radionuclides in workplaces.[14] The same *S* values for marrow were used to assess patient doses from radiopharmaceuticals[15] and were also incorporated in 1989 into the BASIC computer program MIRDOSE2 (Oak Ridge Associated Universities).

*MIRDOSE3 (1994).* Eckerman[16] recalculated the data of Whitwell and Spiers[13] to account for secondary electrons originating within trabeculae or within the marrow cavity after radionuclide gamma-ray interactions with bone. Eckerman considered active red marrow as a separate source organ apart from the total marrow space. *S* values from this work were incorporated[17] into the updated (1994) computer program MIRDOSE3 (Oak Ridge Institute for Science and Education, Oak Ridge, TN). A comparison of marrow doses that result from use of each of these three different *S* values, for activity in marrow irradiating marrow, is given in Table 1 for two radionuclide sources (iodine-131 and yttrium-90).

## Correlations between marrow dose and hematopoietic damage

Numerous studies show correlations between average red marrow dose and average body weight, relative hematopoietic stem cell concentration, and lethality. Larger mammalian species are more susceptible to hematopoietic damage than are smaller species. Breitz et al.[18] studied the correlation of marrow toxicity with marrow absorbed dose in 25 patients treated with rhenium-186-labeled-NRLU-10 monoclonal antibody. Table 2 presents *r* value correlation coefficients, obtained by

**Table 1.** Comparison of marrow dose for two radionuclides ($^{131}$I and $^{90}$Y) obtained for marrow irradiating marrow using three different *S* values

| | *Skeletal average absorbed dose (mGy/MBq-s)* | |
|---|---|---|
| | *Iodine-131* | *Yttrium-90* |
| *S* value source | | |
| MIRD-11/MIRDOSE | $1.7\times10^{25}$ | $6.2\times10^{25}$ |
| ICRP-30/MIRDOSE2 | $2.9\times10^{25}$ | $14\times10^{25}$ |
| MIRDOSE3 | $1.4\times10^{25}$ | $5.9\times10^{25}$ |

**Table 2.** Correlations between dosimetry by the serum measurement method and indicators of toxicity for rhenium-186-NRLU-10 antibody[18]

| | *Correlation coefficients* | | |
|---|---|---|---|
| *Clinical indicator* | *Marrow dose* | *Whole-body dose* | *Administered activity* |
| White blood cell count | 0.69 | 0.74 | 0.76 |
| Platelet count | 0.67 | 0.71 | 0.56 |
| Combined grade | 0.76 | 0.80 | 0.72 |
| Percent baseline white blood cell count | 0.63 | 0.58 | 0.64 |
| Percent baseline platelet count | 0.75 | 0.73 | 0.73 |
| White blood cell nadir | 0.59 | 0.60 | 0.57 |

the paired Student's $t$ test ($P<0.001$) for various pairings of toxicity measure and dosimetry parameter. These results indicated that percent baseline and grade of white blood cell count and platelets all showed a moderate correlation with absorbed dose and amount of activity administered (normalized for body weight). The serum-measurement method was used in calculations of the marrow absorbed dose. Breitz et al.[18] concluded that dosimetry estimates were useful for assessing biodistributions of radiolabeled targeting agents, but were of limited value for predicting the toxicity to individual patients. This was partly due to the substantial variation in the patient-to-patient response of bone marrow to protracted internal radiation.

## Uncertainties in absorbed dose calculations

All estimates of internal dose have uncertainties associated with mismatches between the geometry of human organs and tissues, the geometry of calculational models used to simulate body organs, the actual activity distribution in organs and tissues, and the assumed activity distributions.[15] The likely error associated with direct gamma camera measurements used to determine the amount of activity in a source organ may be as much as a factor of two (± 100%), depending on organ size, position, background count rate, overlying or underlying tissues, and shape.[15] The likely error associated with an application of a mathematical model, such as the MIRD phantom system, to represent the actual size and mass of the patient and the patients internal organs is ±20 to 60%. In contrast, the radiological physics data on the emitted radiations and their transport from one part of the body to another are known with relatively greater accuracy (±20 to 50%).[5] Given these uncertainties, one of the most important elements in successful dosimetry will be consistency in methods used throughout a clinical investigation.

## SUMMARY AND CONCLUSIONS

Red marrow dosimetry is subject to many assumptions, uncertainties, and ever-changing dosimetric models. Despite these uncertainties and the difficulty of marrow dose calculations, studies have shown fair correlations between calculated dose and biologic response. Efforts are in progress to improve calculational internal dosimetry. Methods and parameters used in dose assessment should be consistent within an overall clinical study. Direct measurements of activity in marrow over time provide the most reliable data for marrow dosimetry. A marrow dose assessment will be more reliable if the residence time is determined from direct measurements of red marrow activity over time rather than by merely grouping marrow with remainder tissues as a target tissue.

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# Radioimmunotherapy of Common Epithelial Tumors

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Common epithelial tumors are prone to develop resistance to cytotoxic drugs and have heterogeneous distribution of tumor associated antigens and an inhomogeneous vascular supply. Radioimmunotherapy is an attractive approach to their treatment because resistance can be overcome if a sufficient dose of radiation can be delivered selectively and the range of targeted beta-emitting radionuclides can place non-antigen-producing tumor cells within range of lethal damage. Separate therapeutic strategies are required to treat poorly vascularized tumor areas. A mathematical model based on clinical data indicates that efficiency of antibody targeting depends most on antibody specificity, affinity, avidity, stability within the tumor, antigen density, and flow through and clearance from the blood and extravascular tumor spaces. Design of antibody molecules, choice of radionuclide, and radiobiological considerations can be manipulated to optimize therapy. This will be illustrated in the context of colorectal carcinoma using engineered antibodies to carcinoembryonic antigen of different valency and stability and labeled with $^{131}$iodine or $^{90}$yttrium. The need for and the effects of combining radioimmunotherapy with antivascular therapy using dimethyl xanthenone acetic acid will also be shown. The tools are now available to permit improved radioimmunotherapy of common epithelial tumors, although the problem remains more challenging than the therapy of lymphoid malignancies.

# Radioimmunotherapy for Malignant Lymphoma

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The results of radiolabeled monoclonal antibody therapy for malignant lymphoma will be reviewed. Data (*N Engl J Med* 329:459–465, 1993) on the use of radioactive iodine labeled to anti-B1 antibody suggest that patients can have durable responses with mild or moderate myelosuppression. The background and history of radiolabeled monoclonal antibody therapy is reviewed. Data utilizing Y2B8 (yttrium-90 anti-CD20 monoclonal antibody) are then provided. A multi-institution study for patients with B cell low-grade non-Hodgkin's lymphoma is reviewed. Patients with low-grade lymphoma ≥ second relapse or relapse after anthracycline-regimen were eligible, as were patients with intermediate-grade lymphoma who were in first relapse. Patients with first relapse were eligible for study. Patients were required to have bidimensionally measurable disease and those with a platelet count >100,000 and an ANC of >1500 with less than 25% bone marrow involvement were eligible. Patients were excluded if they had prior myeloablative therapy, prior radioimmunotherapy, CNS lymphoma, AIDS-related lymphoma, or prior external beam radiation therapy to >25% of active bone marrow. There were 39 patients evaluable for response. Twenty-five had low-grade lymphoma, 11 had intermediate-grade disease, and three had mantle cell lymphoma. The complete response rate was 20% in patients with low-grade lymphoma and ~10% in patients with intermediate-grade lymphoma. The overall response rate, including partial responses, was ~80% in low-grade lymphoma and 45% in intermediate-grade lymphoma. Data on dosimetry and correlation of dosimetry with tumor uptake is provided. The major toxicity was mild to moderate myelosuppression, and there was no renal, hepatic, pulmonary, or other organ dysfunction. Radiation exposure to organs was acceptable as determined by dosimetry and lack of significant normal organ toxicity. There was a low incidence (3.5%) of HAMA/HACA. These and other data suggest that radiolabeled monoclonal antibody therapy has a role to play in the treatment of recurrent malignant lymphoma. Better results are seen in patients with low-grade lymphoma, but the role of this treatment in intermediate- or high-grade lymphomas remains to be determined.

# CHAPTER 15
# NEW AVENUES

# Induction of Graft-vs.-Malignancy as Treatment for Malignant Diseases

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## BACKGROUND

Allogeneic bone marrow transplantation (alloBMT) was initially conceived as a means to deliver supralethal doses of chemotherapy and total body radiation for treatment of leukemia. The marrow transplant was given as a means to restore hematopoiesis.[1,2] It has subsequently become clear that the high-dose preparative regimen generally does not eradicate the malignancy, and that the marrow transplant itself confers an important immune-mediated graft-vs.-malignancy effect.

Considerable data support the presence of an allogeneic graft-vs.-malignancy (GVL, from graft-vs.-leukemia) effect (Table 1). This includes the reduced risk of leukemia relapse in patients with graft-vs.-host disease.[3–5] There is a higher risk of leukemia relapse after syngeneic bone marrow transplantation[6–8] and after T cell–depleted allotransplants.[4,9] The most direct evidence supporting the concept of GVL is the observation that many patients who relapse after alloBMT can be reinduced into complete remission by infusing additional donor lymphocytes.[10–13]

Hematologic malignancies differ in their susceptibility to GVL effects. The risk of relapse is increased with syngeneic transplants in both acute and chronic myeloid leukemia (AML and CML), suggesting that allogeneic target antigens are involved.[4] T lymphocytes appear most important in CML, where T cell–depleted transplants are associated with a fivefold increase in the risk of leukemia relapse.[4,9,14–16] In AML, the relapse rate is only minimally affected by T cell depletion, and natural killer (NK) cells have been suggested as mediators of antileukemic activity.[9,17] Among the major leukemias, acute lymphoblastic leukemia (ALL) appears least affected by GVL.[4,7]

Approximately 70% of CML patients relapsing into chronic phase achieve complete remission with donor lymphocyte infusions.[10–13,18–20] Responding patients generally become negative for residual leukemia cells by polymerase chain reaction analysis for *bcr-abl* rearrangement, and these responses are generally durable. Approximately one-third of AML or myelodysplasia patients respond, but these remissions are generally transient, and patients typically recur within the following year. Only rare patients with ALL have benefited. Graft-vs.-malignancy

**Table 1.** Clinical evidence supporting an allogeneic graft-vs.-leukemia effect

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|---|
| Reduced risk of relapse in patients with acute and chronic GVHD |
| Detection of minimal residual disease early posttransplant |
| Increased risk of relapse after syngeneic transplant |
| Increased risk of relapse after T cell–depleted transplants |
| Induction of remission by donor lymphocyte infusion in patients relapsing post-BMT |

responses also affect lymphoid malignancies including chronic lymphocytic leukemia (CLL),[21] lymphoma,[22,23] and multiple myeloma.[24–26]

It is uncertain whether graft-vs.-tumor effects occur against nonhematopoietic malignancies. Pilot studies in patients with breast cancer have reported antitumor responses in patients with graft-vs.-host disease, suggesting a graft-vs.-adenocarcinoma effect.[27,28] Further studies are required to determine if immunodominant tissue-restricted minor histocompatibility antigens are present in nonhematopoietic tumors and whether a clinically meaningful graft-vs.-tumor effect will occur to justify the added morbidity related to allogeneic transplantation.

The effector cells involved with GVHD and GVL are incompletely defined. Both $CD4^+$ and $CD8^+$ T cells participate in the initiation of GVHD; other cell populations, including NK cells, are subsequently recruited, and cytokines participate as mediators of tissue injury.[29–34] In animal models of leukemia, both $CD4^+$ and $CD8^+$ effectors have been described. In many systems, $CD8^+$ cells appear to the principal effectors of GVL.[35–40] In human BMT recipients, both $CD4^+$ and $CD8^+$ cytotoxic antileukemic T cell lines or clones have been described. In patients transplanted for CML, several recent studies have identified $CD4^+$ T cell lines or clones that either inhibit the growth of leukemia progenitors or are directly lytic.[37,41–43] NK cells have also been implicated as mediators of GVL effects.[40,44–47]

In patients relapsing after bone marrow transplantation, the leukemia typically recurs in host-derived cells, but residual normal hematopoiesis and immunity remain largely donor derived. The infused donor lymphocytes are, therefore, not subject to rejection, but acute GVHD is a major problem. In initial studies, patients generally received large doses of T cells, generally $>5\times10^7$/kg, and acute GVHD developed in 50 to 80% of cases, with mortality in up to 20% of recipients. After infusion of donor lymphocytes, there is initially little change in peripheral blood counts, but after a median of 4 months, responding patients may suddenly become hypoplastic followed by recovery from donor-derived hematopoietic cells and return to complete chimerism.[10,18,48] Marrow aplasia may occur unless sufficient donor-derived normal progenitors are present to restore hematopoiesis.[49] Consistent with this premise, patients developing aplasia generally recover after a second infusion of donor hematopoietic stem cells from either marrow or mobilized peripheral blood. A critical factor following donor lymphocyte infusion

is the kinetics of growth of the leukemia; rapid regrowth may outpace the development of an effective immune antileukemic response.

A major challenge is to separate the beneficial GVL effect from the adverse manifestations of GVHD. A number of approaches have been studied as indicated. There is a dose-response effect with higher rates of GVHD as well as antileukemia responses with increasing doses of T cells.[50] MacKinnon et al. performed a study administering graded doses of T cells to patients with CML relapsing into chronic phase after an allogeneic transplant from an HLA-identical sibling. Antileukemic responses and GVHD did not occur at doses of $10^5$ to $5\times10^6$ T cells per kg. Of 21 patients receiving $10^7$ T cells per kg, eight achieved CR and only one developed acute GVHD. At higher doses, there were additional responses but a much increased risk of GVHD. Thus, it may be possible to induce antileukemic responses at a T cell dose below the threshold necessary to produce GVHD. This general approach has been confirmed by others.[20,51]

An alternative strategy is to infuse T cell subpopulations that can mediate GVL with a reduced potential for GVHD. Selective depletion of CD8-positive cells from the allogeneic donor marrow transplants reduces the incidence of GVHD without increasing the risk of relapse in CML.[52–54] Donor lymphocyte infusions using CD8-depleted cells have also been effective to reinduce remission in patients with CML with a low rate of GVHD.[55,56]

GVHD is initiated by alloreactive T cells. A novel strategy to prevent GVHD is to transduce donor T cells with a suicide gene, such as Herpes simplex virus thymidine kinase (HSV-TK), which confers sensitivity to ganciclovir treatment. The viral thymidine kinase phosphorylates ganciclovir into a monophosphate that is subsequently converted by cellular kinases into the cytotoxic triphosphate form. In preliminary studies, TK-transduced lymphocytes are capable of alloreactivity resulting in GVL effects. If the patient develops GVHD, it can be abrogated by ganciclovir treatment, lysing the transduced lymphocytes. This approach has been successful in pilot studies using TK-transduced T cells for donor lymphocyte infusions[57] or combining TK-transduced lymphocytes with T cell–depleted marrow transplants.[58,59] In each setting, acute GVHD could be successfully treated with ganciclovir. Ganciclovir treatment may also abrogate GVL effects, and it is uncertain if this strategy will improve leukemia-free survival.

## INDUCTION OF GVL AS A PRIMARY TREATMENT MODALITY USING NONABLATIVE PREPARATIVE REGIMENS

The high-dose chemotherapy and radiation typically used as the preparative regimen for bone marrow transplantation produces considerable morbidity and mortality and limits the use of this modality to a minority of patients who are young and in good general medical condition.

An alternative strategy is to utilize a low-dose, nonmyeloablative, preparative regimen designed to provide sufficient immunosuppression to achieve engraftment of an allogeneic blood stem cell or marrow graft, allowing for development of a graft-vs.-malignancy effect.

We have evaluated this strategy by using relatively nontoxic, standard-dose chemotherapy as a nonmyeloablative preparative regimen for allogeneic marrow or blood progenitor cell transplantation using chemotherapy regimens active against the patient's malignancy that are only modestly myelosuppressive without marrow transplantation. The regimens were selected to be sufficiently immunosuppressive to prevent graft rejection, allowing engraftment of a marrow or blood stem cell allograft which could then produce a GVL effect against susceptible malignancies.

At M.D. Anderson Cancer Center, we performed pilot trials to determine whether this strategy of nonablative chemotherapy with allogeneic blood stem cell transplantation could induce durable engraftment and remissions in patients with hematologic malignancies. We studied this approach in patients who were ineligible for standard myeloablative preparative regimens because of advanced age or comorbidities. We demonstrated that fludarabine or 2-CDA (purine analogs) containing nonmyeloablative chemotherapy regimens allowed engraftment of HLA-compatible hematopoietic progenitor cells, and extended remissions were observed in some patients with recurrent AML or CML.[60]

Indolent lymphoid malignancies are also affected by graft-vs.-malignancy effects.[22] Fifteen patients with CLL or lymphoma have been treated using a nonmyeloablative regimen of fludarabine/cyclophosphamide or fludarabine, cytarabine, cisplatin.[61] All patients had failed to respond or recurred after primary chemotherapy. Nine patients had CLL in relapse after a prior fludarabine response and six patients had lymphoma. All patients had active disease at the time of transplant, three had a performance status of 3 (according to Southwest Oncology Group [SWOG] criteria), two had elevated liver function tests, and all had received extensive prior therapy. The chemotherapy is known to be nonmyeloablative, and mixed chimerism was anticipated. Eleven of the 15 patients had evidence of engraftment as documented by acquisition of donor type restriction fragment length polymorphisms. The percentage of donor cells in the marrow ranged between 50 and 100% at 1 month posttransplant. One had 75% donor cells in his marrow at 6 weeks posttransplant, and converted to 100% donor cells following a donor lymphocyte infusion. Hematopoietic recovery was prompt and, with the exception of a patient with hepatitis C, no patient had a nonhematologic toxicity of greater than grade 2. The patients failing to engraft recovered endogenous hematopoiesis promptly and had no serious adverse effects. Increasing the intensity of the immunosuppression may increase the rate of engraftment. The minimal toxicity and mild cytopenias indicate the potential feasibility of administering this procedure on an outpatient basis. All 11 patients with engraftment have responded,

and eight have achieved complete remission. Maximal responses are slow to develop and gradually occur over a period of several months to 1 year.

The success of nonablative regimens for GVL induction using allogeneic transplantation requires engraftment of the donor immunocompetent cells and sufficient time to develop an effective antileukemic response. This approach has not been successful in patients with refractory acute leukemias; in this setting, the leukemia generally recurs and progresses rapidly. This strategy may be useful, however, for consolidation of remission in patients at high risk to relapse.[62]

The optimal intensity of the preparative regimen is uncertain, and several factors must be considered, including aggressiveness of the underlying malignancy, immunocompetence of the recipient, and genetic disparity between donor and recipient (Fig. 1). Slavin and co-workers reported use of a more intensive preparative regimen consisting of busulfan 8 mg/kg, fludarabine, and antithymocyte globulin, with encouraging preliminary results.[63] This regimen produces marked myelosuppression and has not been administered without hematopoietic transplantation. Other lower-dose or nonablative regimens have been proposed.[64]

Immunocompromised patients, such as those with advanced CLL, will likely require less immunosuppressive therapy to achieve engraftment than a fully immunocompetent recipient. Indolent malignancies may not require cytoreduction, but it appears necessary to achieve at least a short-term remission in patients with highly proliferative malignancies, such as acute leukemias and aggressive lymphomas, to allow development of an effective GVL response.

The optimal use of posttransplant immunosuppressive therapy is also uncertain. Acute GVHD does occur with these nonablative regimens but has been relatively mild and controllable. Immunosuppressive therapy given early posttransplant to prevent GVHD likely also affects GVL.[65] Effective strategies to separate GVHD from GVL are critical for the success of this approach to treatment.

The role for nonmyeloablative preparative regimens and induction of GVL is uncertain. This strategy allows treatment of older and medically infirm patients

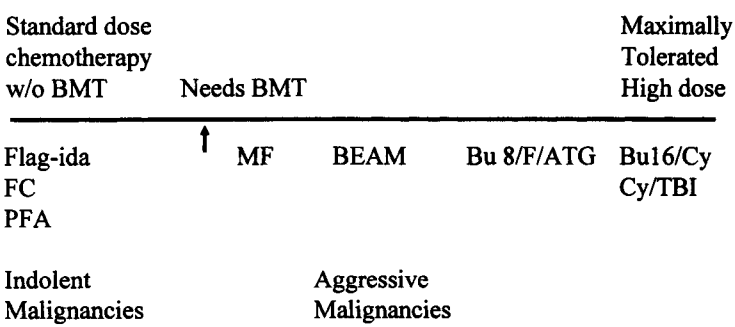


**Figure 1.** *Intensity of preparative regimen.*

who cannot tolerate conventional high-dose preparative regimens. From the preliminary data, morbidity and early mortality appear reduced in high-risk patients. Long-term efficacy must still be determined, and controlled trials are necessary comparing this approach with alternative therapies as well as standard transplantation regimens.

In conclusion, the immune graft-vs.-malignancy effect accounts for much of the benefit of allogeneic blood and marrow transplantation. For susceptible diseases, donor lymphocyte infusions can induce durable remissions in patients relapsing after an allogeneic transplant. Preliminary studies indicate the potential feasibility of inducing graft-vs.-malignancy effects as primary therapy. Use of less toxic, nonmyeloablative preparative regimens may allow transplantation in older patients and those with comorbidities that preclude high-dose chemoradiotherapy. Controlled clinical trials are required to determine appropriate candidates for this approach as well as the relative efficacy of this strategy vs. transplantation using standard myeloablative regimens.

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# Emerging Viral Infections in Blood and Marrow Transplant Recipients: Focus on Respiratory Syncytial Virus

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## ABSTRACT

The spectrum of viruses causing disease in blood and marrow transplant recipients has expanded rapidly in the last decade. Caring for patients with polyviral infections has become the norm. Among the emerging viruses are herpesviruses other than cytomegalovirus (CMV), HSV, and VZV, such as Epstein-Barr virus (EBV) and human herpes virus (HHV)-6; parvovirus B 19; polyomaviruses (BKV, JCV); and multiple hepatitis viruses and gastroenteritis viruses. Opportunistic infections with these viruses are associated with a broad scope of clinical illnesses, including acute and chronic central nervous system disorders with HHV-6 and JCV; cytopenias with parvovirus B 19 and HHV-6; and lymphoproliferative disorders with EBV. Also emerging are the Community Respiratory Viruses: respiratory syncytial virus (RSV), parainfluenza viruses, influenza viruses, rhinoviruses, adenoviruses, echoviruses, and coxsackieviruses. The respiratory illnesses caused by these viruses range from self-limited upper respiratory illnesses to fatal pneumonias depending largely on the type of virus and type, degree, and duration of the underlying immunodeficiency. RSV has been associated with an exceptionally high frequency of fatal viral pneumonia. Some of the community respiratory viruses, such as influenza, adenoviruses, echoviruses, and coxsackiesvirus, cause a wide range of nonrespiratory clinical syndromes as well. Sophisticated diagnostic research tools are gradually being integrated into the clinical diagnostic laboratories, and clinical investigations are being undertaken to define the sensitivity and specificity of these assays and to distinguish shedding of virus from disease. The need for more effective, less toxic, less costly, and easier-to-administer antiviral drugs has become apparent, as has the need for the routine availability of antiviral susceptibility testing. Since many of these emerging infections are highly communicable, infection control strategies are being redefined.

## INTRODUCTION

Infectious diseases in blood and marrow transplant recipients have traditionally been dominated by bacteria, fungi, protozoa such as *Pneumocystis carinii*, and a variety of other opportunistic pathogens. In terms of viruses, the focus has been on several herpesviruses (particularly CMV, HSV, VZV) and an occasional adenovirus. These have been the known pathogens. Equally dominating have been the unknown pathogens manifested in the many unexplained cases of fever, rash, pneumonia, hepatitis, gastroenteritis, meningoencephalitis, etc. These illnesses have frequently eluded extensive diagnostic evaluations and have frequently failed to respond to a spiral of empirical antibiotics. By default, these illnesses have often been attributed to noninfectious etiologies such as conditioning therapy, other drug toxicities, or graft-vs.-host disease (GVHD) or have been classified into a poorly understood "idiopathic" category.

During the last decade, the spectrum of viruses causing serious disease in transplant recipients has grown rapidly, as has our understanding of their wide range of disease manifestations. Caring for patients with polyviral infections has become the norm. This change has occurred in the setting of a growing population of severely immunosuppressed patients at risk for opportunistic viral infections and a rapidly evolving field of virology giving rise to the routine availability of sophisticated diagnostic tools.

The spectrum of emerging viruses causing serious disease in transplant recipients is broad and includes other herpesviruses such as HHV-6 and EBV, polyomaviruses such as BKV and JCV, parvovirus B 19, hepatitis viruses, and gastroenteritis viruses such as rotavirus. Infections with these viruses are associated with a wide range of infectious syndromes, as well as cytopenias and malignancies. For instance, HHV-6, a relatively newly recognized herpes virus linked to roseola (sixth disease) and febrile seizures in young children, is now recognized to cause fevers, bone marrow suppression, encephalitis, and possibility pneumonitis in bone marrow transplant (BMT) recipients. EBV, the most common cause of heterophile positive infectious mononucleosis in young adults, is now recognized to cause lymphoproliferative disorders in immunosuppressed transplant recipients. Parvovirus B 19, the etiologic agent of erythema infectiosum (fifth disease) in young children and arthropathy in healthy adults, is now recognized to cause chronic anemias in immunodeficient patients. Similarly, BKV has been associated with hemorrhagic cystitis and JCV with progressive multifocal leukoencephalopathy.

## COMMUNITY RESPIRATORY VIRUSES

Also emerging are the Community Respiratory Viruses (CRVs), i.e., the viruses which commonly cause respiratory disease in the global community (RSV,

influenza virus, parainfluenza virus, rhinovirus, coronavirus, adenovirus, echovirus, and coxsackievirus).[1–6] In transplant recipients, the respiratory illnesses caused by these viruses range from self-limited upper respiratory illnesses (URIs) to fatal pneumonias depending largely on the type of virus and type, degree, and duration of the underlying immunodeficiency. CRVs such as RSV , parainfluenza virus, and rhinovirus are almost exclusively respiratory pathogens. Other CRVs cause a wide range of other clinical syndromes as well. Influenza infections may also present as encephalitis, myocarditis, and myositis. Echovirus and coxsackievirus infections may also present as fevers, exanthems, meningitis, encephalitis, enteritis, pleuritis, pericarditis, and myocarditis. Adenovirus infections may also present as hemorrhagic cystitis, nephritis, enterocolitis, hepatitis, encephalitis, and disseminated disease. In some centers, adenovirus infections are being recognized in >20% of the BMT recipients.[7]

The CRV are all single-strand (SS) RNA viruses, except for adenovirus, which is a double-strand (DS) DNA virus.

Adenovirus is assembled in the nucleus, and, similar to CMV, morphologic abnormalities can frequently be visualized in clinical specimens with light microscopy as the presence of intranuclear inclusions. The RNA viruses are assembled in the cytoplasm. In some cases, morphologic abnormalities can be visualized in clinical specimens with light microscopy as the presence of intracytoplasmic inclusions. In most cases of pneumonia due to the RNA-containing CRV, however, there are no distinguishing cytological abnormalities. In these cases, the histopathologic abnormalities may be categorized as diffuse alveolar lung damage or "ARDS" unless the clinical illness and viral cultures point to a more specific viral etiology, or more sophisticated diagnostic tools are used, such as electron microscopy or polymerase chain reaction (PCR).

Many of the CRV infections are seasonal, and the temporal occurrence of these infections in transplant recipients tends to mirror their occurrence in the community. Community outbreaks of RSV infections typically occur during the late fall/winter/early spring, and influenza outbreaks typically occur during the winter in temperate climates. Parainfluenza virus infections occur throughout the year, with outbreaks occurring primarily in the spring/summer/fall. The other viruses typically occur year-round, although sporadic outbreaks of all CRVs need to be anticipated.

With the exception of adenovirus, which can also be acquired by endogenous reactivation, the CRVs are all acquired exogenously by the transfer of contaminated respiratory secretions from an infected person to a susceptible person through aerosols, direct contact, or fomites. Because these illnesses are highly communicable, devastating hospital outbreaks may occur. Aggressive infection control measures are crucial and highly effective in controlling nosocomial transmission.[8]

CRVs are a common cause of acute respiratory disease in both allogeneic and autologous BMT recipients, occurring in a minimum of one-third of hospitalized BMT transplant recipients with an acute respiratory illness.[3] Although these infections may occur at any time during the transplant period, either pre- or postengraftment, the severity of these infections has in some instances been temporally related to the time of the transplant.

CRV infections in hospitalized blood and marrow transplant recipients are frequently complicated by life-threatening pneumonias. Approximately 40% of CRV infections have remained limited to the upper respiratory tract and have been characterized by rhinorrhea, nasal and sinus congestion, sore throat, and mild cough. However, 60% of the infections have been complicated by pneumonia, either primary viral or secondary bacterial/fungal pneumonias, and the pneumonia-associated mortality has been approximately 50%. An important clue to the diagnosis of CRV-associated pneumonias has been the finding that 85% of these pneumonias have been preceded by signs and symptoms of a URI, in contrast to pneumonias due to CMV or *Pneumocystis carinii*, which are not typically preceded by a URI. In an era of reasonably effective prophylaxis of CMV disease, CRV-associated pneumonias have assumed a dominating role in BMT recipients, occurring four times more frequently than CMV pneumonia and accounting for four times as many deaths.[4]

There has been considerable diversity in the frequency and severity of CRV infections observed in different transplant centers. This diversity reflects several factors, including the intensity of the surveillance; the time of year when the surveillance was performed and the viruses prevalent in the community; the type and degree of immunosuppression of the patients being evaluated; the type of infection control and influenza vaccination policies; the inclusion of potential as well as actual transplant recipients; the inclusion of outpatients as well as inpatients; the types of viruses routinely assayed for in the laboratory; the multiplicity of laboratory methods used to identify different viruses (e.g., culture, rapid Ag assays, PCR, histopathology); and the definition of a case (i.e., confirmed by culture or by rapid detection assays).

## RESPIRATORY SYNCYTIAL VIRUS

RSV is the leading cause of serious lower respiratory tract disease in infants and children, causing tracheobronchitis, bronchiolitis, and pneumonia. Most persons are infected during the first years of life. Natural immunity is incomplete, and older children and adults experience repeated infections, which are usually manifested as relatively benign upper respiratory illnesses or tracheobronchitis. In the elderly, RSV infections are not infrequently associated with lower respiratory tract syndromes similar to influenza.

Among adult transplant recipients, only sporadic cases of serious RSV disease in adults had been reported until 1988.[9] Since then, studies of RSV infections in BMT recipients reported from several centers have established a fairly consistent picture of the epidemiology, frequency, and clinical course of RSV infections in BMT recipients.[10–13] These studies have established that RSV is a frequent cause of acute respiratory disease in autologous as well as allogeneic pediatric and adult BMT recipients during community outbreaks. Devastating nosocomial outbreaks have occurred, highlighting the need for aggressive hospital infection control strategies. For instance, at M.D. Anderson Cancer Center (MDACC), 19 (17%) of 111 hospitalized adult BMT recipients were diagnosed with RSV infections during a 9-week winter outbreak in 1992–1993. These 19 patients constituted 45% of the adult BMT recipients hospitalized with an acute respiratory illness. Two-thirds of the infections were acquired nosocomially, and nine (56%) of 16 pneumonias were fatal. In subsequent years, the frequency of infections declined dramatically, due to an aggressive multifaceted infection control strategy and a new policy of postponing transplants in patients with URIs.

RSV infections in BMT recipients follow the same clinical sequence as in children: pneumonia is preceded by signs and symptoms of URI such as rhinorrhea, sinus congestion, sore throat, and otitis media. The frequency of progression of URI to pneumonia is highest in patients who are early (<1 month) posttransplant (70–80%). In patients who are late (>2 months) posttransplant or postengrafted, the frequency is reported to be 25–40%. These latter figures are an overestimate, since patients are followed less closely in the late posttransplant period, and primarily severe cases come to medical attention. Once RSV disease has progressed to pneumonia, however, the mortality is more than 80% whether the patient is pre- or postengraftment, and the mortality of patients who have required mechanical ventilation has approached 100% regardless of the therapeutic intervention.

The therapeutic options for RSV disease are limited. The only drug approved for the treatment of RSV disease is aerosolized ribavirin.[14] Aerosolized ribavirin has been shown in controlled trials to be effective in the treatment of RSV pneumonia and bronchiolitis in high-risk children. Controlled trials of therapy for RSV disease in immunocompromised patients have not been conducted. In BMT recipients with RSV disease, favorable responses have been reported primarily when therapy has been initiated at an early stage of the respiratory illness (URIs, tracheobronchitis, or lower respiratory tract disease without radiographic infiltrates).[10] In contrast, in BMT recipients with radiographically visible RSV pneumonia, monotherapy with aerosolized ribavirin has been associated with a 70% mortality.[11]

Because monotherapy with aerosolized ribavirin alone did not appear to be of benefit in established pneumonia, the approach at MDACC since 1992 has been to combine aerosolized ribavirin with IVIG which contained substantial neutralizing

antibody titers to RSV (1:2048 to 1:8192).[13,15–18] The overall mortality in BMT recipients with RSV pneumonia treated with this combination has been approximately 30% among patients in whom therapy was initiated prior to mechanical ventilation. In contrast, the mortality among patients who were treated after the onset of respiratory failure has approached 100%, as has the mortality of patients who were not treated. Because of this favorable response rate during the early years of the study, we have continued to use combination therapy, though in recent years standard IVIG has been substituted for high RSV titered IVIG.

Other treatment regimens for RSV pneumonia have been tried with varying success, including intravenous ribavirin and combinations of aerosolized and intravenous ribavirin.[19–21] The ease of administering intravenous ribavirin compared with aerosolized ribavirin is attractive. However, in an open trial of intravenous ribavirin in 10 BMT recipients with RSV pneumonia, the mortality was 80% and two patients developed severe hemolytic anemia,[20] suggesting that monotherapy with intravenous ribavirin is neither effective nor benign.

In spite of a high cognizance and an intensive surveillance for RSV, many pneumonias are only diagnosed after the onset of profound respiratory failure or death, and even with aggressive therapy prior to profound respiratory failure, the mortality is still high. The need for empiric therapy or for prophylaxis or early therapy of URIs before the onset of viral pneumonia is apparent.

Various approaches to the therapy of RSV-URIs in BMT recipients are currently being investigated, including aerosolized ribavirin and/or high RSV titered IVIG or standard IVIG.[4,5,19,20] Aerosolized ribavirin is being studied at different daily dosages (ranging from 2 to 6 grams), at different concentrations (ranging from 20 to 60 mg/mL) and for different daily durations (ranging from 2 to 18 hours a day). The overall preliminary findings suggest that the frequency of pneumonia and death may be decreased, but far from eliminated, by promptly treating URIs. Whether higher daily doses and longer daily durations of therapy are more effective remains to be determined. Since so many RSV-URIs are self-limited, interpretation of the response to therapy in these studies has been limited by lack of controls.

The decision to initiate therapy with aerosolized ribavirin and/or IVIG for a simple RSV-URI is complex and needs to take into consideration many factors including the patient's risk of developing serious lower respiratory tract disease (with regard to underlying disease, type of transplant, immunosuppressive therapy being administered, and time posttransplant); the presence of underlying lung disease such as bronchiolitis pneumonia obliterans or radiation pneumonitis; the stage of the URI such as whether the illness has already progressed to a deep tracheobronchitis; whether the respiratory illness is improving or worsening; the unproven efficacy of these drugs in transplant recipients; the potential for environmental contamination and exposure of health care workers; the psychological and

physical discomfort to the patients of prolonged aerosol therapy and confinement within a scavenging tent; the adverse effects of aerosolized ribavirin, such as bronchospasm; the high cost of these drugs as well as the intensive respiratory therapy needed to safely administer aerosolized ribavirin; and the need for hospitalization with more frequent or prolonged ribavirin dosing regimens. Studies are underway to define more closely the patients at risk and to identify the regimen that is simplest to administer and least costly yet effective.

Although prompt therapy of URIs with antiviral drugs and/or immunotherapy appears to have had a favorable impact on the frequency of pneumonia and death, the morbidity and mortality associated with RSV infections in these patients is still high. The optimal therapy is prevention. Active immunoprophylaxis is not an option, because an effective vaccine is not currently available. Passive immunoprophylaxis appears promising in high-risk young children and warrants investigation in immunocompromised patients. In children, the monthly administration of RSV-IVIG (Respigram, Medimmune), a human polyclonal IgG immune globulin with high RSV microneutralization titers, has been effective in decreasing the risk of serious RSV disease, as has the monthly administration of monoclonal RSV antibody (MEDI-493, Medimmune), a humanized monoclonal antibody.[22–24] Prevention of infection through an aggressive infection control strategy can be highly effective.[8] At the core of an effective strategy is continuing education of patients, family members, visitors, and staff regarding the potential seriousness of CRV infections, their epidemiology, their modes of transmission, and means of control. In general, infection control strategies need to be designed to prevent spreading by several different modes of transmission, since several different CRVs commonly circulate in the community concurrently and since CRVs usually can be spread by several modes of transmission. These measures may need to be intensified during community or hospital outbreaks of CRV infections. The intensity and duration of the infection control measures need to be tailored according to the risk of serious CRV in different subsets of transplant recipients and the needs of the patient and quality of life issues.

## CONCLUSION

The spectrum of emerging viruses and their disease manifestations in blood and marrow transplant recipients has evolved rapidly. In addition, traditional herpesviruses such as CMV are posing new challenges, particularly in the late posttransplant period, and are becoming the model for a new modality of therapy, adoptive immunotherapy.[25,26] Sophisticated diagnostic research tools are gradually being integrated into the clinical diagnostic laboratories, and clinical investigations are being undertaken to define the sensitivity and specificity of these assays and to distinguish shedding of virus from disease. The need for more effective and less

toxic, less costly, and easier-to-administer antiviral drugs is becoming increasingly apparent, as is the need for the routine availability of antiviral susceptibility testing. The challenges ahead are immense and filled with the excitement of a new frontier.

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# Asymmetric Division as Measurable Parameter of Stem Cells With Self-Renewal Capacity

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The prevailing hypothesis for hematopoietic reconstitution after transplantation presumes that the initial cell division of the stem cells (SC) gives rise to one daughter cell that retains self-renewal capacity and another that differentiates into lineage-committed progenitors. A fundamental issue in developmental biology is if and how such asymmetric division occurs in normal and neoplastic cell clones. We have developed a time-lapse camera system to directly observe cell division on a single-cell basis. Combined with index sorting and single-cell culture systems developed in our laboratory, we have correlated the early replication behavior of the $CD34^+$ subsets as well as cells of the same immunophenotype but of various ontogenic ages. Replication history of individual stem cells was measured using the cell membrane labeling dye PKH26. Symmetry of cell division, division kinetics, and asymmetric division index (ADI) of each cell type were calculated from the singly sorted cells. We have confirmed definitively for the first time that *1*) asymmetric division occurs during the first mitosis of some $CD34^+$ cells and their subsets: e.g., 40% of $CD34^+/CD38^-$ cells showed asymmetric divisions vs. 31% of $CD34^+/CD38^+$ cells; *2*) the % of cells undergoing asymmetric division (ADI) decreases with ontogenic age, i.e., fetal liver > umbilical cord blood > adult bone marrow; *3*) the ADI of $CD34^+$ cells is altered by the cytokines added to the medium: early cytokines (flt3 or thrombopoietin) increased and late cytokines (containing GM-CSF, erythropoietin) decreased ADI. This unique technology will permit precise definition of cytokine and cellular determinants of replication behavior of SC as well as leukemic cells and is a powerful tool for addressing fundamental issues in normal and neoplastic stem cell biology.

# Minimal Therapy Models of Transplantation

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Classic transplantation models involve lethal myeloablation, a setting in which a few or very large numbers of infused marrow stem cells will give 100% donor cells after transplantation. Using a BALB/c male/female marrow transplant model that detects male DNA in host tissues by Southern blot or fluorescent in-situ hybridization (FISH), we have shown that high levels of male BALB/c marrow chimerism can be established in untreated female BALB/c hosts, albeit by infusing relatively high levels of cells. We have shown that exposure to doses of irradiation that cause minimal myeloablation (50–100 cGy) leads to very high levels of donor chimeras, such that relatively small numbers of marrow cells can give donor chimerism in the 40–100% range. Our studies of radiation sensitivity of long-term engrafting cells have shown that 100 cGy, while not myelotoxic, is stem cell toxic, and imply that the final host:donor ratios are determined by competition between host and donor stem cells. In this minimal myeloablation model, we further delineated the basic characteristics of engraftment from marrow infused at different time intervals after 100 cGy whole-body irradiation and engraftment of 100 cGy of marrow cells irradiated in vivo. The results show that infusing cells at longer time intervals after 100 cGy still results in high levels of engraftment, suggesting a failure of renewal of host stem cells at the time intervals evaluated. We also have evaluated secondary engraftment after minimal ablation and the effects of 5-FU and hydrea on engraftment in these models. Finally, we have begun work in an allogeneic model (B6.SJL donor/Balb/c recipient) using donor spleen cell infusions and anti-CD40 ligand followed by low-dose irradiation (100 cGy) as minimal preparative treatment. Transplanted mice were killed at 6 weeks posttransplant, and engraftment was determined by measuring the percentage of donor cells in marrow, spleen, and thymus using fluorescent antibody staining with FITC-CD45.1. The results of these studies will be presented. Ultimately, these studies should provide nontoxic approaches for improved stem cell engraftment in clinical gene therapy, or stem cell expansion strategies, and should form a base for the development of an approach to nontoxically create allochimeras for therapy of hemoglobinopathies or for cellular immune therapy of cancer.

# Neuropoiesis

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Neuropoietic stem cells exist both during embryonic murine development and in adult animals. We have investigated progenitor populations from the striatal tissue of day 15 embryo BALB/c mice. Striatal tissue is triturated to form a single-cell suspension which is then grown in liquid culture with 20 ng epidermal growth factor. Under these conditions, the cells aggregate to form neurospheres and can be passaged over many weeks. We have studied the first one to four passages, looking at clonal populations within the neurospheres and the receptor phenotype. Neurosphere cells express receptors by RT-PCR for 17 different growth factors. Cultured neurosphere-derived cells were triturated, filtered through a 35-μm filter, and single-cell deposited in microtiter wells using a MoFlo cell sorter. Under these conditions and in the presence of 20 ng epidermal growth factor, ~1.3 to 2.0% of the wells showed clones at 12 to 14 days. These clones evidenced neuronal, astroglial, and oligodendrocyte lineages as determined by neurofilament, GFAP, or 04 antibody staining. The epidermal growth factor was washed from the colonies at day 14, brain-derived neurotrophic factor was added, and the majority of colonies became trilineage. The cycle status of colony-forming cells was determined using high specific activity tritiated thymidine suicide. These results showed a mean killing of 41.5%, indicating that the cells were in active cell cycle. These data indicate the presence of a complex neural progenitor system, capable of forming neuronal, oligodendrocyte, and astroglial lineages in the presence of EGF.

# CHAPTER 16
# SUMMARIES

# Summary: Leukemia

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## ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

### Allogeneic bone marrow transplantation (alloBMT)

In the last decade the significant advances in chemotherapy-based treatment for adult ALL, together with a well-publicized International Bone Marrow Transplant Registry (IBMTR) ALL study group analysis showing no advantage for allografts in first remission, reduced enthusiasm for first-remission BMT in adult ALL. In the light of recent results, however, there has been a shift toward alloBMT rather than chemotherapy as postremission treatment. The trend was reflected in the question posed by Hoelzer in this meeting: "Which adult ALL patient should *not* be transplanted?" His current indication for allotransplants includes patients with any one of the following: $Ph^+$, t4;11, pro B-ALL, pro T-ALL, remission induction time >6 weeks, evidence of minimal residual disease after 6 months of remission. The indication for allotransplantation in T-ALL is also stronger, because several recent studies have not reproduced the favorable results of chemotherapy earlier reported by the German Cooperative Group. There is therefore a growing consensus to offer postremission intensification chemotherapy only to low-risk ALL patients—young adults presenting with low count, Ph-negative common ALL—and to consider all other categories for alloBMT in first remission from a matched sibling. All comparative studies between chemotherapy and alloBMT agree that relapse rates are lowest following the allograft. However, procedure-related mortality is higher after the transplant. Further reduction in treatment-related mortality (TRM) may be achieved by the use of peripheral blood stem cell transplants (PBSCT).

### Autologous BMT

The results of autoBMT in ALL have been disappointing. The relapse rates can reach 60–70%, and there is therefore little evidence that myeloablative dose intensification is superior to more prolonged chemotherapy. In many comparative studies, marginal advantages for autotransplants are lost in terms of leukemia-free survival because of a higher TRM. The use of PBSCT in place of marrow transplantation may reduce TRM: As reported by Powles, PBSCT shortened recovery time and permitted the early introduction of posttransplant maintenance treatment. Furthermore,

although there is no clear evidence that the high relapse rate after autoBMT reflects inadequate purging, circulating ALL cells are less frequent in the blood and may reduce relapse rates. The precise role of autografting in ALL remains unclear, but for some high-risk subgroups the autotransplant may offer the only chance of prolonged survival. For example, in $Ph^+$ ALL some more promising results were presented with about 20% of patients having prolonged leukemia-free survival.

### New developments

There is a continuing interest in augmenting the weak graft-vs.-leukemia (GVL) effect seen after allogeneic transplants in ALL. This means identifying antigens on B and T cells that are not necessarily unique to leukemic cells, but that could serve as targets for alloreacting T cells. A promising strategy is the use of immunotoxins targeting B cell antigens. In a phase I trial, Uckun described full and partial remissions in refractory and relapsed ALL given anti-CD19 immunotoxins.

## ACUTE MYELOID LEUKEMIA (AML)

### Which postremission therapy?

The choice of postremission therapy in AML is becoming better defined, in particular following the results of the MRC 10 trial reported by Burnett. In this very large multicenter study, it was possible to identify treatment outcomes for all age groups and risk categories. The consensus is to offer allografts to all patients in first remission under 55–60 years old with an HLA-identical sibling except AML with t15;17, t8;21, and inv 16.

### AutoBMT

The place of the autograft in AML is less well defined. Some things are evident, however: relapse rates after autoBMT are lower than with chemotherapy. The disease-free survival is significantly in favor of the autograft in trials and subgroups where the transplant-related mortality is low. Thus, the future of the autograft in AML treatment largely rests in reducing the TRM, which can be as high as 20% in some series. However, hopes that the use of PBSCT might improve the outcome of the autograft have not yet been fulfilled.

What is the place of purging in AML autografts? Purging appears to be favorable in a number of studies both in the United States and in Europe. However, the procedure risks prolonged cytopenia, and since eligibility for the autograft depends on a reasonably cellular marrow, it is important to consider the possibility that biological selection of a more favorable AML subgroup explains the superior outcome.

### New directions

A role for interleukin (IL)-2 postautograft still remains unclear with the study reported by Blaise showing no benefit. Conflicting results from IL-2 studies could reflect complex interactions between IL-2 and other cytokines; Simonsson presented data suggesting that histamine augments the effector cell stimulatory function of IL-2. Results of combination trials of IL-2 and histamine will be of interest. One agent that has not fulfilled its earlier promise as an immunostimulant is linomide. Results of a Swedish phase III trial show no benefit for this agent in preventing relapse after ABMT. New areas of development are the use of radioimmune conjugates to target bone marrow and enhance antileukemic effects. Finally, the efficacy of a new generation of AML vaccines (transduction of B7 costimulatory molecules and IL-2 or -12 genes) awaits clinical trial.

## CHRONIC MYELOID LEUKEMIA (CML)

### AlloBMT

The patient newly diagnosed with chronic-phase CML with an HLA-identical family or unrelated donor is faced with the dilemma of the safer but unreliable option of seeking prolonged disease control with interferon or proceeding directly to allogeneic transplantation, which has an up-front mortality in the region of 15% but an 80% chance of eventual cure, either from the transplant itself or from subsequent transfusions of donor lymphocytes. Risk for HLA-identical transplants can be readily calculated using the scoring system, obtained from European Group for Blood and Marrow Transplantation (EBMT) data, by Gratwohl. A score is allotted for degree of donor match, age, disease stage, sex, and interval from diagnosis to transplant. Low scores indicate transplant as the most favorable option. The consensus is to offer allogeneic BMT from an HLA-identical sibling to individuals <40 years. With the extremely promising results reported by the Seattle group for well-matched unrelated donor transplants in CML, it is also appropriate to offer unrelated donor transplants as initial treatment, if risk factors are favorable. These include molecular matching, cytomegalovirus (CMV) seronegativity, and younger age. The cytotoxic T lymphocyte precursor frequency is a reliable but not necessarily independent predictor of outcome.

### AutoBMT

There is now a considerable body of data on autologous stem cell transplants for CML. Updated results from the original analysis by McGlave indicate prolonged survival but no evidence of a plateau. Five-year survival rates are comparable to the results obtained with interferon. New data from the EBMT suggest a benefit for the

use of posttransplant interferon but no definite benefit from current purging techniques. The new flexibly designed EBMT study (by Carella and Apperley) will randomize patients to receive interferon-based or autologous transplant–based therapies. In the interferon arm, investigators can elect to use additional cytosine arabinoside (Ara-C). In the autograft arm patients may be mobilized using ifosfamide, carboplatin, etoposide (ICE) chemotherapy as used by Carella to select Ph-negative CD34 cells or receive unmanipulated transplants. The use of improved and highly sensitive polymerase chain reaction (PCR) techniques described by Goldman has made it possible to precisely evaluate the clinical significance of extremely low levels of minimal residual disease in CML after autoBMT. Patients at the lowest levels of detection after autoBMT appear to have long disease-free intervals.

### New treatment approaches

The unique susceptibility of CML to the GVL effect of alloreacting lymphocytes makes this disease the obvious starting point for evaluating the curative effect of nonmyeloablative transplants. The presentation by Carella of a successful outcome after autologous stem cell transplant followed by a "mini" allotransplant represents an important step in treatment. The success of nonmyeloablative conditioning treatments and their low toxicity as reported by Giralt and Slavin begs the question whether the only maneuver required to cure CML is to establish a donor immune system using just enough conditioning to achieve full donor lymphocyte chimerism. Another promising area is the continuing effort to target the *bcr-abl* fusion product with specific tyrosine kinase inhibitors. So far progress has been slow, and antisense strategies have fallen out of favor. However, the tailored tyrosine kinase inhibitors (tyrophostins) described by Carlo-Stella may soon find a place first in purging of the autograft.

## FUTURE DIRECTIONS

The next few years are likely to see an explosion in the use of allogeneic stem cell transplants using "minitransplants"—low-intensity, nonmyeloablative preparative regimens. The preliminary data presented at this meeting establish that such transplants are associated with low morbidity and mortality and that they may confer a GVL effect. As technology improves, advances in immunotherapy can be anticipated. In the field of allogeneic transplantation, improved ways of manipulating the transplant, T cell selection and expansion, and Tk gene insertion should make targeted T cell therapy a realistic possibility. In the autograft field, immunologic and pharmacologic purging should become more feasible, but perhaps more importantly, antitumor responses may be elicited using dendritic cell therapy, gene-modified tumor vaccines, nonspecific activation with cytokines and sophisticated humanized toxin–conjugated monoclonal antibody based treatments.

# Autologous Blood and Marrow Transplantation After High Dose Chemotherapy for Solid Tumors: Current Barriers and Future Strategies

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## INTRODUCTION

This summary of two scientific sessions on the topic of solid tumors at this international symposium is different from others that have been written in the past. Prior to arriving at the symposium, this author developed a list, which he hoped was nearly complete, of barriers that stood in the path to more successful disease-free survival (DFS) for patients with solid tumors being treated with high-dose cytotoxic therapy and autologous stem cell transplants. The challenge was to identify areas where research needed to be done. The goal was to learn from the oral presentations what research was being done and to match that research with the identified areas of needed research. This was the first priority of the author.

The second priority was to sense, from the presentations and often heated discussions afterward, what were the underlying themes, concerns, ideas, and questions of the assembled clinical scientists. They may be predictive of future directions, interests, initiatives, and research. Four such areas were identified and will be discussed.

The third priority was to impart a little bit of philosophy while treating the first two priorities from the vantage point of being in the field of stem cell transplantation after high-dose therapy for malignancies for 30 years as of the date of this symposium. This is also the time from the first successful allogeneic marrow transplant of the modern era until the present.

Finally, although science is objective, fulfilling these priorities was subjective. Despite the subjective nature of this process, the author has gone to great lengths to tabulate his thoughts to improve the clarity of his presentation and, hopefully, to ease comprehension of the material.

## STRATEGIES TO OVERCOME BARRIERS TO MORE SUCCESSFUL OUTCOMES

Table 1 presents the author's "top ten" list of barriers to overcome to improve DFS for patients with solid tumors receiving high-dose therapy and autologous

**Table 1.** Strategies to overcome barriers to better DFS for patients with solid tumors *(continues*

| *Barrier* | *Strategies* |
|---|---|
| 1) Drug resistance (native and acquired) | • Identify molecular basis of resistance and develop bypass of resistance mechanism<br>• Explore new drugs in high dose therapy (HDRx)<br>• Use HDRx in first complete remission (CR1)<br>• Give noncytotoxic therapy after HDRx<br>• Develop novel therapies |
| 2) Sanctuary site | • Introduce irradiation therapy (RT) into the treatment plan<br>• Include drugs that penetrate blood–brain barrier |
| 3) Tumor burden | • Administer more chemotherapy before HDRx<br>• Add RT to sites of "former" bulk disease after HDRx<br>• Try HDRx in CR1<br>• Use maintenance therapy after HDRx<br>• Explore novel Rx in setting of minimal residual disease (MRD) |
| 4) Cell cycle dependent drug sensitivity (see native drug resistance) | • Give multiple courses of sub-maximal tolerable dose (MTD) HDRx<br>• Try molecular regulators of cell cycle<br>• Add maintenance therapy after HDRx |
| 5) Dose limiting toxicity (DLT) of HDRx regimens | • Explore drugs to reduce DLT of MTD |
| 6) Tumor contamination of stem cell product | • Reevaluate purging *in vivo*:<br>- Positive selection<br>- Negative depletion |
| 7) Tissue oxygenation | • Subvert resistance due to hypoxia<br>• Produce tumor cell hypoxia |
| 8) Neutropenic infections | • Shorten aplasia<br>• Use antibiotic prophylaxis |

*across page)*

| *Examples* | *Contributors* |
|---|---|
| • Undermine inhibitor of apoptosis<br>• Try new antimetabolites & antifols as well as new alkylators & topoisomerase inhibitors<br>• Treat patients with ovarian cancer in CR1<br>• Use immunotherapy: vaccines, donor lymphocyte infusions (DLI), dendritic cells loaded with tumor-specific antigens<br>• Evaluate: hyperthermia, thalidomide, rationally designed drugs | Demonstration of barrier: Rosti, Gluck, McCarthy (male breast cancer), & Viens (inflammatory breast cancer);<br>Fields (paclitaxel & topotecan)<br>Stiff |
| • Consider brain RT for small cell lung cancer, breast cancer<br>• Use Ara-C, BCNU, methotrexate, Etc. in HDRx | Elias |
| • Compare with "total rx" plan for myeloma pre-HDRx<br>• Deliver to sites where $>10^{10}$ cells may still remain<br>• Treat because up to $10^{10}$ cells may easily be left in body<br>• Compare with alpha-interferon for CML after HDRx<br>• Evaluate rationally designed drugs, e.g., anti-Her2Neu antibody Rx | Dicke & Blumenschein (breast cancer), Takaue (neuroblastoma) |
| • Try "tandem" HDRx, multiple courses of intermediate dose Rx<br>• Recruit & synchronize cells into S1/M/S2 phase<br>• Explore something like bcr/abl thymidine kinase inhibitor in CML | Vahdat, Prince, Hohaus, Abonour (germ cell tumors), Leyvraz (small cell lung cancer) |
| • Deliver drugs such as amifostine, IL-11, etc. | Rizzoli (for PBSC harvest) |
| • Give more drugs *in vivo*<br>• Use CD34 column<br>• Use anti-NBL magnetic beads | Demonstration of barrier: Kvalheim, Hood, Moss |
| • Use hypoxic sensitizer for RT and maybe for chemotherapy, too<br>• Try anti-angiogenesis factors | |
| • Growth factors (GF), Chemo-GF PBSC, GF-bone marrow<br>• Use quinolones and fluconazole | |

*Continued on next page*

**Table 1.** Continued from previous page

| *Barrier* | *Strategies* |
|---|---|
| 9) Competition with community physicians: will we see enough patients to do our research? | • Explore new strategies<br>• Treat high risk patients<br>• Extend indications or HDRx options |
| 10) High cost of research: will the care of our patients be reimbursed? | • Reduce research costs: shorten hospital stays |

blood or marrow transplants. Although the table is long and appears to be complicated, it should be easy to follow. Further, although hoped to be nearly complete, it cannot be counted on to be comprehensive, exhaustive, or all-inclusive. Columns 1–3 present barriers to success, potential (but not all possible) strategies to overcome the barriers, and a limited number of examples pursuing specific strategies.

Each solid tumor session abstract usually recognized one (but sometimes more than one) barrier to success and presented data exploring one of the methods to pursue a potential strategy to overcome the barrier. The fourth column lists the last name of the first author of each abstract on the same line as the strategy that the abstract's authors were pursuing or the strategy about which the presenter made his or her most salient comments. Although many strategies appear to remain unexplored, it should be remembered that the symposium had only a limited number of invited investigators to discuss their work.

## THEMATIC AREAS FOR CONSIDERATION

During the course of listening to the oral presentations in both scientific sessions, the author recognized four major questions that had been discussed but remained unresolved, predominantly because of limitation of time. Table 2 is the author's attempt to crystallize these questions and formulate concise, logical answers that pertain to the circumstances which represent the current state-of-the-art.

Tables 3 and 4 are necessary to complete the author's response to the fourth question: "Should just phase III trials be done?" Table 3 presents the meanings of phase I, phase II, and phase III trials for high-dose therapy and hematopoietic progenitor cell transplant. Table 4 presents the author's recommendation of what type of study to do under what set of conditions. Consideration of *1*) issues about the ease of doing the study (number of patients needed, expected accrual rate of

| *Examples* | *Contributors* |
|---|---|
| • Reduce HDRx doses and give something like DLI after allogeneic BMT<br>• Treat small cell lung cancer patients<br>• Try allogeneic BMT for breast cancer looking for graft antitumor effect | |
| • Do outpatient BMT, mini-BMT, several courses of attenuated dose therapy | Rodenhuis |

**Table 2.** Thematic areas for consideration

*Group's question*

1) Restaging in breast cancer: Now that we can identify tumor cells in marrow and blood specimens from patients with stage III and even high risk stage II breast cancer, should we now restage these patients as stage IV?

*Author's response*

NO! Stage creep and its outcome consequences.

i) We are already treating these patients with systemic, including high dose, cytotoxic therapy.

ii) It is still not clear what extra risks these findings mean for patients receiving high dose therapy.

iii) Were we to do this, the results for both stage IV (in CR or PR because of microscopically detectable tumor cells) and high risk stage II/III patients receiving high dose therapy would improve; i.e., this would give rise to the "Will Rogers effect."

*Group's question*

2) Chemoresistant patients: Now that we have sufficient follow-up data for patients who have failed at least two combination regimens for their malignancy and who have subsequently received high dose therapy, should we now stop treating these patients because of their poor DFS (≤10% at 5 years)?

*Author's response*

QUALIFIED NO! What's true for some is not true for all.

i) Because of the poor DFS for these patients, community physicians utilizing published protocols should stop treating this class of patient with those protocols.

ii) On the other hand, academic physicians should be encouraged to develop new clinical research initiatives to improve outcome for these genuinely needy patients.

iii) Community physicians should learn to contemplate high dose therapy for their high risk of relapse patients earlier in the course of the disease.

*Continued on next page*

**Table 2.** Continued from previous page

iv) Third party payers should welcome the opportunity to fund the clinical research to improve outcomes for their customers in this difficult situation at academic centers focusing on this particular problem.

*Group's question*

3) Private sector competition: Many community physicians are now giving high dose therapy to their patients, which reduces referrals to our transplant centers. This is especially true of patients with high DFS probability, e.g., adjuvant breast cancer patients. Should we do anything about this decentralization of medical technology?

*Author's response*

NO! Translation is a goal; decentralization is dissemination.

i) In the course of performing their assigned duties, academic physicians
   a) train young doctors to be at least as good clinicians as their teachers and
   b) explore new ideas to improve clinical outcomes for patients in specific circumstances all the while rendering the best clinical care they know how for their patients. Success in academic jobs means former trainees are in the community practicing the new state-of-the-art medicine that their teachers have developed.

ii) In a public health sense, transfer of technology into the community is a goal and should only result from good science demonstrating efficacy and safety as well as masterable technology.

iii) The extent to which decentralization occurs is a fundamental measure of how valuable clinical research is.

iv) Community physicians doing transplants should be drawn into academic center networks to participate in clinical research and to report data on outcomes.

*Group's question*

4) Phase III Trials. Considering that resources are always limited and that formal proof (level one data) requires a prospective, randomized trial, should we not devote all of our energies to performing phase III trials?

*Author's response*

QUALIFIED NO! There is a time for everything.

i) Phase III trials confirm or deny the hypothesis that a new treatment plan is better than an old treatment plan, for instance.

ii) Phase III trials require considerable time and resources to plan, draft, gain all approvals, initiate, monitor performance, collect and validate data, evaluate and, finally, communicate results to the clinical science arena.

iii) Phase III trials do not make new discoveries.

iv) There are times when phase III trials are not appropriate but other phase trials are (see Tables 3 and 4).

v) Finally, there are times when well designed phase III trials asking highly relevant clinical questions and moved along as fast as humanly possible are out-of-date by the time their results are communicated because biotechnology growth and development have voided the need for the answer to the question the phase III trial posed in the first place.

**Table 3.** Types of clinical trials in high-dose chemotherapy.

| *Type of trial* | *Characteristics of trial in high-dose therapy* |
|---|---|
| Phase I | Usually modifying an old regimen of HDRx because of addition of new drug but could be putting all new drugs together for HDRx; essential is dose escalation until MTD is reached and DLT are known; toxicities are the unknown being evaluated; responses are collected to guide phase II study design; all patients are treated alike; highest patient risk in this type of study because drugs have not been given in these high doses before. |
| Phase II | For any HDRx regimen; essential is exploration by disease of response rates (PR and CR) for patients who have evaluable disease or relapse rates and time to relapse for patients receiving HDRx in any CR or in adjuvant setting; response rates are the unknown being evaluated but significant expectation of response exists; more data on toxicities are being collected; all patients treated alike. |
| Phase III | For any HDRx regimen; essential is the head to head comparison (by stratifying and randomizing) of response rates or relapse rates and toxicity rates for patients with a specific disease and specific stage produced by the HDRx regimen and either conventional chemotherapy or another HDRx regimen; null hypothesis (no difference in outcomes) is being evaluated; if antitumor results are different, then one therapy is better than another; if antitumor results are not different, then treatment strategy would be chosen by toxicity profiles or costs of therapy; randomization to one of at least two different treatment strategies. |

patients, number of years to accrue patient goal, minimum number of years of follow-up of the last treated patient before statistical analyses can clinically meaningfully be done), *2*) probable risks and potential benefits, and *3*) clinical value of the study once completed (incremental improvement in DFS that can be demonstrated [e.g., 90 vs. 85%] with a reasonable sample size in a reasonable period of time) determined the code numbers. The assignment of a code number for each type of trial and each range of DFS is strictly the opinion of the author. This opinion may not be shared by the editors, let alone the reader.

## CONCLUSION

Much remains to be done for patients with solid tumors; however, major success has been achieved for patients with high risk of relapse, locally advanced breast cancer, and twice relapsed but still chemosensitive testicular cancer. Improvements can be anticipated in small cell lung cancer and ovarian cancer if both receive high-dose therapy as part of the *initial* therapeutic plan. It is worth noting that many strategies remain unexplored and it is anticipated that many new strategies will be

**Table 4.** Guide to selection of appropriate phase for BMT clinical trials

| *Current DFS* | *HDRx example* | *Phase I* | *Phase II* | *Phase III* |
|---|---|---|---|---|
| >85% | Young, uninfected, untransfused patient with severe aplastic anemia | 0 | 0 | 0 |
| 70–85% | Young CML patient in first chronic phase <1 year from diagnosis | 0 | 0 | 1 |
| 30–70% | Young AML in CR1 | 0 | 1 | 2 |
| 15–30% | Young AML in CR2 | 1 | 2 | 1 |
| <15% | CML in blast crisis | 2 | 1 | 0 |

*0, inappropriate circumstance to do this type of trial in this DFS group; 1, satisfactory circumstance to do this type of trial in this DFS group; 2, most appropriate circumstance to do this type of trial in this DFS group.*

discovered. As more and more success comes to this endeavor for solid tumors, it may appear that further success is harder to achieve. It is therefore worth remembering that, in any trial, failures are seen first and successes take a lot longer, because success is the continued and continuing absence of failure.

# Strategies in Autologous Bone Marrow Transplantation

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## PRETRANSPLANT TREATMENT

Further escalation of currently available chemotherapy and radiotherapy likely will not further decrease relapse rates posttransplant. To overcome drug resistance, new approaches will have to be incorporated into the pretransplant "conditioning." Even if chemoprotective agents (such as amifostine) will allow dose intensification, it is uncertain whether this will result in greater tumor cell kill. Since chemotherapy and radiation represent only two modalities of cancer therapy, non–crossreactive modalities such as angiogenesis inhibitors, cytokines, monoclonal antibodies, and cellular treatments will be part of an attempt to achieve maximum disease reduction immediately before stem cell infusion. By incorporating these modalities, it will become possible to decrease the total amount of chemotherapy and radiation. This will help to reduce regimen-related toxicities.

## PURGING THE GRAFT

Genetic marking studies in autologous transplantation for acute myeloid leukemia have shown that residual cells in the graft can contribute to relapse. Conversely, grafts that do not have detectable (at the molecular level) lymphoma cells provide longer disease-free survival after bone marrow transplant (BMT). Although many investigators believe that relapse is predominantly caused by residual cells in the host, the gene marking data suggest that one of the objectives should be to improve purging to obtain molecular remissions. When we arrive at a point where we consistently can eliminate residual disease in the patient, then the availability of a "clean" stem cell product will become a necessity.

## MANIPULATION OF THE GRAFT

Purging represents only one aspect of graft manipulation. Other approaches include the addition, activation, or removal of cells, which can have various functions (immune-activation, suppression, etc.). For some tumors, selection of stem cells is the first step toward graft manipulation. Additional methods include the ex vivo culture and activation by cytokines such as interleukin (IL)-2, IL-12, or

IL-15. The non-$CD34^+$ fraction, which in most cases contains both immune cells and tumor cells, could potentially be used to differentiate tumor cells into antigen-presenting dendritic cells, or stimulate tumor cells with anti-CD40. Tumor cells may also be genetically transfected to render them better antigen-presenting cells.

## POSTTRANSPLANT TREATMENT

A manipulated graft may require that certain cytokines are given after BMT to maintain the activity of the immune cells that are present. To achieve an allogeneic response, T lymphocytes from a closely HLA-matched family member will be considered. Autologous T cells, ex vivo expanded and directed against patient-specific tumor antigens, should be useful once additional tumor antigens have been defined. Cloned, genetically manipulated T cells and NK cells/lines will also be available for cellular treatment posttransplant. Eradication of persistent minimal disease after bone marrow transplant could also be achieved with monoclonal antibodies that have been harnessed by additional cytotoxic "entities" (drugs, radiation emitters, or cytolytic cells).

When cytokines that produce rapid recovery of graft function after transplantation become available, the challenges of an autologous stem cell transplant will shift from the management of complications related to high-dose chemotherapy to posttransplant patient-specific treatments combined with sophisticated graft engineering methods. The role of the "transplanter" in the future will consist of providing expertise in these technologies combined with the ability to administer them.

# Summary: Lymphoma

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Although there were no major advances in autotransplants for lymphoma in the last few years, there has been some progress, albeit limited. Moreover, future prospects are particularly promising.

Indications for autotransplants continue to be explored. The issue of the role of autotransplants in relapsed follicular lymphoma requires resolution. Unfortunately, the only prospective trial to address this question—the European CUP trial—was closed prematurely because of inadequate accrual (140 instead of 300). That study (see page 161) compared chemotherapy to autotransplant with purged vs. unpurged autografts. Interim analysis shows no difference in survival among the three arms. Although an adequate prospective trial is necessary to definitively address the role of autotransplant for low-grade lymphoma, it is no no longer clear that such a study is feasible. A further factor that will require inclusion in any study design is the availability of anti-CD20 antibody for clinical use. A somewhat less daunting prospect is evaluating autotransplants in mantle cell lymphoma. The Omaha group (page 184) observed continuing relapses among autotransplant recipients (n=39) while in contrast, five of seven allotransplant recipients (follow-up of 3–45 months) are progression-free. Further studies with greater numbers are required in this disease, and here too, the role of an antibody such as Rituxan should be investigated.

Timing of autotransplant is a key issue and also applies to the optimal management of intermediate-grade non-Hodgkin's lymphoma. A prospective trial was conducted by the Italian group comparing VACOP-B with VACOP-B and intensive therapy and autotransplant as frontline treatment for patients with aggressive advanced intermediate- and high-grade NHL (page 192). At a median follow-up of 37 months, the 6-year progression-free survival is similar for the two groups (47 vs. 60% for chemotherapy alone and with autotransplant, respectively; P=0.4). When patients with high-risk disease as identified by the International Prognostic Index were compared, disease-free survival for the autotransplant recipients was superior. This outcome is similar to the results of the GELA study (see reference 7, page 190) and is supported by the outcome of a single-arm study by Schenkein and colleagues (page 185). A prospective trial of autotransplant as frontline therapy for high-risk patients is now under construction.

Patterns of relapse after autotransplant for NHL were described from data derived from the Lymphoma Registry of the EBMT and the University of Southampton (page 166). While median time to relapse was shorter in patients with local vs. distant relapse, there was no difference in survival. Moreover, survival was inversely correlated with histologic grade. Since median survival after relapse was 15 months, new strategies are required to improve the outlook of this group.

The treatment of patients relapsing after autotransplant has received insufficient attention and lacks a coordinated approach. A step in the right direction is the "Samosh protocol" from the St. Bartholemew's group (page 139). They provide evidence that patients with Hodgkin's disease relapsing after autotransplant have acceptable quality of life and superior survival after a multiagent regimen consisting of lomustine, chlorambucil, s/c bleomycin, vinblastine, and methotrexate compared with local radiotherapy and single-agent chemotherapy.

More attention is now deservedly directed to investigating late nonrelapse mortality after autotransplant. In a study of autotransplant recipients with Hodgkin's disease, Reece and colleagues documented an overall nonrelapse treatment-related mortality rate of 19%. Importantly, 9% of the deaths were late (>100 days) and due to a variety of causes including secondary malignancies, infection, and pulmonary fibrosis. More extensive investigations from other centers and database registries may help to identify risk factors and reduce such treatment failures.

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