Autologous Bone Marrow Transplantation

Proceedings of the Third International Symposium

EDITED BY

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The University of Texas
M. D. Anderson Hospital and Tumor Institute at Houston

Autologous Bone Marrow Transplantation

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PROCEEDINGS OF THE THIRD INTERNATIONAL SYMPOSIUM

Edited By
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To our colleagues, Drs. R. Lee Clark and Dick van Bekkum, who set the examples for endurance and originality, and who attained achievements so necessary in science.

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Preface

The Proceedings of the Third International Symposium on Autologous Bone Marrow Transplantation was very successful. It has become evident that the use of high-dose cytoreductive therapy with autologous bone marrow is advantageous both to patients with leukemia and to patients with lymphoma and neuroblastoma.

It is remarkable how these symposia have been instrumental in communications among clinical scientists. They have led to the organization of a worldwide international randomized study called "The Parma Study in Relapsed Non-Hodgkin's Lymphoma," with other studies in leukemia and Hodgkin's disease to follow. The enthusiasm of the investigators in this field is apparent. Without their motivation, these major achievements would not have been made.

But let's be realistic. Much, much more needs to be done. Our patients continue to wait for that magic therapeutic answer: high cure rate with minimal mortality. Can we give this to them? In view of the strides made in the field of genetic engineering, we can be optimistic about the progress made in growth factors and improvement of cytoreductive therapy.

This symposium is in tribute to Dr. Dirk van Bekkum, Director of the Radiobiological Institute TNO, Holland, and Dr. R. Lee Clark, President Emeritus of The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston. We greatly acknowledge Dr. van Bekkum's major contributions to the field of bone marrow transplantation and Dr. Clark's initiative to start the first independent cancer center. Our thanks and appreciation are due to these two original and extraordinary physicians.

I would like to paraphrase part of my statement from our first symposium: without the courage of our patients, progress in medicine cannot be realized. Therefore, this symposium is also a tribute to the patient undergoing a bone marrow transplant.

Karel A. Dicke

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The success of this symposium is due to the efforts of our entire team. I want first to acknowledge Joanne Taylor for the overall organization of the meeting. I especially wish to thank Diane F. Bush, Managing Editor, who played a major role in the realization of these proceedings. Her editorial skills and the skills of her assistants, editors Beth W. Allen, Barbara Reschke, and Lore Feldman have proved invaluable. Many thanks also to Le Foster and Ellan Stewart for their assistance during the meeting.

IA. Clinical Studies in First-Remission Leukemia

Autologous Bone Marrow Transplantation in Patients With Acute Myeloid Leukemia in First Remission

B. Lowenberg, J. van der Lelie, R. Goudsmit, R. Willemze, F. E. Zwaan, A. Hagenbeek, W. J. L. van Putten, L. F. Verdonck, and G. C. de Gast

The use of high-dose chemotherapy and radiotherapy followed by allogeneic bone marrow transplantation (BMT) has proved to be an effective treatment for patients with acute myeloid leukemia (AML). When the combination is used during first remission in particular, approximately 50% of patients will remain in continuous remission. The major limitations of the treatment relate to the complications of interstitial pneumonia, infections, and graft-versus-host disease; as a consequence, its use is currently restricted to patients younger than 50 years who have a genotypically human leukocyte antigen (HLA)-identical donor (1-4).

To circumvent these limitations, current research programs deal with the development of new therapeutic strategies. One of these modalities is the use of autologous bone marrow taken from the patient during full remission (5,6). It is assumed that, at this stage of the disease, the bone marrow is contaminated with only minimal numbers of malignant cells and that the reinfusion of small numbers of those cells will not necessarily lead to relapse following intravenous infusion. In addition, autologous bone marrow transplantation (ABMT) can be

used in patients older than 50 years and thus is less subject to strict age limits than allogeneic BMT. On the other hand, since ABMT is not associated with graft-versus-host disease and interstitial pneumonia, these advantages should be weighed against the higher probability of relapse of leukemia after transplantation.

Pilot studies carried out at several centers in first (5,6) and subsequent (7) remissions of AML suggest promising results from marrow ablative cytotoxic therapy followed by ABMT in patients with AML. These results now await confirmation in prospective trials, since one could argue that patients who were particularly good risks were selected for transplantation in those studies. To assess the value of ABMT in patients with AML, we initiated a study in which patients with newly diagnosed AML would receive ABMT or allogeneic BMT following attainment of complete remission. Patients received allogeneic BMT when they had an HLA-matched sibling donor and were between 15 and 45 years old; in all other instances, patients received ABMT.

STUDY DESIGNS AND METHODS

Patients received one or two courses of remission-inducing chemotherapy (daunomycin and β-cytosine arabinoside) and, after attaining complete remission, a course of consolidation chemotherapy. Subsequently, the patients received ABMT or allogeneic BMT unless early relapse interfered. During complete remission, autologous bone marrow was harvested, cryopreserved, and reinfused after conditioning chemotherapy and radiotherapy. Pretransplant conditioning included two separate regimens, the first consisting of β-cytosine arabinoside (1 g/m^2 × 4) and amsacrine (115 mg/m^2) and the second of cyclophosphamide (120 mg/kg) and total body irradiation of 8 Gy (lung dose, 7 Gy). The study was begun in December 1984, and our most recent analysis was completed in June 1986. Bone marrow was collected from the iliac crest and pelvic spine in 2- to 4-ml aspirates and collected in bottles containing heparinized Hanks' balanced salt solution. The buffy coat (prepared by centrifugation at 2,000 *G*) or Ficoll Isopaque-separated cells were filtered through a nylon gauze and then through a glass filter. Nucleated cells were counted in Turk solution. Samples from the graft were sent for bacteriological and granulocyte-macrophage colony-forming unit (CFU-GM) cultures. Cells were frozen in 10% dimethyl sulfoxide and 20% fetal calf serum using a controlled-rate freezer and stored at -196°C in liquid nitrogen (8). On 2 subsequent days, cyclophosphamide (60 mg/kg) was administered to the patients in saline during a 1-hour infusion under conditions of forced diuresis (125 ml/hour). Following the start of the cyclophosphamide infusion, mesna was given in divided portions at -10 minutes, +4 hours, +8 hours, and +12 hours up to a total dose of 48 mg/kg on each of 2 days.

The marrow graft was thawed on day 0 (8) and then reinfused for 30-45 minutes. The marrow graft was tested with CFU-GM cultures, which were

performed by a double agar layer technique with a leukocyte feeder as described previously (9).

Blood products for transfusion were irradiated (15 Gy); leukocyte-poor cotton-wool filtrated red cells were given; platelet transfusions were given when the platelet counts dropped below 20×10^9/l.

RESULTS

Of 104 patients entered into the study, 73 (70%) have reached complete remission at this time. Of these complete responders, 17 (23%) received allografts and 29 (40%) received ABMT. Twenty-seven patients (37%) had not received either mode of transplantation because of early relapse (n = 11), refusal (n = 4), or other causes (n = 7); five patients who are at an early stage of remission are still candidates for grafting.

The ABMT patients were between 16 and 57 years of age (median, 40 years) and included 18 females and 11 males. Their grafts contained an average of 1.5×10^8 nucleated cells per kilogram of body weight or 4.3×10^4 CFU-GM (myeloid progenitors) per kilogram of body weight. The median times of recovery to granulocyte counts of 0.5×10^9/l and platelet counts of 20×10^9/l were 40 and 65 days in the ABMT recipients. To date, 11 ABMT patients have experienced AML relapse at a median of 115 days (range, 36-293 days) posttransplantation. Neither the survival curves (Fig 1A) nor remission duration (Fig 1B) show any significant differences between ABMT and allogeneic BMT to date (data not shown).

DISCUSSION

Our initial experiences with the use of nonpurged autologous bone marrow in patients with AML in full remission indicated that durable remissions can be obtained without additional maintenance chemotherapy (5). Our present observations demonstrate the feasibility of using ABMT in a cooperative prospective study currently under way. However, hematopoietic regeneration of platelets and granulocytes may be delayed. This remains a major drawback of ABMT in patients with AML.

We assume that the marrow ablative antileukemic therapy used in both ABMT and allogeneic BMT is the major factor in preventing relapse after remission. Thus, the type of conditioning treatment is probably critical to the outcome of ABMT in patients with AML. Comparative data on different schedules of pretransplant antileukemic therapy are not available, but encouraging results have been obtained with total body irradiation (5,6), mega-intensive chemotherapy, or both (7). The question of the need to purge the autologous marrow of residual leukemic cells has not been settled. Since a high incidence of relapse is also seen following allogeneic BMT, it is likely that a significant proportion of AML recurrences stem from surviving AML cells in the

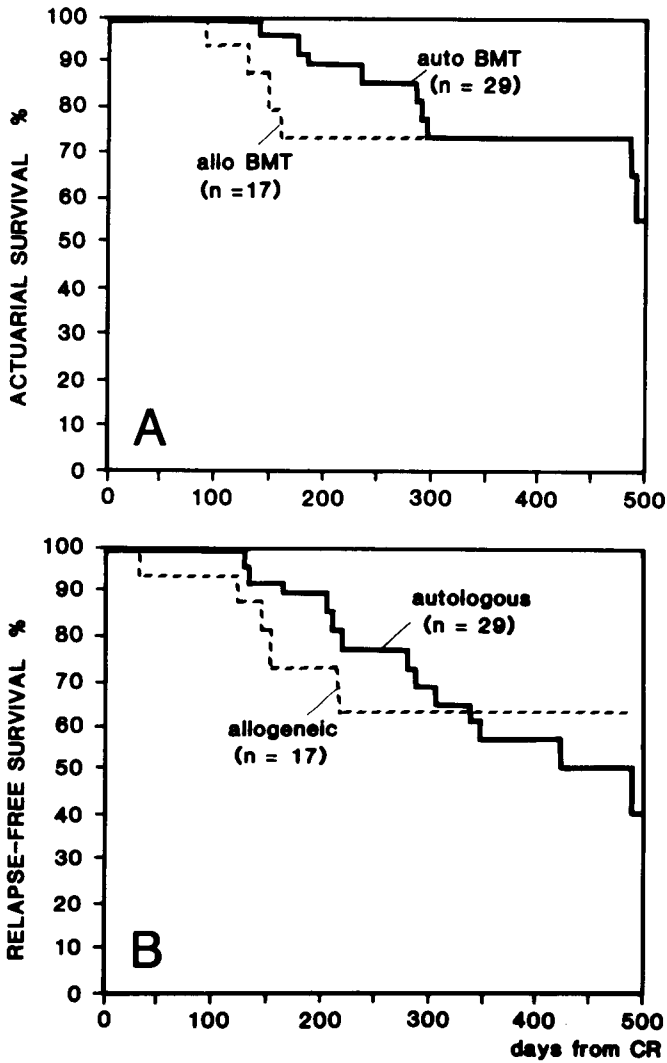

Figure 1. *Actuarial survival* (panel A) *and actuarial disease-free survival* (panel B) *in patients with AML in first remission following autologous versus HLA-matched bone marrow transplantation in a prospective comparative study.*

host rather than from residual AML cells in the autograft. Registration data have not indicated a survival benefit for patients treated with purged marrow as compared to results with nonpurged bone marrow. This fact argues further against the effectiveness of purging and casts doubt on the contribution of the grafted contaminant AML cells to relapse. We decided to determine the natural history of ABMT with nonpurged marrow in first remission AML. At present, our results show continuous remission at a median of 300 days of follow-up in more

than 50% of patients. The study does not reveal differences in survival or remission durations between ABMT and allogeneic BMT, but longer follow-up is clearly needed.

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BAVC Regimen and Autologous Bone Marrow Transplantation in Patients With Acute Myelogenous Leukemia in Remission

Giovanna Meloni, Paolo de Fabritiis, Alessandro Pulsoni, Antonella Sandrelli, Francesco Malagnino, Maria Concetta Petti, Alfredo Covelli, and Franco Mandelli

High-dose radio- and chemotherapy followed by autologous bone marrow transplantation (ABMT) probably represents the most effective approach for relapse of acute myelogenous leukemia (AML). The majority of patients achieve a second complete remission (CR2), which is unfortunately of short duration (1): less than 5% are long-term survivors. However, a second course of treatment with marrow rescue can result in long-term CR2 (2). Recently, following the model of allogeneic bone marrow transplantation (BMT) to prolong the disease-free survival of AML patients (3), high-dose cytoreductive therapy and ABMT, in an early phase of the disease, have been anticipated as being a very intensive consolidation program during first complete remission (CR1) (4).

Following a previous experience of AML patients in first relapse (5), AML patients in CR1 or CR2 were entered into a clinical trial of ABMT and BCNU (carmustine), amsacrine (*m*-AMSA), VP-16-213 (etoposide), and cytarabine (ara-C) (BAVC)-conditioning regimen at the Institute of Hematology in Rome. From March 1984 to November 1986, 39 patients were accrued.

PATIENTS AND METHODS

The techniques of marrow collection, cryopreservation, and thawing have been previously described (5). Pretransplant chemotherapy consisted of the BAVC regimen (Fig 1): BCNU (carmustine) (800 mg/m^2 on day 1), amsacrine (150 mg/m^2 i.v. on days 2 to 4), VP-16-213 (etoposide) (150 mg/m^2 i.v. on days 2 to 4), cytarabine (150 mg/m^2 i.v. every 12 hours from days 2 to 4). Marrow cells were reinfused 24 hours after the last dose of chemotherapy.

Of the 39 AML patients who underwent ABMT, 30 (11 were females and 28 were males) were in CR1. The median age was 27 years (range, 1-14 years). Nine patients (four of whom were females) were treated while in CR2. Two patients in this group also had M_3 leukemia. The median age for patients in CR2 was 24 years (range, 10-47 years). The median durations of CR before transplantation were 5 months (range, 1-14 months) and 4 months (range, 1-25 months) for patients receiving treatment in CR1 and CR2, respectively.

Eighteen patients (60%) who underwent ABMT during CR1 received the same induction and consolidation treatment, which consisted of two cycles of daunorubicin (DNR) plus cytarabine (3 + 7 and 2 + 5, respectively) followed by four courses of DNR plus cytarabine plus 6-thioguanine, with an escalating dose of cytarabine from cycles 1 to 4. The other 12 patients received the same induction but different consolidation therapies, which, in 6 of them, consisted of high-dose cytarabine plus DNR. Marrow was collected and cryopreserved immediately before starting pretransplant chemotherapy. Four patients, whose leukemic clonogenic cells totally reacted to an antilactofucopentaose III S4-7

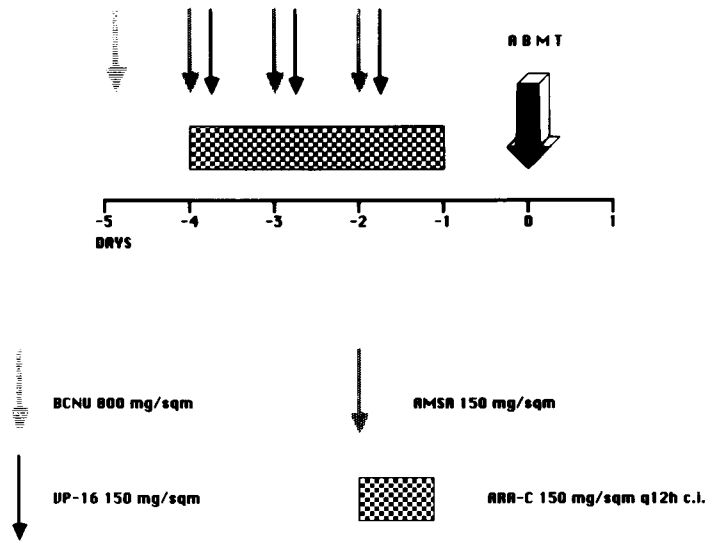

Figure 1. *BAVC regimen.*

monoclonal antibody (MAb), underwent ABMT while in CR1 after in vitro purging with S4-7 MAb and complement (6).

All patients were treated in single rooms and received bowel decontamination with nonabsorbable antibiotics. All blood products were irradiated before infusion to prevent possible graft-versus-host reaction. Broad-spectrum antibiotics were given for fever during aplasia, and amphotericin B was added for documented systemic fungal infection or for persistent fever during aplasia. Patients who were at risk for the recurrence of herpes simplex virus infection received prophylactic oral acyclovir. Before starting pretransplant chemotherapy, a central venous catheter was inserted for the administration of fluids, antibiotics, and blood products in all patients. Maintenance chemotherapy after ABMT was not employed.

RESULTS

All 39 patients obtained hematologic reconstitution (Table 1). No relationship was observed between the time of recovery of granulocytes and the number of transplanted marrow cells, which contrasts with our experience that platelet recovery seems to be related to the number of reinfused mononuclear cells. A longer delay in platelet recovery was observed in the four patients who received marrow-purged cells.

Toxic reactions to the BAVC regimen were acceptable with no life-threatening problems (Table 2). Nausea and vomiting were common and seven patients developed severe mucositis, which made them unable to eat. Thirty-two patients were febrile during aplasia (in 14 [44%], fever was associated with positive cultures for bacteria) and responded to broad-spectrum antibiotic therapy. In one case of resistant fever, fungal pneumonitis was identified and successfully treated with amphotericin B. Clinical symptoms of pulmonary distress, including rapidly progressing dyspnea, dry cough, and tachypnea were observed in five out of nine patients younger than 15 years old 2-3 months post-ABMT. Pulmonary function test revealed arterial hypoxemia, marked

Table 1. Hematologic Recovery After Autologous Bone Marrow Transplantation in AML Patients With BAVC Conditioning

Remission Status	Mononuclear Cells Infused × 10^8/kg		PMN >500 μl (in days)		PLTS >50,000 μl (in days)		Hospitalization (in days)	
	median	(range)	median	(range)	median	(range)	median	(range)
CR1 (30 patients)	1.2	(0.13-2.8)	18	(10-31)	46	(16 - >250)	26	(21-42)
CR2 (9 patients)	1.7	(0.25-3)	15	(11-34)	38	(18 - >100)	26	(21-39)

Abbreviations: AML, acute myelogenous leukemia; BAVC, BCNU, amsacrine, VP-16-213, cytarabine; CR1, first complete remission; PMN, polymorphonuclear neutrophils; PLTS, platelets; CR2, second complete remission.

Table 2. Toxic Reactions to BAVC Regimen

Reactions	No. of Patients
Nausea and vomiting	39
Diarrhea	13
Mucositis	
Mild	20
Severe	7
Dysrhythmia	3
Transient liver alteration	9
Fever (>38° C)	32
Pneumonitis (fungal)	1
Positive cultures	
Blood	
Staphylococcus	5
Escherichia coli	1
Streptococcus	2
Urine	
Pseudomonas	1
Escherichia coli	1
Streptococcus	1
Klebsiella	1
Proteus	1
Staphylococcus	1
Nasal	
Aspergillus	2

Abbreviation: BAVC, BCNU, amsacrine, VP-16-213, cytarabine.

reduction of residual volume and total lung capacity, and decreased CO_2 diffusion; a pattern of diffuse interstitial infiltration was observed in chest x rays of three patients. Bacterial, viral, and fungal etiologies were excluded in all patients by culture and serologic tests. Low-dose steroid treatment was given, resulting in disappearance of all clinical signs and x-ray normalization in all patients. Periodic lung-function studies showed progressive improvement of restrictive pattern with return to pretransplant values after 6-10 months.

As of November 1986, the conditions of 29 of the 30 patients treated in CR1 (Fig 2) were evaluable for the duration of the CR, because of one early death (pulmonary thromboembolism on day 21 after ABMT). Twenty-two patients (73%) are alive in unmaintained continuous complete remission (CCR), with a median follow-up of 7 months (range, 1-33 months). Seven patients (23%) experienced a relapse at 1, 4 (two patients), 7 (two patients), 11, and 25 months: three achieved a CR2 and were consolidated with a second ABMT, which employed a different conditioning regimen with busulfan plus Cytoxan (8). Two patients obtained complete hematologic reconstitution and are in unmaintained CCR 8 and 4 months after the second ABMT. The third patient died of cerebral hemorrhage 2 months after the second ABMT, with

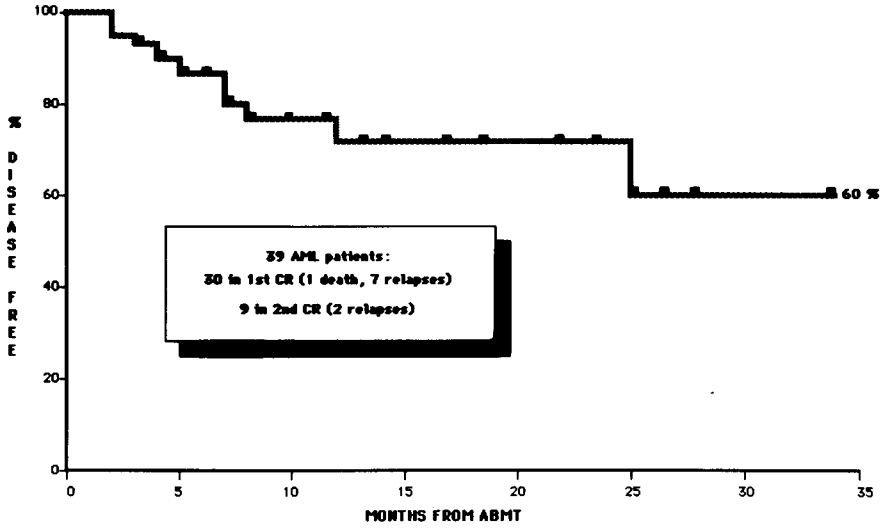

Figure 2. *Autologous bone marrow transplantation in 39 patients with acute myelogenous leukemia.*

normal marrow but persistent thrombocytopenia. None of the patients who received marrow treated in vitro with S4-7 MAb had a relapse. Of the nine patients who underwent ABMT while in CR2, two relapsed after 3 and 6 months, and seven patients (77%) are in unmaintained CCR with a median follow-up of 12 months (9).

DISCUSSION

In our experience, high-dose chemotherapy regimen, BAVC, produces very few toxic reactions. Preliminary results are encouraging, especially for patients undergoing ABMT in CR2. A longer follow-up is necessary to assess the antileukemic efficacy of BAVC in CR1 AML patients: 73% of 30 patients who underwent ABMT are in CCR, but median follow-up is only 7 months.

The value of pharmacologic purging has not been demonstrated (10); very few reports have been published (11) on immunologic purging techniques in AML, and it is too early to evaluate their applicability and efficacy. Our preliminary experience with S4-7 MAb purging in four patients seems satisfactory, but the number of patients is too small and follow-up is too short to be able to draw any conclusion.

We are now undertaking a pluricentric cooperative trial in which AML patients, after achieving CR, will be randomly allocated to either ABMT or aggressive conventional chemotherapy.

Although promising, results of ABMT in AML patients who are in CR1 are difficult to evaluate, mainly because of the small number of cases reported from

various institutions (4). Moreover, several other issues contribute to jeopardizing data analysis: different conditioning regimens (with or without total body irradiation); whether marrow purging is valuable; time to transplant from CR; and different induction or consolidation therapy or both before ABMT. Finally, the main question to be answered still remains the value of ABMT versus conventional chemotherapy or allogeneic BMT or both. Prospective randomized trials are necessary to clarify this important issue.

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Use of Bone Marrow Incubated With Mafosfamide in Adult Acute Leukemia Patients in Remission: The Experience of the Paris Saint-Antoine Transplant Team

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Transplantation of allogeneic bone marrow from siblings with identical human leukocytic antigens has recently been shown to improve the prognosis of patients with acute leukemias (1-3). Autologous bone marrow transplantation (ABMT) may offer a similar chance to most patients who have no available donor. But, a major impediment to effective ABMT may be the persistence of leukemic cells in the marrow, even though it is collected when the patient is in complete remission (CR). It is generally assumed, therefore, although not yet proved (4), that the collected marrow should be cleansed before reinfusion.

Following the initial report on the efficacy of cyclophosphamide (CY) derivatives in eliminating residual marrow leukemic cells in the brown Norway myelocytic leukemia rat leukemia model (5), we studied the in vitro effect of Asta Z 7557 (International Nonproprietary Name, mafosfamide); kindly provided by Professor N. Brock (Asta-Werke, Bielefeld, Federal Republic of Germany) on human leukemic and normal hematopoietic stem cells (6) and initiated a therapeutic trial using ABMT with marrow incubated with this drug as

consolidation therapy for adults with acute leukemia in remission. We had two goals: 1) to treat each patient's marrow with the highest possible dose of mafosfamide to achieve maximum antileukemic activity, but also to spare enough normal stem cells for successful engraftment; and 2) to assess the potential benefit of high-dose consolidation treatment followed by ABMT with cleansed marrow, in terms of duration of remission and disease-free survival.

We report here the current status of our clinical trial with 57 patients treated while they were in CR with CY and total body irradiation (TBI), followed by the reinfusion of autologous bone marrow treated with mafosfamide at a dosage predetermined to spare 5% colony-forming units granulocyte-macrophage (CFU-GM LD_{95}).

MATERIALS AND METHODS

Details on in vitro studies, determination of the dose of mafosfamide for each patient (by preincubation test [PIT]), marrow incubation, marrow freezing, pretransplant regimen, and ABMT procedure have been described previously along with the preliminary clinical results in a first series of 24 patients (7). In short, for each patient's PIT a 10-ml marrow aspirate was taken 15 days before bone marrow collection. The sensitivity of remission CFU-GM to increasing doses of mafosfamide was studied and a dose-response curve established. The optimal dose for later incubation of the marrow was defined as the dose sparing 5% CFU-GM as measured by the PIT. This dose was selected from previous continuous liquid culture studies as the highest possible dose that would spare enough normal stem cells to ensure consistent engraftment; the residual amount of 5% CFU-GM was considered a safe margin beyond which further cytotoxicity would not be measurable, at least by conventional laboratory means.

Marrow collection took place in patients in CR after one to three consolidation courses. A total volume of 600-1,300 ml of bone marrow was collected from the posterior iliac crests under general anesthesia. A volume corresponding to 0.5×10^8 nucleated cells/kg was saved and directly cryopreserved to serve as a back-up marrow. The remaining volume was further processed with mafosfamide for incubation. The buffy coat was collected on a Haemonetics H-30 cell separator (Haemonetics, Plaisir, France) and adjusted with TC 199 medium to a final cell concentration of 2×10^7 cells/ml with a final hematocrit of 5%. The suspension was finally incubated with mafosfamide, at the concentration previously established from the PIT, for 30 minutes in a water bath at 37°C and with gentle shaking. In the freezing procedure, dimethyl sulfoxide was used at a final concentration of 10% in TC 199 medium, Teflon-capton DF 1000 Gambro bags (Gambro, Paris, France), and a Nicool 316 programmed biological freezer (CFPO, Sanssenage, France). The cooling program followed modern principles of stem-cell cryopreservation, as described by us and others (8).

All patients received the same basic, intensive pretransplant regimen consisting of CY, 60 mg/kg/day × 2 plus mesna, 60% of the CY dose; and TBI, 10 Gy with lung shielding at 8 Gy.

Twenty-four hours after TBI, the bags of frozen purged marrow were thawed rapidly in a water bath at 37°C and the marrow infused immediately. Day 0 was defined as the day of marrow infusion. No maintenance chemotherapy was used after autografting.

Between January 1983 and October 1986, 57 patients entered the study; 37 were men. The patients' age range was 6-55 years, the median age being 35 years; 15 patients were older than 40 and 4 younger than 15 years of age. Of acute lymphocytic leukemia (ALL) patients in first remission (CR1), 5 were considered standard-risk and 13 were considered high-risk patients; 3 ALL patients were in second remission (CR2). Of acute myelocytic leukemia (AML) patients in CR1, 26 were considered standard-risk and 6 high-risk patients; 4 of the AML patients were in CR2. For patients in CR1, the median interval from remission to ABMT was 6 months (range, 2-15 months), and for patients in CR2 the median interval was 4 months (range, 0.5-12 months).

Criteria for the high-risk classification were: *At diagnosis*—leukocytes >25 × 10^9/l, mediastinal enlargement on chest x ray, presence of a Philadelphia chromosome, extrahematopoietic localization (other than in CNS), and secondary leukemia; *before ABMT*—CNS involvement at any time; *at ABMT*—evidence of partial remission and no CR, lactic dehydrogenase of 1,000 U.

Of 20 patients in the high-risk category, 12 had two or more of these conditions. By usual transplantation standards, the population of patients was relatively old since 15 patients were over 40 years of age. Twenty-one patients had ALL (CR1, 18; CR2, 3), and 36 had AML (CR1, 32; CR2, 4). The median intervals from diagnosis and remission to ABMT were 6 months for the ALL and 4 months for the AML patients. Longer delays arose mainly from difficulties in harvesting rich CR marrow with no residual leukemic cells detectable by appropriate means and from inappropriate incubation procedures with mafosfamide that led to residual CFU-GM fractions not in keeping with the study design.

RESULTS

Patients With Acute Myelogenous Leukemia

Of the 26 patients with standard-risk AML who received autografts during their CR1, 18 (69%) have remained disease free so far, and they have been followed up a median of 11 months (range, 4-42 months). Seven patients have a follow-up of longer than 1 year, five beyond 2 years, and three beyond 3 years. Eight patients died of toxic reactions, five during the ABMT procedure (two as a result of sepsis, one of viral infection, one of graft failure, one of liver veno-occlusive disease [VOD]), and three patients died while in continuous complete

remission (CCR) at 4, 6, and 16 months after transplant (two of viral infections and one of lung fibrosis).

Interestingly, except for one patient in whom leukemic cells were detected in the liver, no other leukemic recurrence or infiltration was documented.

Disease-free survival and the disease-free probability (DFP) since remission were 58% and 93%, respectively, at 4 years (Figs 1 and 2).

Of the six patients in the high-risk category in CR1, one had persisting leukemia and died 3 months after ABMT, one died of severe thrombocytopenia and gastrointestinal bleeding at 12 months after transplantation, and four are in CCR at 1, 1, 6, and 7 months afterward.

Of the four patients in CR2 who received grafts, one died of cytomegalovirus (CMV) infection in CCR at 5 months, two relapsed at 2 and 4 months, respectively, and one patient who has AML M_3 has remained disease free for more than 26 months.

Patients With Acute Lymphocytic Leukemia

Of the 18 ALL patients in CR1 who received autografts, 10 (55%) are alive and disease free after a median of 8.5 months (1-41 months). Among four patients followed longer than 1 year are three with a follow-up beyond 2 years.

Two patients died of toxic effects during the ABMT procedure (one of sepsis and one of VOD). Six patients relapsed at 3, 5, 8, 14,19, and 29 months after transplantation. The three relapses after 1 year post-ABMT occurred in patients in the standard-risk category, who had the longest follow-up. Disease-free survival and DFP at 3 years were 40% and 45%, respectively (Figs 1 and 2).

Of the three patients in CR2 who received grafts, one relapsed at 4 months and two remain in CCR 4 and 5 months after undergoing the procedure.

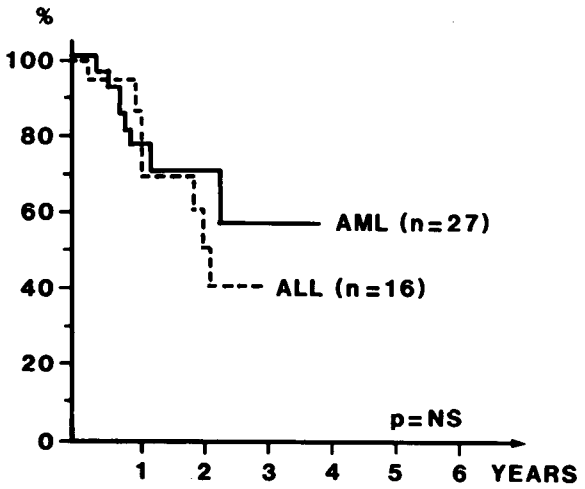

Figure 1. *Comparison of disease-free survival of patients with acute lymphocytic leukemia and acute myelogenous leukemia in first remission.*

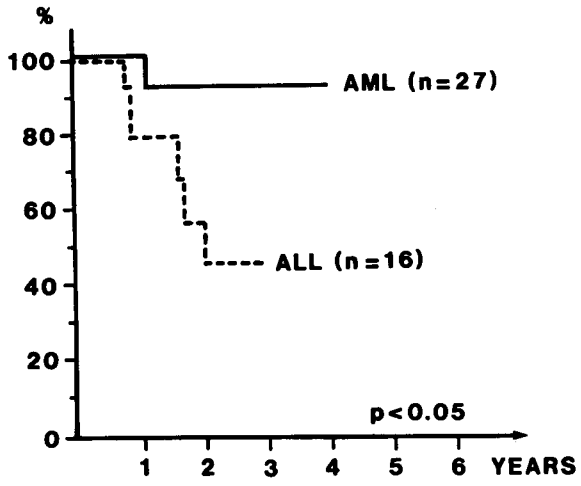

Figure 2. *Comparison of disease-free probability for patients with acute lymphocytic leukemia and acute myelogenous leukemia in first remission.*

Kinetics of Engraftment

The kinetics of engraftment were considerably delayed in patients with AML compared with those for the ALL group (Table 1). Thrombocytopenia, arising from hemorrhage or transmission of CMV or herpesvirus by unscreened donors, contributed to the death of five AML patients. In contrast, recovery of blood cell counts occurred rapidly in patients with ALL, none of whom died of viral complications while in CCR.

Complications

During the ABMT procedure and immediately afterward, the spectrum of complications was dominated by bacterial and viral infections (Tables 2 and 3).

Table 1. Differences in Kinetics of Engraftment in Relation to Diagnosis

	Median No. of Days to Recovery (Range)		
	AML	ALL	*P*
WBC >10^9/l	30	19	
	(13 - 66)	(15 - 30)	<.001
Platelets >50 x 10^9/l	100[a]	47	
	(45 - 485)	(15 - 90)	<.01
Reticulocytes >0.1%	20	15	
	(12 - 39)	(11 - 25)	<.01

[a] *Includes five patients with prolonged thrombocytopenia (150, 150, 180, 475, and 485 days).*

Table 2. Patients With Acute Leukemia in Complete Remission: Early Complications of Transplantation Period (n = 57)

Infections	No. of Patients
Bacterial	
Sepsis	47
Other (localized)	6
Viral	14
Fungal	5
Liver veno-occlusive disease (3 deaths)	3
Other	6
PNO	2
Hematuric cystitis	2

Table 3. Patients With Acute Leukemia in Complete Remission: Late Complications of Transplantation Period (n = 57)

Infections	No. of Patients
Viral	14
Cytomegalovirus	4
Herpes zoster	7
Bacterial (7 pneumonitis)	7
Pneumocystis carinii	1
Toxoplasmosis	1
Fungal	8
Pneumonitis	5
Aspergillosis	4
Liver veno-occlusive disease (1 resolutive)	2
Other	14
Autoimmune disorders	7
Cataract	2
Hypothyroidism	1
Chromosomal abnormalities	3

The bacteria involved were mainly gram-positive (67%), with *Staphylococcus aureus* accounting for 44% of all infections. Among gram-negative bacteria, *Escherichia coli* was responsible for 17% of all infections. We had only one incident of *Pseudomonas*-caused sepsis.

In the late posttransplantation period, viral infections were the predominant risk factor. We observed seven autoimmune manifestations; these included monoclonal IgG spiking, three cases that were resolved; autoimmune pancytopenia and peripheral neuropathy, one, resolved by steroid therapy; autoimmune hemolytic anemia, one; hepatitis with graft-versus-host pathologic features,

one; and primary biliary cirrhosis, one. Except for the patient with graft-versus-host disease features, who died of gastrointestinal bleeding, all other autoimmune manifestations were resolved either spontaneously or with steroid therapy.

Three patients developed chromosomal abnormalities that, in two of them, involved chromosome 1. Two of these patients are disease free at 11 and 41 months after transplantation: one still has chromosomal abnormalities in a small faction of marrow cells; in the other, these disappeared 10 months after the surgery. The third patient, who had ALL, relapsed and died 19 months after transplantation without resolution of the chromosomal abnormalities. We postulated that marrow incubation with mafosfamide is responsible for the genesis of these aberrations.

CONCLUSIONS

We drew the following conclusions from the present status of our clinical trial:

1. In adult patients with acute leukemia in first remission and who do not have identical siblings, ABMT may offer a chance similar to that of allogeneic bone marrow transplantation.

2. Results at the moment look more favorable for AML than for ALL patients, with disease-free periods of 58% at 4 years for the former and 40% at 3 years for the latter. Strikingly enough, the very low incidence of leukemic relapses in standard-risk patients with AML in first remission (<5%) contrasts with a higher rate (>35%) as well as a later occurrence of relapse in patients with ALL.

3. The design of our study precludes any estimation of the value of our marrow-purging procedure—mafosfamide at dosage determined according to the sensitivity of stem cells in each patient. Obviously, an evaluation would have required a randomized trial to compare ABMT with purged and nonpurged marrow. Such trials are in progress in several institutions (9). Although results of the European Bone Marrow Transplantation Group (EBMTG) registry do not at the moment indicate any advantage of marrow purging over nonpurging (10), the low rate of leukemic relapses in our AML patients contrasts with the 42% incidence rate in the EBMTG study, suggesting that our study, which involves a small number of patients, should be pursued.

4. In AML, prolonged delays in platelet recovery increase the rate of deaths from toxic effects, which makes urgent a rapid evaluation of the antitumor effect of our marrow-purging procedure in order to consider reducing the dose of mafosfamide used (CFU-GM LD_{90} or lower).

5. Finally, late relapses in ALL may justify some increase in the therapeutic maneuvers. Consideration may be given to an increase in the pretransplantation consolidation regimen, perhaps with additional cytosine arabinoside; the introduction of maintenance chemotherapy after ABMT; and double autografting.

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Autologous Transplantation of Unpurged Bone Marrow During First Remission of Acute Myeloid Leukemia

Alan K. Burnett, Stephen Mackinnon, and Ann Morrison

In recent years, we and others have been investigating the use of ablative chemoradiotherapy or chemotherapy alone with autologous marrow infusion as an alternative strategy of consolidating the first remission in patients with acute myeloid leukemia (AML). We are encouraged by the prospect of using autologous bone marrow transplantation (ABMT) because of the 70-90% of patients remaining leukemia-free after allogeneic bone marrow transplantation. We recognize that potentially important components are involved in the allograft that would not be present in the autologous situation. One in particular is the loss of the postulated graft-versus-leukemia effect. Such an effect has been demonstrated experimentally, usually in lymphoid leukemia models (1), and a statistical prediction of a higher prospect of remaining leukemia-free along with a higher grade of graft-versus-host disease (GVHD) was shown by the Seattle group (2) in patients with relapsed leukemias and those with acute lymphoid leukemia in remission but not in patients with AML in remission. This association may be difficult to demonstrate, however, when the overall relapse rate is as low as 20%. Some evidence suggests that chronic GVHD may be associated with a lower relapse rate (3). Perhaps the more important evidence to indicate an allogeneic effect is the increased relapse rate (50%) observed in

syngeneic transplants for AML in first remission (4). More recent experience with the use of T-lymphocyte depletion, which provides protection from GVHD, may show higher rates of relapse in AML similar to those already observed in chronic granulocytic leukemia after the use of this technique (5).

For many researchers and clinicians, the major conceptual objection to autografting in acute leukemia is the assumption that, because relapse of disease seems inevitable, the autograft is certain to contain occult leukemia cells that will inevitably result in disease recurrence. This traditional view may not be inconsistent with the possibility of achieving a measure of success. In addition, a proportion of the leukemic blast cells are lost during storage. Few clonogenic cells may remain to be reinfused if it is considered that no more than 1-2% of marrow cells are harvested from the patient in remission who may have, at most, a similar percentage of blast cells present, of which fewer than 10% are clonogenic, and they may even seed to the irradiated marrow microenvironment less efficiently than normal hematopoietic precursors. Although it cannot be denied that residual leukemic cells contaminating the marrow may result in relapse, perhaps the major problem remains eradication of the disease from the patient. There should be little doubt that this is the major obstacle to success, as indicated by the fact that currently used regimens failed to eradicate the disease in half the syngeneic grafts.

In the majority of clinical studies done in recent years, no attempt was made to remove occult disease from the autograft. Whether there are effective ways of doing this remains debatable. These studies indicated that about 50% of patients remain disease free (6,7). Although these patients require longer follow-up, the negligible difference from syngeneic results suggests that residual disease in the graft may currently be a minor issue.

A valid criticism of the autograft data so far is that several of the procedures were done for patients who had already been in remission for several weeks and therefore had an improved prognosis anyway.

There is little doubt that, to ensure the best results, first remission is the optimum time for autograft. But timing is still one of the important remaining issues: When in first remission should the autograft be incorporated into the overall treatment protocol?

THE GLASGOW AUTOGRAFT EXPERIENCE—AN UPDATE

Since 1981 in Glasgow, patients with AML in first remission have been offered ablative chemoradiotherapy as remission consolidation. If they were younger than 40 years and had a donor with identical homologous leukocytic antibodies, allogeneic marrow transplant was the treatment of choice. Those who did not have a donor and were up to 55 years old were offered autograft.

Twenty-three patients have received ABMT. Before the procedure, all patients received induction and consolidation chemotherapy involving daunorubicin, cytosine arabinoside, and 6-thioguanine, but the number of courses and the durations of remission before the autograft differed.

The first 13 patients received two doses of 50 mg/kg of cyclophosphamide and 950 cGy as a single fraction in total body irradiation (TBI), at a low-dose rate of 5.5 cGy/minutes. The autologous marrow was stored at 4°C for 54 hours before reinfusion. To improve the antileukemic effect, the next 10 patients were given 110 mg/m^2 of melphalan, 6 x 200 cGy of TBI with lung shielding to 1,000 cGy, and the cryopreserved autologous marrow.

Overall, nine patients died, eight of recurrent leukemia and one of a cerebral hemorrhage after 36 months of complete remission (CR) following the graft. Of the 14 living patients, one relapsed but has been in second remission for 12 months, and one patient had a leukemic regeneration but is stable 3 months later. The other 12 patients remain in CR, 8 between 24 and 60 months after the autograft. The predicted overall survival rate is 52%, and leukemia-free survival rate is 54% at 5 years (Fig 1).

The leukemia-free survival rate of patients in the cyclophosphamide plus TBI group was 49% and in the melphalan plus TBI group, 51%, although the latter patients have had shorter follow-up. Those who had received more chemotherapy before autograft (but who were in remission longer) tended to have fewer relapses. Of the 13 patients who received autografts within 5 months of achieving CR, 7 have relapsed, whereas of the 10 patients autografted beyond 5 months of CR, 3 have relapsed.

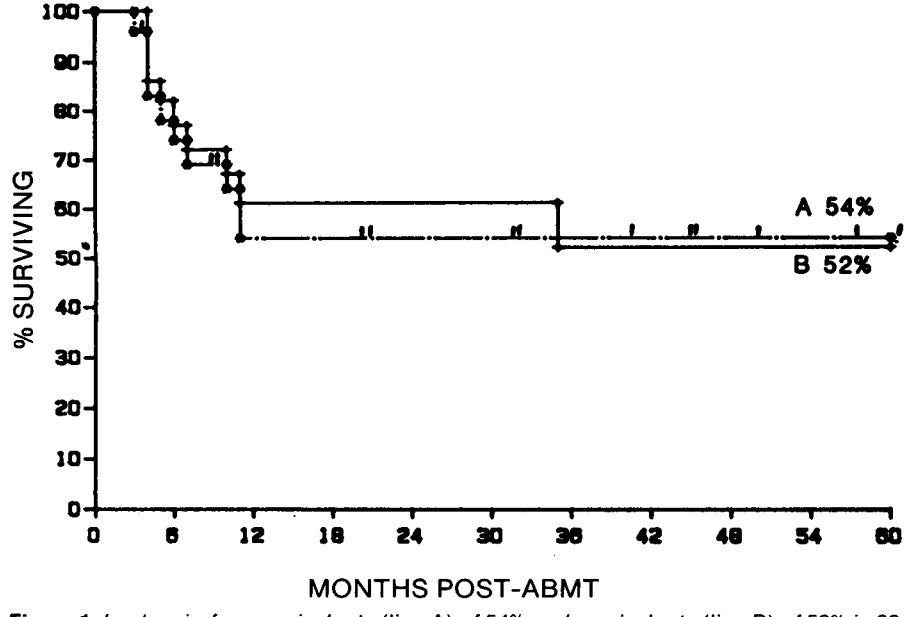

Figure 1. *Leukemia-free survival rate* (line A) *of 54% and survival rate* (line B) *of 52% in 23 patients with consecutive autografts for acute myeloid leukemia in first remission. Data from the Glasgow study (6).*

RELATIONSHIP OF PREAUTOGRAFT INTERVAL TO LEUKEMIA-FREE SURVIVAL

To examine the relevance of preautograft delay, we reviewed our experience combined with that of six other series that have been reported.

The data were from 95 patients whose characteristics are shown in Table 1. Cyclophosphamide plus TBI was the chosen ablative protocol for 58 patients; 23 received high-dose combination chemotherapy, and 14 underwent double autografts and high-dose chemotherapy. No measures were taken to treat the autograft in vitro in an attempt to remove residual leukemia. Twenty-nine patients experienced relapse, all but two of these occurring within 12 months of the autograft. Five patients died of causes other than leukemia. Figure 2 shows the overall predicted survival and leukemia-free survival rates at 5 years.

The period of remission that had elapsed before the autograft was variable, even within treatment centers, in this group of patients. To assess the influence of preautograft delay on leukemia-free survival, the patients were divided into groupings whose delay was 0-4 months, 5-8 months, and 9-12 months. Excluded from the analysis were patients whose delays were longer than 12 months and patients who died of reactions.

Predicted disease-free survival rates shown in Figure 3 are for the 31 patients in the 0- to 4-month delay group, 40 patients in the 5- to 8-month delay group, and for the 14 patients in the 9- to 12-month delay group.

DISCUSSION

As a strategy for maintaining remission in AML and an alternative to traditional chemotherapy, ablative treatment necessitating ABMT clearly merits consideration. Although 40-50% of patients have relapsed, relapses have been uncommon beyond 12 months after the autograft, suggesting that the pattern of relapse previously observed with chemotherapy is altered. Since the relapse rate so far is not higher than that of syngeneic-graft recipients with AML in first remission, there is little reason to believe that problems of leukemia contamination of the autograft will be a priority in the immediate future. Preliminary clinical results of efforts to purge the marrow do not yet show an advantage over the unpurged results (7).

Current results of autograft are encouraging and the toxicity is acceptable. Autograft may be more tolerable for patients and a better use of treatment resources than the alternatives of prolonged intensive chemotherapy (13,14).

Improved methods of eradicating residual disease in the patient seem to be a concern of immediate priority. Although augmented TBI has been relatively ineffective in reducing the relapse rate of patients who received allografts during relapse (15), these may be more successful if applied during remission. Equivalent chemotherapy used with allografting during first and second remissions has an impressive record of eradicating residual disease (16).

Table 1. Autologous Bone Marrow Transplantation for AML Patients in First Remission Using Unpurged Marrow

Center	No. of Patients	Age (range)	Protocol	Pre-ABMT Interval (wks)	Relapse (no. of patients)	Deaths From Toxicity
Glasgow (Burnett and Mackinnon [6])	23	14-53	Cyclo + TBI Melph + TBI	16-90	10	1
London (Anderson, Linch, and Goldstone [8])	16	19-55	BACT × 1 (10) BACT × 2 (6)	4-43	5	0
Rotterdam (Lowenberg *et al.* [11])	17	15-57	Cyclo + TBI	4-52	4	0
Seattle (Stewart *et al.* [9])	13	13-38	Cyclo + TBI	4-56	8	2
Rome (Mandelli *et al.* [10])	13	12-37	BAVC	22-60	1	1
Genoa (Carella *et al.* [12])	5	22-44	Cyclo + TBI	13-37	0	1
Marseilles (D. Maraninchi, personal communication, 1986)	8	10-52	Melphalan × 2	9-26	1	0

Abbreviations: Cyclo, cyclophosphamide; Melph, melphalan; TBI, total body irradiation; ABMT, autologous bone marrow transplantation; BACT, carmustine, cytarabine, cyclophosphamide, 6-thioguanine; BAVC, BCNU, amsacrine, VP-16-213, cytarabine.

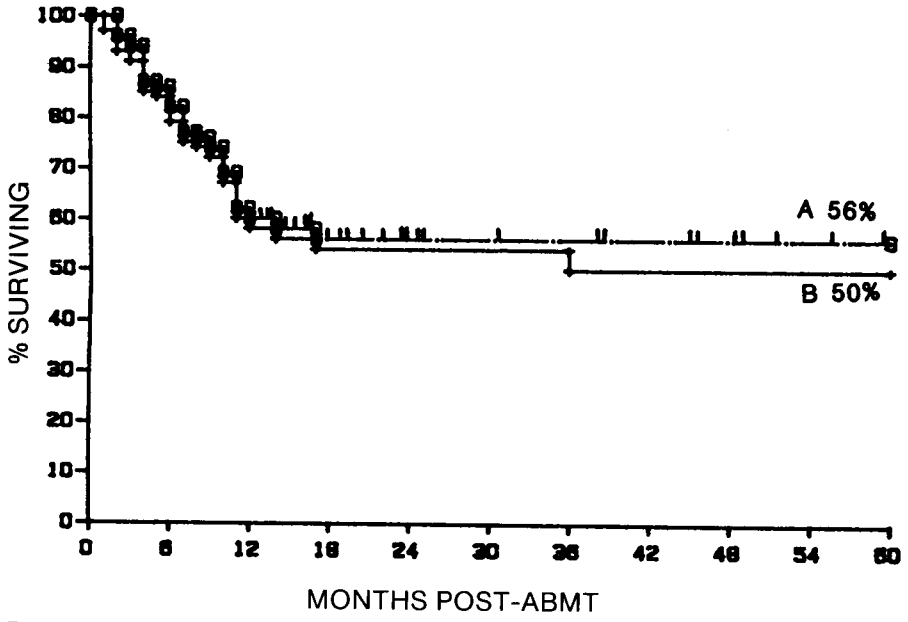

Figure 2. *Pooled data from seven centers (Table 1) of autograft using unpurged marrow from patients in first remission. Leukemia-free survival rate* (A) *is 56% and overall survival rate* (B) *is 50%.*

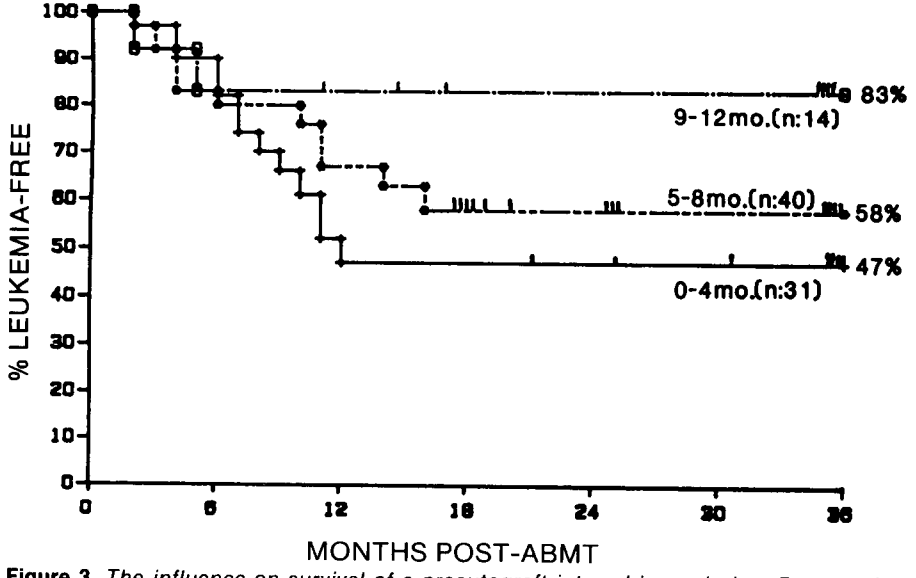

Figure 3. *The influence on survival of a preautograft interval in remission. Data are for predicted leukemia-free survival.*

The results achieved by autograft so far may be biased by the selection of patients who have been in remission (and are selected according to absence of relapse) for several weeks. The analysis suggests that timing of the autograft in first CR may affect outcome. This effect could be due to either selection or additional cytoreduction before the autografting procedure. Timing may be a relevant consideration in protocols aimed at elucidating the contribution of autograft in a randomized trial.

ACKNOWLEDGMENT

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Autologous Bone Marrow Transplantation for Acute Myelogenous Leukemia:
24 Unselected Patients in First Remission

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High-dose cytoreductive therapy followed by autologous bone marrow transplantation (ABMT) has been used in various pilot studies as an alternative to conventional therapy for acute myelogenous leukemia (AML) in first complete remission (CR1). In our study, each AML patient was administered the same induction regimen of daunorubicin, cytosine arabinoside, and 6-thioguanine (DAT). Each patient lacking a sibling with identical human leukocytic antigens (HLA) was included in the study if remission was obtained.

PATIENTS AND METHODS

Twenty-four patients with AML in CR1 were included in this pilot study: 14 adults with a median age of 29 years (range, 17-44 years) and 10 children with a median age of 5.7 years (range, 2-14.5 years). Each patient achieved CR1 following chemotherapy with DAT. Consolidation chemotherapy was administered with one course of daunorubicin and cytosine arabinoside. VP-16-213 (etoposide) or 1-(2 chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) was added after randomization. Only one patient received two courses of high-dose cytosine arabinoside as consolidation regimen.

Of 24 patients, 11 were at high risk of relapse owing to the following features at diagnosis: eight patients had the M_4-M_5 FAB subtypes (two with high WBC count and one with secondary leukemia), two had high WBC count (30×10^9/l), and one had initial CNS leukemia. Bone marrow was collected as soon as possible after consolidation therapy (average: 3.3 months after CR1). All but two harvested marrows were purged in vitro, using mafosfamide (Asta Z 7557) (1).

The preparative regimen consisted of high-dose polychemotherapy for 17 patients (6-thioguanine, aracytine [cytarabine], CCNU, cyclophosphamide [CY] [TACC]-modified), fractionated total body irradiation (TBI) (12 Gy, 6 fractions) followed by CY for six, and busulfan and CY for one (2).

RESULTS

Early complications have been previously described (3) with three early deaths occurring within the first month after transplant. Relapse occurred in 14 patients, 13 during the first year (12 in the group conditioned with chemotherapy and 3 in the group treated with TBI and CY). Seven patients are still in unmaintained continuous complete remission: 68+, 68+, 51+, 29+, 27+, 17+, 9+ months after ABMT, with a disease-free survival rate of 30% as shown in Figure 1.

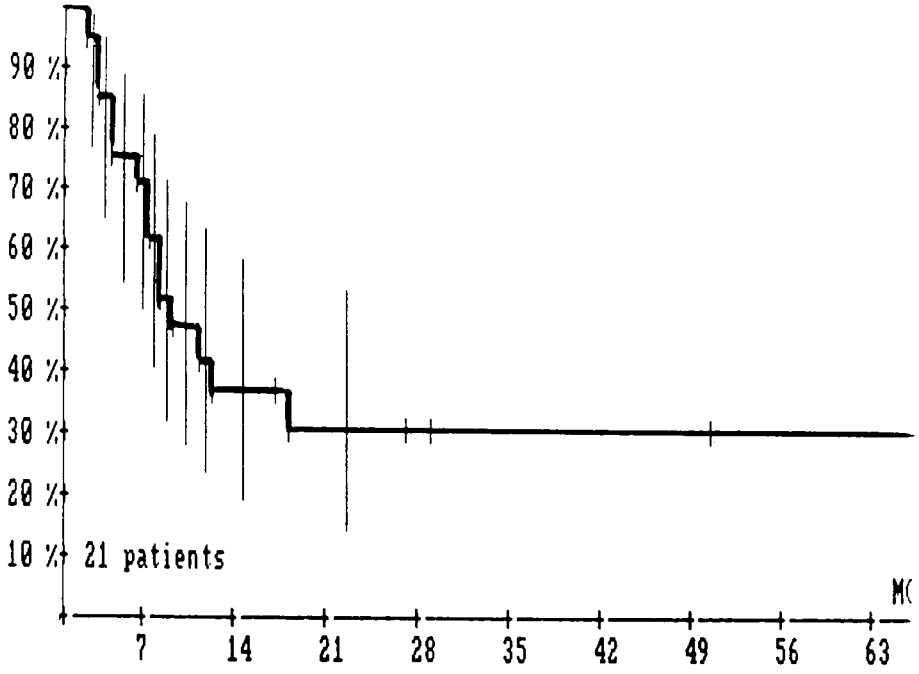

Figure 1. *Autologous bone marrow transplantation for acute myelogenous leukemia in first complete remission disease-free survival.*

DISCUSSION

The present study indicates that results of ABMT in CR1 are not different in this group of *unselected* patients from those expected following conventional therapy, with a relapse-free survival rate of 30%. These results differ from those published (4) and may be due to various reasons. The conditioning regimen with TACC chemotherapy may be inadequate. In vitro treatment may be insufficient, but the relevance of pharmacologic purging remains to be proved. Each patient was grafted as soon as possible after remission, and none relapsed between CR1 and the conditioning regimen. These problems must be considered and analyzed prospectively in randomized trials to determine the real impact of ABMT for ANLL in CR1. One year ago, a randomized study was begun in our cooperative study group, to compare chemotherapy versus TBI and CY followed by ABMT in patients lacking HLA-identical donors.

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Autologous Bone Marrow Transplantation in Acute Lymphocytic Leukemia

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In the last 10 years, conventional therapy for acute lymphocytic leukemia (ALL) in adults has not been satisfactory. Ninety percent of patients with high-risk ALL achieve complete remission (CR) with induction therapy, but no more than 20-30% of them are long-term survivors (1). Recently, several reports have demonstrated that intensive chemoradiotherapy followed by bone marrow transplantation is capable of either prolonging the CR phase in a substantial number of patients or curing the leukemia (2-4).

Between 1982 and 1986, an Italian Study Group started using autologous bone marrow transplantation (ABMT) in "poor-risk" ALL in first complete remission (CR1) and in ALL in second complete remission (CR2) for the patients in whom allogeneic bone marrow transplantation (BMT) was not available (5). However, it was clearly demonstrated that in the remission phase of ALL, the collected marrow may contain residual viable leukemic cells. Attempts to remove occult clonogenic malignant cells from autologous bone marrow have been made by physical, immunological, and pharmacological methods (6-11).

In our study, we evaluated the therapeutic efficacy of ABMT in 27 ALL patients using marrow "ex vivo" treated with Asta Z (mafosfamide) and

compared the results with those obtained in a group of patients autografted with unpurged marrow.

PATIENTS AND METHODS

Data on 27 ALL patients (16 CR1 and 11 CR2) who received autografts with purged marrow and 6 ALL CR1 patients who received transplants with untreated marrow were collected from eight Italian institutions. The criteria for CR1 patients in this study were: the hypercellularity at diagnosis (leukocyte count $>100 \times 10^9$/l), chromosomal abnormality, L_3 French-American-British subtype, extramedullary disease (with or without CNS involvement), and the patients' poor response to induction therapy with difficulty in achieving CR. The induction, consolidation, or maintenance therapy was heterogeneous in all patients; however, the median interval from the onset of CR to marrow collection was 2.5 months (range, 1-4 months). The characteristics of ALL patients are summarized in Table 1.

Preparative Regimen

The majority of patients (90%) received cyclophosphamide and total body irradiation (TBI) as a pretransplant ablative regimen: 60% received fractionated TBI (3 Gy × 4 days) and 40% received TBI (single dose of 10 Gy). The cyclophosphamide was administered at 200 mg/kg within 4 days (48% of patients) or 120 mg/kg within 2 days (52% of patients).

Bone Marrow Collection and Purging

Cells were aspirated from multiple sites on the posterior iliac crests of patients under general anesthesia. At least $2\text{-}3 \times 10^8$ cells/kg body weight was collected. The suspension was filtered and then centrifuged at 2,500 rpm for 15 minutes. The buffy coat was removed, and the cells were counted and resuspended in 20% autologous plasma and 80% TC 199 medium. To purify bone marrow suspension from the malignant cells, we used mafosfamide at a final concentration of 80-100 μg/ml.

Cells were resuspended at a concentration of 2×10^7/ml with the hematocrit ranging between 6% and 15%. Mafosfamide (80-100 μg/ml) was added to the bone marrow cells and the suspension was incubated for 30 minutes at 37° C in a water bath; the cells were kept at 4° C for 5 minutes and then centrifuged at 3,000 rpm for 10 minutes. The pellet was gently resuspended, and the cells were counted and diluted at a final concentration of 4×10^7/ml in 55% autologous plasma, 35% TC 199 medium, and 10% dimethyl sulfoxide for cryopreservation. Cells were transferred to Gambro hemofreeze bags for cryopreservation and frozen in a Nicool 316 programmed biological freezer at 1° C/minute until they reached 4° C, 2° C/minute until they reached 40° C, and finally, 5° C/minute until they reached 140° C. The bags were then stored in the liquid phase of a liquid nitrogen freezer.

Table 1. Patient Characteristics

	ABMT in ALL[a] (No. of Patients)	ABMT in CR1 ALL[b] (No. of Patients)
ABMT remission		
CR1	16	
CR2	11	
FAB classification		
L_1	5	1
L_2	13	2
L_3	4	1
ND	5	2
Immunologic class		
pre-B B	3	—
T	3	1
C	7	2
non-T	8	3
non-B	8	3
ND	6	—
Mean age at diagnosis (yr)	25	28
Range	17–56	22–44
Preparative regimen		
CY + TBI	24	6
Other	3	

Abbreviations: ABMT, autologous bone marrow transplantation; ALL, acute lymphocytic leukemia; CR1, first complete remission; CR2, second complete remission; FAB, French-American-British; CY, cyclophosphamide; TBI, total body irradiation.

[a] *Mafosfamide-treated marrow received by 27 patients.*

[b] *Untreated marrow received by six patients.*

Bone Marrow Transplantation

Before reinfusion, the cells were thawed by a rapid immersion in a water bath of 37° C. The viability of cells was evaluated by the trypan blue dye exclusion test and the cells were infused into patients 24 hours after completing the conditioning regimen and were reinjected at doses ranging from 0.9 to 2 × 10^8/kg. Engraftment was documented by the daily evaluation of hematologic recovery. None of the patients received chemotherapy after ABMT.

Supportive Care

All patients were hospitalized in laminar air flow rooms. During post-transplant aplasia, patients were supported with platelets and packed RBC transfusions that were previously irradiated (15-30 Gy). A broad spectrum of antibiotics, antimycotic and antiviral drugs, and gamma immunoglobulin

were administered until the patients were free of fever and the total leukocyte count rose above $1 \times 10^9/l$ with absolute neutrophils more than $0.5 \times 10^9/l$.

Statistical Analysis

Statistical calculations were performed according to the Kaplan and Meier method (12). The analysis of disease-free survival (DFS) included both patients who, before they could be discharged, had a relapse and patients who died during ABMT. The significance of data was calculated by the Wilcoxon rank sum test.

RESULTS

Figures 1-3 give the clinical results for DFS for ALL CR1 and CR2 patients. Among the 16 ALL CR1 patients with purged marrow, 6 had a leukemic relapse, 1 died of leukemia, 3 obtained CR post-ABMT with conventional therapy, and 1 will receive a second transplant in the near future. Two other patients died of renal failure and severe hemorrhage with sepsis during ABMT.

The DFS for ALL CR1 patients was 49.9% at 54 months. Six other ALL

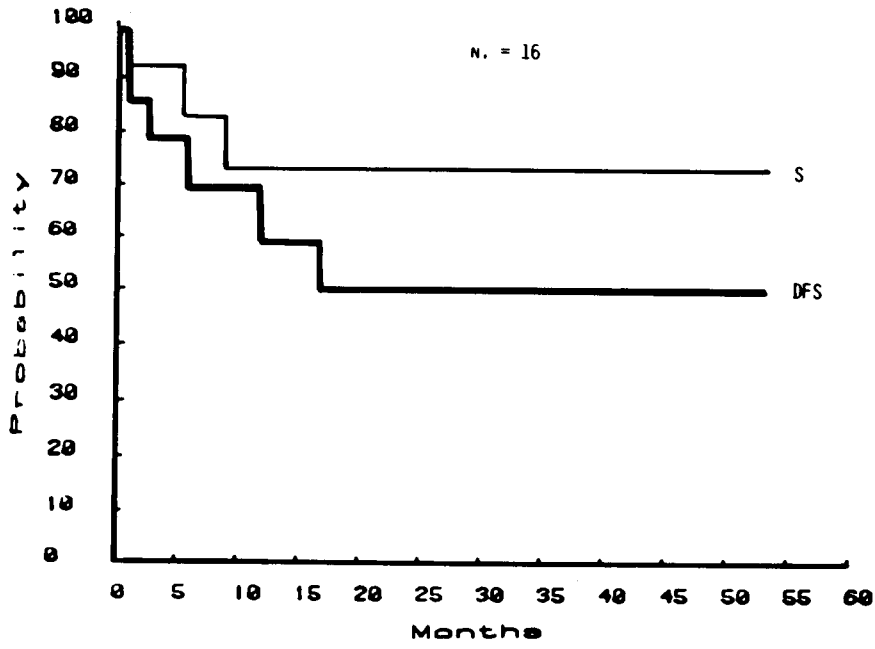

Figure 1. *Autologous bone marrow transplantation in acute lymphocytic leukemia (first complete response): survival and disease-free survival. Autograft was with mafosfamide-treated marrow.*

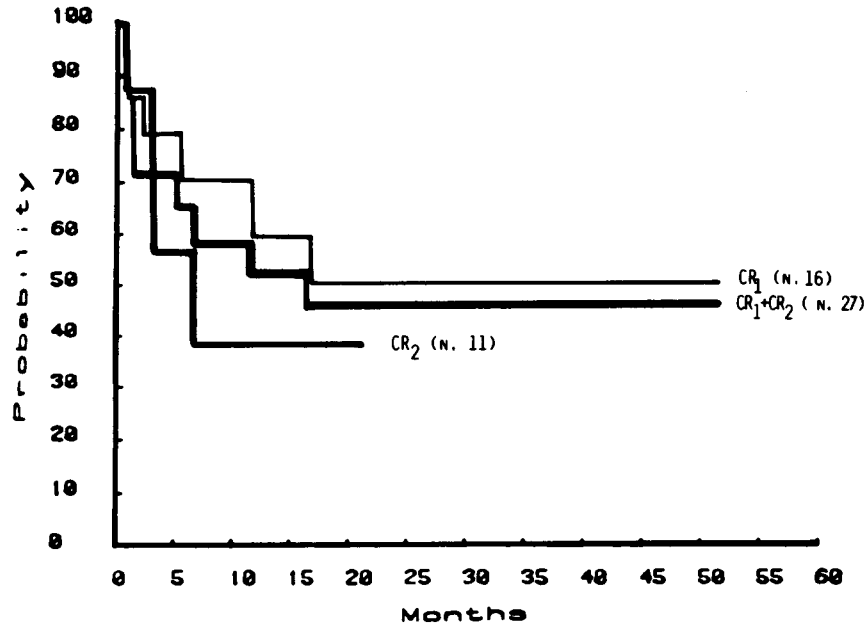

Figure 2. *Disease-free survival acute lymphocytic leukemia: first complete response vs. second complete response. Autograft was with mafosfamide-treated marrow.*

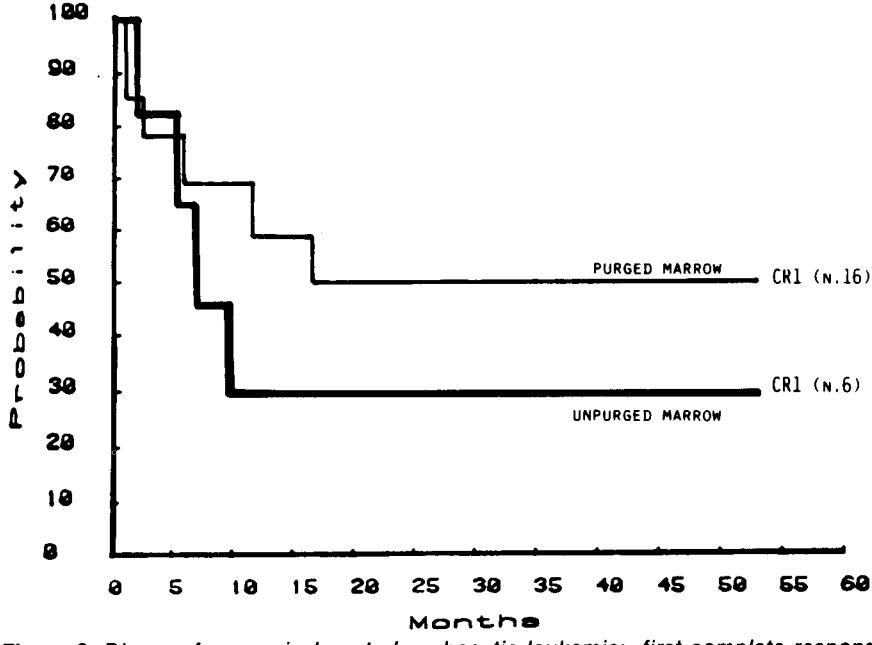

Figure 3. *Disease-free survival acute lymphocytic leukemia: first complete response. Autograft was with mafosfamide-treated marrow and untreated marrow.*

CR1 patients received transplants with unpurged marrow: 3 relapsed at 2, 6, and 8 months post-ABMT and died of leukemia. One patient died of sepsis during persistent aplasia. The DFS of these patients was 29% at 54 months. The statistical analysis of these data does not show the significant difference between the two groups; however, it does show a trend in favor of purged grafts.

The DFS of ALL CR2 patients was 38.9% at 21 months. Relapses occurred in four patients, two of whom died of leukemia. One patient who had a relapse achieved CR with conventional therapy and is undergoing a second ABMT. One patient died of sepsis before being discharged.

DISCUSSION

The ablative chemoradiotherapy with ABMT in ALL has improved the prognosis of poor-risk ALL patients. Recently, many authors (13-16) demonstrated that allogeneic BMT between siblings with identical homologous leukocytic antibodies has major advantages in the prognosis of ALL, when it is performed in CR1. Our results and that of others (17-20) confirm this finding in ABMT.

The technical approach of cleansing marrow pharmacologically is still under investigation. Following bone marrow treatment with mafosfamide, there is a need for a reliable method to measure the eradication of residual leukemia, which does not exist at the moment. On the other hand, the in vivo kinetics of hematologic recovery after purged ABMT is relatively short and does not show any significant difference with unpurged transplants. Delay of engraftment is essentially observed on platelets that reach $100 \times 10^9/l$ over the 65 days after transplantation. In vitro studies of mafosfamide toxicity on bone marrow demonstrate that the drug reduces the committed and pluripotent stem cell compartment but spares the totipotent hematopoietic progenitors (21).

The evaluation of the number of relapsed patients suggests the hypothesis that complete eradication of the leukemic cells in the host is not possible with the pretransplant regimen of cyclophosphamide and TBI. Santos and colleagues reported encouraging results in a group of ALL patients in third CR treated with chemotherapy alone (unpublished data, 1986).

The present report on 27 ALL patients autografted with mafosfamide-purged marrow does not demonstrate any significant difference between ABMT in CR1 and CR2; there is a trend in favor of CR1 ALL, but the number of cases is small and the follow-up is very short for patients transplanted in CR2 (Fig 2). Likewise, we observed a favorable trend for ALL CR1-purged ABMT compared with ALL CR1 unpurged grafts (Fig 3), but more observations are needed.

On the basis of our data, we conclude that ABMT-purged marrow in poor-risk ALL is a consolidation therapy of high efficacy. The DFS plateau

(46.5% for ALL and 49.9% for ALL CR1 at 3 years) (Fig 1) is better than both the reported results of ABMT and the allogeneic transplantation. It also defines ABMT as a valuable approach to the management of ALL.

The evaluation of real efficacy of ex vivo bone marrow treatment with mafosfamide needs more randomized clinical studies for a clear demonstration of the possibility to eradicate minimal residual disease.

ACKNOWLEDGMENT

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Studies of High-Dose Intensification and Unpurged Autologous Bone Marrow Transplantation in First Complete Remission Acute Leukemia

Karel A. Dicke, Sundar Jagannath, Leonard J. Horwitz, Gary Spitzer, and Kenneth B. McCredie

The treatment of acute leukemia with high-dose cytoreductive therapy in conjunction with autologous bone marrow transplantation (ABMT) has been studied since 1975. In 1979 we published the first results for patients who had relapsed (1). In this patient population we used a combination of piperazinedione (PIP) and total body irradiation (TBI). The complete remission (CR) rate was high, 78% in those patients with good performance status (Zubrod's scale); however, the long-term disease-free survival rate (DFS) was 0%. Only two patients stayed in remission longer than 12 months after ABMT. The CR rate of patients with a poor performance status (Zubrod's scale, 2) was low (35%). The main reason for failure to achieve CR was an immediate posttransplantation mortality of 20%. Therefore, we decided to use the high-dose combination chemotherapy program, cyclophosphamide (Cytoxan), BCNU (carmustine), and VP-16-213 (etoposide) (CBV), which proved to be less toxic than the PIP plus TBI program (2). Besides being used in relapse and second complete remission (CR2), the program was used as an intensification protocol in first complete remission (CR1). The reason for applying the high-dose cytoreductive

program in CR1 is the relatively low number of leukemic cells remaining in the recipient and the small chance of drug resistance of the leukemic cell population in CR1. This program is the subject of this chapter.

PATIENT POPULATION

The patients selected for transplantation were those who achieved CR and stayed in remission for at least 6 months. Their median age was 35 years (range, 17-50 years). The induction and consolidation programs for acute myelogenous leukemia (AML) and acute lymphocytic leukemia (ALL) are shown in Figures 1 and 2. It should be noted that in AML as well as in ALL, high-dose ara-C (cytarabine) was used for consolidation immediately after achieving CR. Three courses of chemotherapy, AD-OAP (Adriamycin-Oncovin [vincristine], ara-C [cytarabine], prednisone) for AML and MTX-DOMP (methotrexate-daunorubicin, Oncovin [vincristine], mercaptopurine, prednisone) for ALL, were administered before high-dose intensification with CBV and ABMT. During the chemotherapy

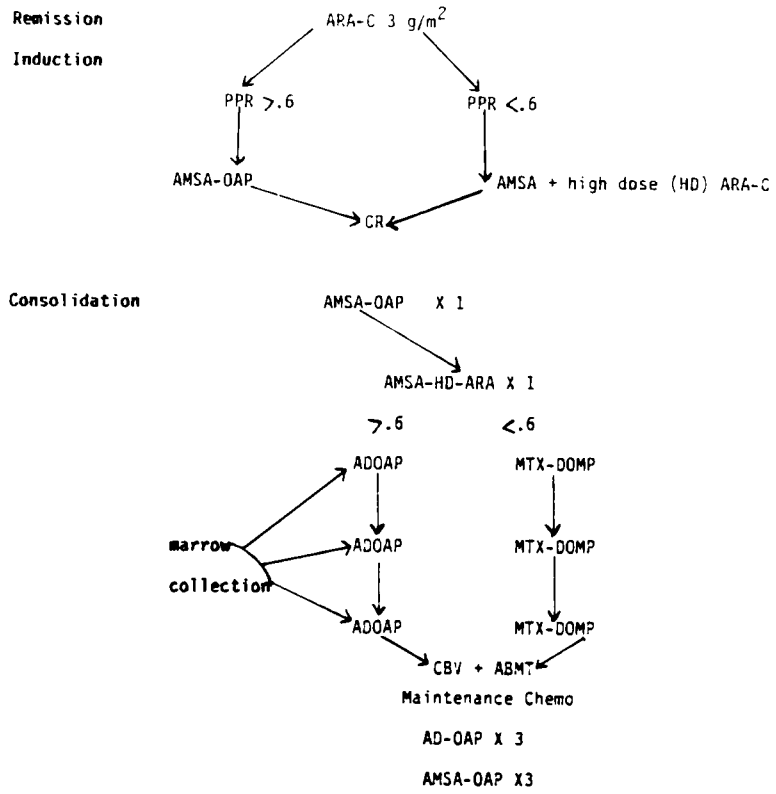

Figure 1. *Treatment schema for AML in first complete remission.*

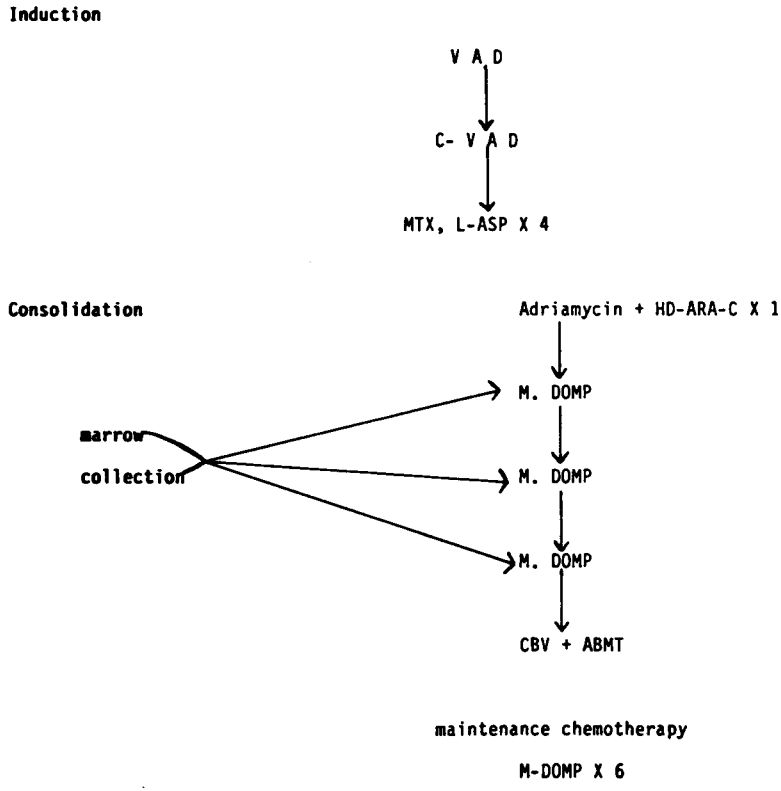

Figure 2. *Treatment schema for ALL in first complete remission.*

after high-dose cytarabine, bone marrow was collected for transplantation. The median period between onset of remission and transplantation was 6 months for AML and 7 months for ALL. After transplantation, when hematopoietic recovery was complete, maintenance chemotherapy was restarted. After transplantation six courses of AD-OAP were administered for AML and six courses of MTX-DOMP for ALL.

The study of AML was originally designed as a randomized study between transplantation and continuation of normal-dose chemotherapy without high-dose intensification. However, the randomization failed because patients refused to enter the protective environment for the second time (remission induction occurred in the protective environment) and because of the potential risk involved in the CBV program. For this reason, patients were selected on a voluntary basis. The study of ALL was a one-arm study that included treatment with CBV.

For the first 10 days after transplantation, patients were treated with acyclovir and with prophylactic antibodies to decontaminate the intestinal tract.

RESULTS

The AML investigation proposed to study the biologic role of intensification with CBV and unpurged ABMT by comparing the results with those of a concurrent nonrandomized control group. We proposed a 3- to 4-year follow-up period. Once the study began, no additional patients were to be entered. The results of the 17 patients entered in the CBV program have now been compared with the control group who, except for the CBV regimen, were treated identically. The patient characteristics matched in the two groups. The results are depicted in Figure 3. The Kaplan-Meier plot shows a 75% projected 18-month

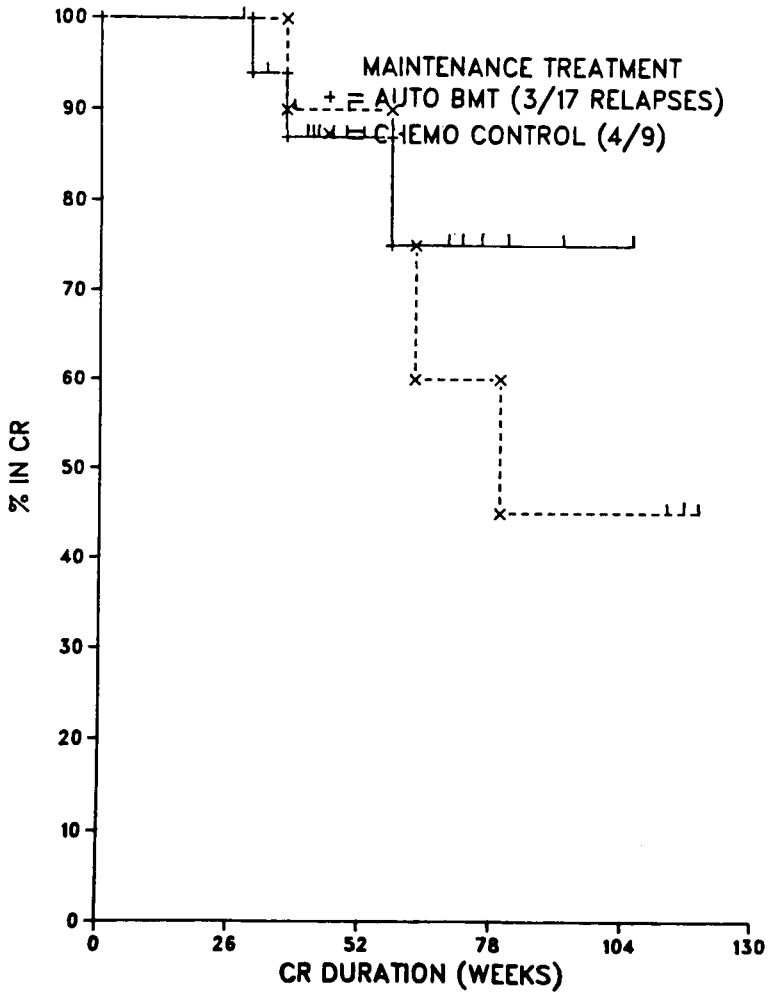

Figure 3. *Updated results of CBV (cyclophosphamide, BCNU [carmustine], VP-16-213 [etoposide]) and unpurged autologous bone marrow transplantation in first complete remission.*

DFS rate for the CBV-treated group and a 50% projected DFS rate for the control group. Since the study was not randomized, a *P* value was not determined.

The mortality immediately after transplantation was 0. The median recovery to 500 absolute granulocytes was 34 days, and the recovery to 50,000 platelets was 41 days after transplantation.

The ALL investigation also proposed to study the biologic role of intensification with CBV and unpurged ABMT. All patients under 60 years were to be entered into the BMT protocol, of whom 25 or 30 would receive the intensification regimen; since the study is one-armed, the data were to be compared with a nonconcurrent control group achieving the best available chemotherapy results. Only 11 patients have been transplanted so far. Seven are alive and in continuous CR after a median follow-up from the onset of CR of more than 18 months (range, 8-30 months). Three patients have relapsed. The immediate posttransplantation mortality in this group is 0; however, one patient in CR died of herpes zoster 5 months after ABMT. Additional patients have been entered in the study, but it is too early to evaluate the results. The median time to recovery to 500 granulocytes is 24 days, and to 50,000 platelets, 34 days. Hematopoietic recovery is significantly faster for ALL than for AML ($P = .05$).

DISCUSSION

Our results for AML in remission are identical to the European results (3): Gorin reports a 60% DFS rate in ALL and AML in the European studies (see Gorin *et al.* "Use of Bone Marrow Incubated With Mafosfamide in Adult Acute Leukemia Patients in Remission: The Experience of the Paris Saint-Antoine Transplant Team," in this volume). Although the data are encouraging, interpretation is difficult. Patient selection may be the cause of the high DFS rate after ABMT. A randomized study to compare the results of ABMT and normal-dose chemotherapy is necessary. In a randomized study between ABMT and allogeneic bone marrow transplantation in CR, Lowenberg reported no difference in results (see Lowenberg *et al.* "Autologous Bone Marrow Transplantation in Patients With Acute Myeloid Leukemia in First Remission," in this volume).

Despite the shortcomings in interpreting the data, we noted an unexpectedly high DFS rate after unpurged ABMT, raising questions about the potential of infusing leukemic cells and, therefore, the role of purging in CR1.

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Leukemia: First Remission

K. A. Dicke and B. Lowenberg, Chairmen

DR. K. A. DICKE: We would like to ask the audience if there are specific questions for the various speakers, and then we would like to address a couple of main issues. Now, are there any specific questions for Dr. Lowenberg?

DR. A. BURNETT: What is the survival curve of the patients treated with autograft when you include the loss of patients between remission and time of transplantation? It can't be much more than 35%.

DR. B. LOWENBERG: The loss before transplantation is indeed a very important point. What I can say is that we compared the overall duration of remission for all patients with a complete response, irrespective of what happened afterwards. And, at this stage, there was no difference between any of the modalities, most likely because of the short follow-up.

DR. V. RIZZOLI: Is there a difference in the outcome between the patients whose marrow was harvested and transplanted early after achieving remission and the group of patients whose transplants were later, after onset of remission?

DR. LOWENBERG: There is no difference.

DR. P. HERVE: Bob (Dr. Lowenberg), how do you explain that you have a very long delay in obtaining platelet recovery?

DR. LOWENBERG: There is no definite answer to that. It seems to be disease related, because we have seen several reports today, all reporting delayed recoveries in acute myelogenous leukemia (AML). It may also be due to

the intensive chemotherapy at the time of marrow harvest. The latter possibility is not likely because acute lymphocytic leukemia (ALL) treatment is probably and quite similarly intensive in this disease and autologous bone marrow transplantation (ABMT) is not associated with delayed recovery.

DR. F. BABAPULLE: Dr. Lowenberg, your patients experienced prolonged pancytopenia following the autograft and you showed a correlation between this phenomenon and granulocyte-macrophage colony-forming unit (CFU-GM) numbers. Do you think you could have circumvented this problem by actually increasing the cell dose or giving them a supplementary autograft, maybe 14 days after the first?

DR. LOWENBERG: At this time, we cannot solve that question. We gave all the cells we harvested immediately after finishing the conditioning regimen, so we had no additional cells left for a second transplant.

DR. C. AUGUST: This may be a naive question and I will ask it to whomever wants to answer it. In the last 3-5 years there has been an extraordinary improvement in the results of this endeavor of using both purged and unpurged marrows. I would like to ask to what do you attribute this?

DR. DICKE: The question is not naive. Let me try to answer it. First, ABMT is done in an early stage of the disease, so that drug resistance will be minimal. Second, the time of marrow harvest, after relatively aggressive chemotherapy, may minimize the presence of leukemic cells. Third, the improved results might be due to patient selection. Randomized studies will answer this.

Dr. Mandelli, what was the mortality immediately after transplantation in your first-remission studies?

DR. F. MANDELLI: Only one death of pulmonary embolism at 21 days after bone marrow infusion.

DR. BURNETT: Dr. Mandelli, how many patients do you know you lost between achieving remission and doing the autograft who are not actually postautograft patients?

DR. MANDELLI: Our program was instituted 2 years ago and I think that the loss of patients was almost between 20% and 25% before bone marrow transplantation.

DR. DICKE: Are there any questions for Dr. Gorin?

DR. BURNETT: Claude (Dr. Gorin), we've done several in vitro studies on purging and have not been able to demonstrate that cells prepared as a mononuclear concentrate from bulk marrow have a reproducible dose-response curve. We do two harvests, and we always store one as a back-up. We have not had the same dose response on a full mononuclear bulk-prepared marrow as we had the second time. My questions are: Do you know whether it is fair to make your assumption on an aspirate rather than on your final product? Was your actual kill, the second time, what you anticipated?

DR. N. C. GORIN: I think the two questions are related. I can tell you that in 95% of the cases we find exactly what we expected in the treated marrow. If I may, and there are no other questions, I would like to make a comment about the differences in the kinetics of recovery in AML and ALL. We tried to investigate this problem 2 years ago. First, we compared the duration of aplasia following conventional chemotherapy at the time of diagnosis between AML and ALL. There was no difference. The second point we studied was the dose of CFU-GM infused in AML and ALL. We found that the dose of CFU-GM infused in AML was lower, although there was no statistical difference between the two groups. Some other investigators have studied this problem and demonstrated that in AML, at any time in the course of the disease following remission induction chemotherapy, the CFU-GM level in the marrow is lower than in ALL. Therefore, the dose you infuse is lower. The third point is the cryopreservation efficiency. We check the cryopreservation efficiency in each patient. From time to time, the cryopreservation is not very good and, I think that if we add the cryopreservation injury to an in vitro treatment, we will destroy stem cells. I also think that the best way to study the marrow, of course, is not CFU-GM, but probably CFU megas. Finally, I wanted to say that we have studied CFU-GM for the kinetics in relation to the pretransplant regimen, total body irradiation, and regarding the treatment of the marrow. Total body irradiation delays recovery, treatment in vitro delays recovery, and higher doses of in vitro treatment delays recovery again for a longer period. So I think we have at least four variables. First, the disease (AML); second, cryopreservation; third, the in vitro treatment; and fourth, the conditioning regimen.

DR. T. PHILIP: Claude, you have reported one graft failure. Can you give us details on that please?

DR. GORIN: It was a patient who had herpes virus infection with encephalitis on day 14, posttransplant. We saw all the counts going down just at the time when he was engrafted. So, we believe it was related to virus infection.

DR. LOWENBERG: Karel (Dr. Dicke), how do you select the patients for transplantation? What is the reason why certain patients are grafted and others are not?

DR. DICKE: Initially, the study was designed to be randomized between chemotherapy and transplantation; however, in the beginning, the patients refused bone marrow transplantation. They did not want to go for another round in the protective environment. Therefore, we had to give up the randomization. We were dealing here with a group of volunteers and I stress the nonrandomization of the study. I do not want to give any statistics on the differences between the transplantation group and the control group because the results may be due to patient selection. One thing I do know is that, according to Michael Keating's prognostic factors, in terms of cytogenetic analysis, this particular patient population was not a prognostically favorable group.

DR. GORIN: Tony (Dr. Goldstone), the slide you showed emphasizes the advantage of a double autograft. When we wrote the European study, half of the patients having double autografts came from your institution, and we cautioned against some kind of premature conclusion because of a possible selection bias. Do you think double autografting is indeed beneficial or not?

DR. A. GOLDSTONE: It is clear that the small group of AML patients in first remission, who managed to get through two autografts, is doing well. Those were patients who were fit, went in remission early, had cellular marrows with good counts, recovered soon after transplantation and, therefore, tended to enter the group that became eligible for a second graft.

DR. D. BUCKNER: I just have one comment on study design. I think that if you are going to do these studies, you ought to store the marrow on everybody in first remission and randomize them to get their transplant either in second remission or at time of relapse. That is the only way you are going to be able to get the salvage rate plus an accurate reflection of the chemotherapy cures. We can salvage 30% of patients with allografts in first relapse. We ought to be able to do the same with autografts.

DR. LOWENBERG: We have come to the end of this session. It is clear that what we need is more comparative studies. The various studies presented to us today have at least indicated that there are several options for improvement of autografting. We feel that, even though the results are encouraging, we continue to deal with a high relapse rate. We need to address the question of more effective conditioning regimens, certainly in future trials. It should probably also be done in comparative settings. And, of course, the question of purging, which, at present, is very important, is still an open issue. Thank you all for your contributions.

IB. Clinical Studies in Second- or Subsequent-Remission Leukemia

A Study Design to Determine the Role of Transplantation in Acute Leukemia

Karel A. Dicke, Sundar Jagannath, Leonard J. Horwitz, Gary Spitzer, and Kenneth McCredie

Autologous bone marrow transplantation (ABMT) in a patient with acute leukemia entails the possible infusion of leukemic cells into the recipient. The role of these infused leukemic cells in the recurrence of leukemia after ABMT plus high-dose cytoreductive treatments is not yet clear.

ABMT in relapsing patients does not result in long-term disease-free survivals (DFS) (1), whereas patients treated with allogeneic transplantation or syngeneic transplants achieve a 20% DFS rate (2,3). On one hand, evidence suggests that in a certain percentage of patients the post-transplantation leukemia recurrence originated from the infused leukemic cells. On the other hand, data concerning ABMT during first remission do not indicate that infused potentially leukemic cells have an active role in recurrence.

We reported during the first ABMT symposium a low DFS rate in the patient group treated with cyclophosphamide (Cytoxan), BCNU (carmustine), and VP-16-213 (etoposide) (CBV) and unpurged marrow in a second complete remission (CR2) or a subsequent remission (4). In this chapter we report an update of our transplantation data in second and subsequent remissions and describe a realistic study design to determine the effect of purging on recurrence.

RESULTS OF VARIOUS TREATMENT MODALITIES IN ACUTE LEUKEMIA AFTER FIRST COMPLETE REMISSION

After CR1, remission is induced in 40-60% of patients. The long-term DFS rate is below 10%. We define DFS as a remission lasting longer than 12 months. Inversion, defined as a duration of CR2 longer than that of CR1 or a duration of third complete remission longer than that of CR2, also occurred in less than 10% of patients. Our results at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston are similar. Remission reinduction in acute myelogenous leukemia (AML) is 50% and in acute lymphocytic leukemia 75%. Our continuous complete remission rate is less than 10%.

Figure 1 diagrams the results of other treatments. Patients treated with high-dose cytoreductive therapy and allogeneic or isogeneic bone marrow transplantation have a 35-40% chance of long-term DFS (3). The inversion rate is identical to the long-term DFS rate. In the patient group treated with 4-hydroperoxycyclophosphamide (4-HC)-purged autologous marrow reported by Johns Hopkins Hospital, the long-term DFS rate is 30% (5). However, the inversion rate in that population is 23%. Ramsay has reported that 6 out of 22 patients treated in CR2 or a subsequent remission with monoclonal antibody-purged marrow plus cyclophosphamide plus total body irradiation (TBI) have achieved long-term DFS status (6). The inversion rate in this study is 27%.

The updated status of the control group for our forthcoming study, patients treated in second and subsequent remissions with unpurged marrow plus CBV, is depicted in Figure 2. Only 4 of the 22 patients had a remission of

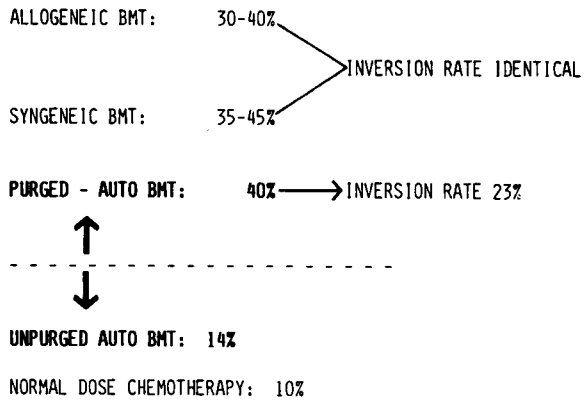

Figure 1. *Continuous complete remissions after various treatment modalities in second and third remissions of acute leukemia. BMT = bone marrow transplantation; auto = autologous.*

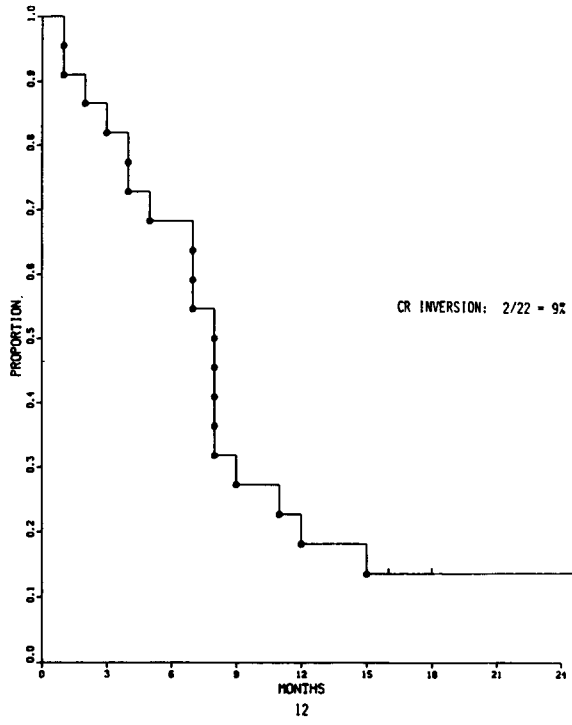

Figure 2. *Updated results of CBV (cyclophosphamide, BCNU [carmustine], and etoposide) plus unpurged ABMT in second and subsequent remissions. Complete remission inversion indicates subsequent remissions longer than the preceding ones.*

12 months or longer. In two, the transplantation remission was longer than the preceding remission, producing an inversion rate of 9%. Because the 95% confidence limit extends from 0 to 21%, we consider a study positive when the inversion rate is at least 22%. According to these criteria, Yeager's and Ramsay's studies are promising.

In our opinion, better results can be obtained when the two methods of purging—the chemoseparation technique and a monoclonal antibody-based technique—are combined. This combination of methods reduces the influence of drug resistance and of antigenic heterogeneity. Therefore, we have initiated a transplantation program for second and subsequent remissions in which we can ultimately combine the two technologies.

Before we can do this, phase I studies of both chemopurging alone and of the magnetic affinity colloid (MAC) technique need to be carried out separately to determine the influence of both on hematopoietic recovery. In chemopurging, the marrow cells are incubated with 4-HC plus vincristine, inhibitory concentrations 75 and 90. The MAC technique is different for AML and non-T-cell ALL. For AML, it employs SBA, CF1, PM81, and MY7

antibodies; for non-T-cell ALL, it employs SBA, CF1, BA1, BA2, BA3, and B4 antibodies. The monoclonal antibody technique, based on magnetizing a pool of monoclonal antibodies and lectin, is discussed in detail by Reading *et al.* (see "Magnetic Affinity Colloid Elimination of Specific Cell Populations From Bone Marrow," in this volume). Horwitz *et al.* (see "4-Hydroperoxycyclophosphamide and Vincristine as Ex Vivo Bone Marrow Treatment for Acute Leukemia in Second Remission," in this volume) discusses the chemoseparation procedure in detail. Both procedures are different from the techniques so far reported in the literature.

Study Design

After completing the phase I studies of each separate technique, we will combine the two procedures and initiate a phase II study for patients in CR2. We will use the CBV conditioning regimen. The level of immunosuppression induced allows consistent engraftment of allogeneic histocompatible marrow. The immediate posttransplantation mortality is low: 8% after transplantation during relapse or in CR2 (54 patients), 0 in CR1 (29 patients); and its antileukemic effect is equivalent to that of the piperazinedione-plus-TBI program and to the International Bone Marrow Transplantation Registry results. Antileukemic effect, myelosuppression level, and low toxicity are the most important factors to determine the choice of a conditioning regimen.

We will compare the phase II data with the data from the unpurged marrow control group. If the results of the purged-marrow group are satisfactory, we will initiate a randomized study for patients in CR1.

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Autologous Bone Marrow Transplantation in Acute Nonlymphocytic Leukemia Using Marrow Treated With 4-Hydroperoxycyclophosphamide

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Although current intensive chemotherapy regimens have resulted in prolonged first remissions and potential cures in patients with acute nonlymphocytic leukemia (ANLL) (1-3), the chances of leukemia-free survival are minimal in those patients who have had one or more hematologic relapses. In such patients, allogeneic bone marrow transplantation (BMT) may be curative, but other threats exist. Graft-versus-host disease and opportunistic viral interstitial pneumonitis account for most of the mortality after allogeneic transplantation. Leukemic relapses are uncommon (4-7). In addition, 60-75% of otherwise suitable allogeneic marrow transplant recipients lack histocompatible related donors.

Autologous bone marrow transplantation (ABMT) allows the patient to receive intensive myeloablative antileukemic therapy followed by infusion of his own marrow, which has been collected in remission and cryopreserved. Because of the concern that autologous bone marrow obtained from patients with leukemia in second or subsequent remission may contain viable occult leukemia cells, strategies to purge the marrow of residual tumor cells before

cryopreservation are warranted. Preclinical studies have shown that rat leukemia cells are eliminated from marrow suspensions by incubation ex vivo with 4-hydroperoxycyclophosphamide (4-HC) (8), a congener of cyclophosphamide and an active alkylating agent in aqueous solution (9). A phase I trial of autologous transplantation with marrow treated with 4-HC in patients with leukemia and lymphoma has demonstrated that hematopoietic repopulating ability is not impaired after incubating marrow with up to 100 μg/ml 4-HC (10). In the study reported here, ABMT was carried out in 40 patients with ANLL in second or third remission using marrows incubated with 60-100 μg/ml 4-HC to determine if ABMT with 4-HC-treated marrow can provide long-term disease-free survival. We also evaluated the toxicities of this regimen and the hematologic engraftment parameters after infusion of 4-HC-treated marrow.

METHODS OF STUDY

Informed Consent

All protocols for marrow treatment and pretransplantation myeloablative therapy were reviewed and approved by the Joint Committee on Clinical Investigation of The Johns Hopkins Medical Institutions. Informed consent was obtained from all patients and from the parents of patients who were children.

Patients

Forty consecutive patients (21 males and 19 females) were included in this study. Median age was 30 years (range, 4-54 years). The diagnosis of ANLL was confirmed in all patients by examination of bone marrow aspirates. Histologic types of leukemia were classified according to guidelines of the French-American-British (FAB) Cooperative Group (11). The patients had the following FAB morphologic classifications: 17, M_1; 4, M_2; 4, M_3; 12, M_4; and 3, M_5. All patients had had at least one hematologic relapse of leukemia and had received intensive chemotherapy to induce initial and subsequent remissions. The median duration of first remission was 15 months (range, 2-96 months). At the time of marrow collection and transplantation, 30 patients were in second and 10 were in third complete hematologic remissions. Two patients had histories of extramedullary leukemia: one patient in second remission had CNS involvement at diagnosis, and one patient in third remission had both CNS and testicular involvements at the time of first relapse. However, no patients had active extramedullary leukemia at the time of marrow collection and transplantation.

Marrow Collection, Processing, and Infusion

The patients underwent marrow collection at a median of 2 months (range, 0.5-6 months) after attainment of second or third remission. Marrow was aspirated from the posterior iliac crests according to previously published

methods (12). For each patient, an attempt was made to obtain at least 4×10^8 nucleated marrow cells per kilogram of body weight.

Approximately 70% of the collected marrow was treated ex vivo with 4-HC at a concentration of 60 μg/ml in one patient in second remission, 80 μg/ml in one patient in second and in one patient in third remission, and 100 μg/ml in the remaining 37 patients. The mean number (± 1 SD) of nucleated marrow cells thus treated was $3.0 \pm 0.47 \times 10^8$/kg (range, $1.6\text{-}5.3 \times 10^8$). In 33 patients, the remainder of the collected marrow was treated with a lower dose of 4-HC (40 or 60 μg/ml) or remained untreated as a reserve marrow to be infused in the event that engraftment did not occur with the fully treated autologous marrow. Because of suboptimal collection, seven patients had no reserve marrow available.

The buffy coat fraction was removed from the collected marrow suspension by centrifugation in standard blood transfer packs in a Sorvall RC 3B centrifuge with an HG-4L head at 2,900 rpm for 10 minutes; the process was then repeated to obtain a second buffy coat layer. These two fractions of nucleated cells were pooled and mixed with autologous plasma and heparinized tissue culture medium (TC 199) (GIBCO, Grand Island, NY) to obtain a concentration of 2×10^7 cells/ml. A solution of 4-HC was freshly prepared in phosphate-buffered saline, and an appropriate volume was added to the marrow cell suspension to obtain the desired final concentration of the drug. The cells were incubated with 4-HC in a water bath at 37°C for 30 minutes, after which the cell suspension was rapidly cooled to 4°C and centrifuged for 10 minutes. The 4-HC-treated cells then were resuspended in 45% TC 199, 45% autologous plasma, and 10% dimethyl sulfoxide at a concentration of 4×10^7 cells/ml. Aliquots (50 ml) of the cell suspension were placed in polyolefin bags, frozen in a controlled-rate freezer at −1°C/minute to −50°C and at −10°C/minute to −70°C, and transferred to the liquid phase of a liquid nitrogen freezer. At the time of marrow infusion (designated as day 0), each bag was rapidly thawed in a 37°C water bath, and the thawed cell suspension was infused through a central venous catheter at a rate of 10-15 ml/minute.

Hematopoietic Progenitor Cell Assays

For each patient, 4-HC-treated marrow samples were assayed for granulocyte-macrophage colony-forming units (CFUs-GM) in a soft agar culture system, as previously described (10). In brief, marrow mononuclear cells were cultured in 35-mm plastic tissue-culture dishes that contained 0.3% agar, 15% fetal bovine serum, and McCoy's 5A medium; human placenta-conditioned medium was added as colony-stimulating factor. After incubation for 10-14 days at 37°C in 7.5% CO_2 in humidified air, culture dishes were examined under magnification (X35-40) for the presence of granulocyte-macrophage colonies. Aggregates of more than 40 cells were considered colonies. The total number of CFUs-GM infused into each patient was calculated from the dose of nucleated marrow cells infused and from the frequency of colonies in culture.

Preparative Regimens

The preparative regimens used in these patients were indentical to those employed for allogeneic BMT at this institution (7,13). Thirty-eight patients received busulfan (1 mg/kg/dose by mouth every 6 hours for 16 doses) on days −9, −8, −7, and −6, and cyclophosphamide (50 mg/kg/dose i.v.) on days −5, −4, −3, and −2. Autologous marrow was infused 48 hours after the last dose of cyclophosphamide. Two patients (one in second and one in third remission) with a history of CNS leukemic involvement received a preparative regimen of cyclophosphamide (50 mg/kg/dose) on days −8, −7, −6, and −5, followed by total body irradiation (3 Gy/day, with lungs shielded after 9 Gy) on days −4, −3, −2, and −1. Autologous marrow was given 24 hours after the last dose of radiation. Prophylactic consolidation chemotherapy was initiated 50-70 days after bone marrow transplantation and consisted of five doses of intrathecal methotrexate (10 mg/m^2 of body surface area; maximal dose, 12 mg), administered twice weekly over a period of 2½ to 3 weeks. Patients with a history of leukemia involving the CNS also received monthly intrathecal injections of methotrexate for a total of 12 additional doses.

Supportive and Posttransplantation Care

Patients were nursed in single rooms with high-efficiency particulate air filtration systems that provided 32 nonlaminar air exchanges per hour. Before marrow transplantation, one or two indwelling central venous catheters were placed in each patient for administering fluids, antibiotics, and blood products (14). All patients at risk for the recurrence of herpes simplex infection (manifested by an IgG antibody titer of $\geq$ 1:8) received prophylactic intravenous acyclovir (15). When absolute neutrophil counts fell below 0.5×10^9/l, reverse isolation using masks and good handwashing practices was employed. Broad-spectrum antibiotics were given for fever during aplasia, and amphotericin B was added for documented systemic fungal infections or for persistent fever during aplasia. Antibiotics and isolation procedures were discontinued when the patients were afebrile and when absolute neutrophil counts consistently exceeded 0.5×10^9/l. All blood products were irradiated with 15-30 Gy before induction to prevent possible graft-versus-host reaction.

Statistical Analysis

Routine statistical calculations were performed with a hand-held calculator. Survival analysis was performed according to the methods of Kaplan and Meier (16) using a microcomputer and statistical software packages developed by the Biostatistics and Information Systems Division of the Oncology Center of The Johns Hopkins University School of Medicine.

RESULTS

Posttransplantation Clinical Course

Most patients receiving busulfan and cyclophosphamide had moderate oral mucositis, which responded to good oral hygiene but which on occasion required the topical application of agents such as lidocaine and diphenhydramine. Mucositis generally resolved within 2 weeks after transplantation. No episodes of severe life-threatening hemorrhage were observed. All patients had fever during aplasia. Six patients died of overwhelming sepsis during aplasia, 8-24 days after marrow rescue: two with *Pseudomonas aeruginosa*, one with *Streptococcus viridans*, two with *Candida tropicalis*, and one with aspergillus. One patient died with multiple organ system failure and presumptive sepsis 34 days after transplantation, though no specific organism was identified in blood cultures. One patient, who had nonfatal sepsis with *Klebsiella pneumoniae* at the time of marrow infusion, had persistent marrow hypoplasia and died with gram-negative sepsis 155 days after BMT. One patient died with interstitial pneumonitis owed to cytomegalovirus while in third remission 95 days after BMT, and another patient in second remission died with idiopathic interstitial pneumonitis 249 days after BMT. One patient developed nonfatal pneumonitis attributable to *Pneumocystis carinii* 130 days after transplantation. Two patients, both in third remission, died with hepatic veno-occlusive disease, one 35 and the other 47 days after infusion of 4-HC-treated autologous marrow.

Hematologic Reconstitution

Marrow samples from most patients had no detectable CFUs-GM after incubation with 4-HC. A mean of 0.59 ± 0.20 (range, 0-5.1) $\times 10^3$ CFUs-GM/kg was infused. Thirty-three patients were evaluable for engraftment (the seven patients who died with sepsis during aplasia were excluded from analysis). One patient with leukemia in second remission had *Klebsiella pneumoniae* sepsis, acute renal failure, and hemodynamic instability at the time of autologous marrow infusion. Although she recovered from that episode of sepsis, she had persistently low levels of leukocytes, neutrophils, and platelets after transplantation (total leukocytes $< 0.4 \times 10^9$/l, neutrophils $< 0.1 \times 10^9$/l, and platelets $< 20 \times 10^9$/l). The patient had no reserve marrow available for infusion after failure to engraft with the 4-HC-treated fraction and died with gram-negative bacterial sepsis 155 days after autologous marrow infusion. Hematologic reconstitution occurred in the other 32 patients, and none required infusion of reserve marrow. In these patients, the median time required to attain a neutrophil count greater than 0.5×10^9/l was 29 days (range, 14-63 days) after BMT. Six patients had recovery of neutrophils but were thrombocytopenic (platelets, 10-35 $\times 10^9$/l) at the time of leukemic relapses, 73-176 days after autologous marrow rescue. One patient remains in second remission with

persistent thrombocytopenia (platelet counts, 30-35 x 10^9/l) 249 days after transplantation. In the remaining 25 patients, the median time to attain a platelet count greater than 50 x 10^9/l was 63 days (range, 23-330 days) after autologous marrow infusion.

Leukemic Relapses and Disease-free Survival

Of the 28 evaluable patients, leukemic relapses were observed in 12 of 22 patients undergoing ABMT in second remission and in three of six patients undergoing transplantation in third remission. The median time until relapse was 176 days (range, 60-321 days) after autologous marrow infusion, for an actuarial relapse rate of 53%. All relapses were hematologic in nature, and no episodes of nervous system or gonadal involvement were apparent at the time of relapse. The relapses occurred in nine patients with FAB morphologic class M_1, two in class M_2, one in class M_3, and three in class M_4. There was no significant difference in length of first remission between those who relapsed (median duration, 13 months; range, 3-53 months) and those who remained free of disease (median duration of first remission, 19 months; range, 2-41 months). The intervals between attainment of remission and time of marrow transplantation were similar in both groups. Attempts at reinduction therapy were successful in 5 patients; the other 10 have died with leukemia in relapse or of complications of its treatment. Thirteen patients remain in unmaintained second (10 patients) or third (3 patients) remission at a median of 572 days (range, 228-1,869 days) after transplantation with 4-HC-treated autologous marrow. In 7 of these 13 patients, the duration of remission after transplantation exceeds the duration of first remission; in contrast, only 1 of the 15 patients who relapsed had a posttransplantation remission that exceeded the duration of first remission. Actuarial survival analysis in these patients indicates an overall survival of 41% and an event-free survival of 31% (Fig 1).

DISCUSSION

Intensive chemoradiotherapy and infusion of histocompatible marrow from a normal donor may be curative in many patients with acute leukemia whose chances of disease-free survival are negligible with current conventional chemotherapy. Transplantation with autologous marrow may provide some advantages over the use of allogeneic marrow. For example, the risks of acute graft-versus-host disease, a major cause of morbidity and mortality after allogeneic BMT, are greatly minimized with autologous marrow. However, experiences with syngeneic BMT for acute leukemia have demonstrated a high (50-60%) relapse rate (17), suggesting that the antileukemic effects observed after allogeneic transplantation are attributable, at least in part, to graft-versus-leukemia effects (18,19). One might therefore expect that ABMT would at best provide antileukemic effects that are similar to those observed after syngeneic transplantation (i.e., a higher incidence of leukemic relapses but a lower

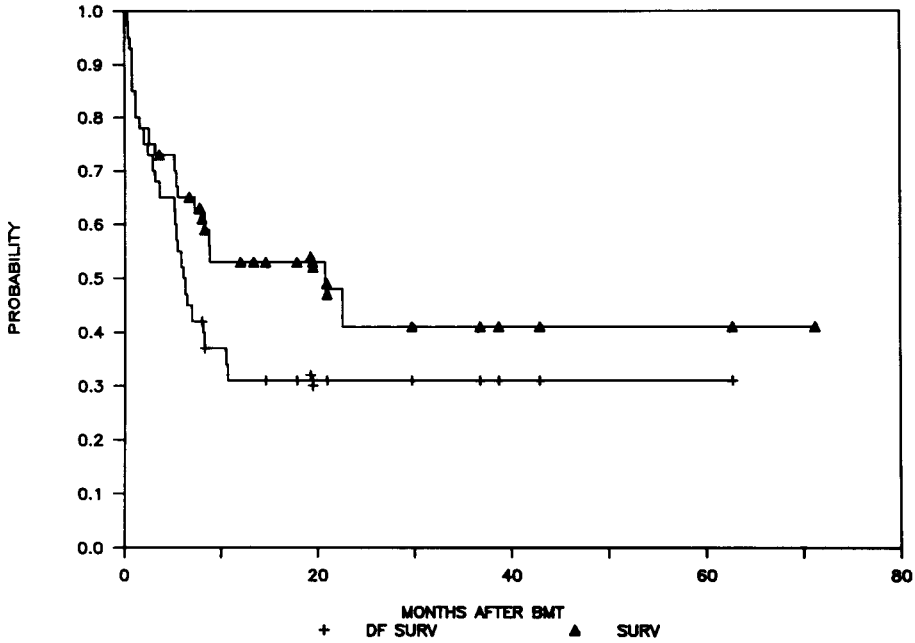

Figure 1. *Survival analysis of 40 patients undergoing autologous bone marrow transplantation for acute nonlymphocytic leukemia in second remission (30 patients) or third remission (10 patients). Overall survival (SURV)* (triangles) *and disease-free survival (DF SURV)* (crosses) *are shown; each symbol represents a living patient.*

incidence of transplant-related complications). Analysis of leukemia-free survival after ABMT must thus encompass similar studies of recipients of syngeneic marrow grafts.

Two problems must be addressed in ABMT for acute leukemia in second or subsequent remission: eradication of residual leukemia in vivo and of clonogenic tumor from the marrow suspension ex vivo. Attempts to remove occult leukemic cells from autologous marrow suspensions by physical methods such as density-gradient centrifugation have been unsuccessful (20). Although results with ex vivo immunologic treatment of autologous marrow with monoclonal antibodies in patients with acute lymphocytic leukemia is encouraging (21,22), the lack of satisfactory monoclonal antibodies that react with myeloid blast cells has limited the applicability of such techniques for ex vivo immunologic purging in acute nonlymphocytic leukemia.

A transplantable murine lymphoma system demonstrated the differences between the sensitivity to antitumor agents of normal and neoplastic cells (23), suggesting that incubation of autologous marrow with selected pharmacological agents might eradicate residual leukemic cells yet spare normal hematopoietic stem cells. Phase I clinical studies with the alkylating agent 4-HC have shown that the ex vivo incubation of autologous marrow with up to 100

μg/ml of 4-HC does not interfere with reasonably prompt hematologic reconstitution after infusion of drug-treated marrows in patients with leukemias and lymphomas (9,10). However, aplasia occurred in three of seven patients who received autologous marrow treated with 120 μg/ml of 4-HC, suggesting that that dose of 4-HC was toxic to normal human hematopoietic stem cells (10).

Exposure to 4-HC substantially inhibits the growth of committed (10,24) and multilineage (24) human hematopoietic progenitor cells in vitro, though more primitive blast cells may be less sensitive to the drug (25). In our study, the number of CFUs-GM infused in 4-HC-treated marrows was greatly reduced, but hematologic reconstitution nevertheless occurred in 32 of 33 evaluable patients. The time to recovery of neutrophil and platelet counts in recipients of 4-HC-treated marrow was significantly slower than time to hematologic reconstitution in recipients of untreated allogeneic or syngeneic marrow. However, similar delays in recovery of leukocytes and platelets have been described in patients undergoing ABMT for acute leukemia with untreated (26,27) or drug-incubated (28-30) marrow. Persistent thrombocytopenia (for 4 to more than 10 months after ABMT) was noted in several patients, a finding similar to that reported for recipients of untreated marrow autografts for ANLL in first remission (27). It is not known whether these patients have had damage to the megakaryocytopoietic stem cell population or have a poorly compensated thrombocytolytic state after autologous marrow transplantation. On balance, the hematopoietic toxicity observed after marrow treatment with 4-HC appears acceptable and is similar to that seen in patients receiving intensive remission-induction chemotherapeutic regimens in acute leukemia (1-3).

Twelve of the 40 patients (30%) died of nonleukemic causes. Eight deaths were attributable to bacterial or fungal sepsis during aplasia, a frequency much higher than that reported after allogeneic (7,31) or syngeneic (17) BMT for ANLL. Two deaths from sepsis with *Pseudomonas aeruginosa* occurred about the same time and were owed to a strain of that organism resistant to multiple drugs, and one death from gram-negative sepsis occurred in the context of neutropenia and persistent marrow hypoplasia. The occurrence of fatal interstitial pneumonitis (one cytomegalovirus, one idiopathic) is similar to that seen in syngeneic marrow transplant recipients (32) and is much less frequent than in allogeneic transplant recipients (4-7). Hepatic veno-occlusive disease, a well-recognized complication of marrow transplantation (33), was fatal in two patients, both in third remission at the time of marrow transplantation. Hepatotoxicity from previous chemotherapy, infectious agents, or both may have predisposed these patients to this complication (33). The prospective identification of and particular attention to those patients at high risk for these kinds of posttransplantation complications may further improve survival after ABMT.

This study demonstrates that intensive antileukemic therapy followed by autologous transplantation with 4-HC-treated marrow may be associated with

long-term disease-free survival in patients with ANLL in second or third remission in whom conventional therapy is not curative. The actuarial relapse rate (53%) is similar to that seen with syngeneic transplants for leukemia (17), and the disease-free survival rate (31%) is comparable to that observed after allogeneic BMT (6,7,31,34). Patients who relapse after ABMT for acute leukemia may have had an intensive pre-BMT conditioning regimen that failed to destroy residual leukemia in vivo or the ex vivo pharmacological treatment meant to eliminate leukemic cells from the marrow cell suspension may have been incomplete. Current techniques do not allow one to determine which of these aspects of the preparative regimen was inadequate. However, the early recurrences of leukemia (within 3-6 months after transplantation) suggest a failure to eliminate tumor from the marrow inoculum, while the later relapses (6-12 months after transplantation) are likely owed to residual disease in vivo (as is the case in leukemic recurrence after syngeneic BMT). The administration of more intensive chemotherapeutic regimens before autologous marrow rescue may therefore be required to eliminate more leukemic cells in vivo and to offset the loss of the allogeneic graft-versus-leukemia effect. Strategies for ex vivo marrow treatment with single or multiple antileukemic drugs and combined immunopharmacological methods may further eradicate residual leukemic cells from autologous marrow suspensions in patients with acute leukemia.

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Transplantation Trial With Marrow Treated With Antimyeloid Monoclonal Antibodies and Complement

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Chemotherapeutic regimens in current use for patients with acute myelogenous leukemia (AML) successfully induce complete remissions (CRs) in up to 70% of patients (1). The majority of patients relapse eventually, however, and become increasingly resistant to chemotherapy. In up to 50% of patients who relapse, a second CR (CR2) that lasts an average of 4-5 months can be induced (2). Long-term survivors are described after initial remission-induction chemotherapy, but they are rare. Considerable attention has been focused recently on the use of bone marrow transplantation (BMT) to improve upon the results of chemotherapy (3). Allogeneic BMT performed in first CR (CR1) results in long-term survival and perhaps cure in 30-70% of patients, depending on age (3). Allogeneic BMT in CR2 is less successful because of a higher relapse rate of up to 50% in some series (4). Since most patients do not have a suitable donor or are older than 40, the age at which the complications of allogeneic BMT become increasingly common, alternative approaches to allogeneic BMT such as the use of autologous bone marrow are of interest. In theory, bone marrow obtained from patients in remission from AML may

contain viable leukemic cells capable of causing relapse. Therefore, we and others have developed autologous bone marrow transplantation (ABMT) strategies that use in vitro treatment of autologous bone marrow in an effort to remove leukemic cells (5,6). We are using complement (C')-fixing murine monoclonal antibodies (MAbs) PM-81 (7) and AML-2-23 (8), which react with myeloid antigens (9,10) expressed on leukemic cells and their progenitors (11-13), to treat bone marrow from patients in remission before ablative therapy and reinfusion of cryopreserved marrow. In this report we describe and update the experience of the Dartmouth-Hitchcock Medical Center (DHMC) and the Scripps Clinic and Research Foundation (SCRF) with 16 patients who underwent transplantation at various stages of the disease.

MATERIALS AND METHODS

Monoclonal antibodies PM-81 (IgM) and AML-2-23 (IgG2b) were purified from ascites as described previously (14). All MAb preparations were tested for sterility by culture in trypticase-soy broth and for endotoxin by the amebocyte lysate assay.

Leukemic cells obtained from the bone marrow of the majority of patients in this study at the time of diagnosis or first relapse were separated by Ficoll-Hypaque gradient centrifugation and incubated with purified MAbs (14). Surface-bound MAbs were detected by the addition of a fluoroscein isothiocyanate-labeled goat antimouse immunoglobulin and the cells analyzed on an Ortho Cytofluorograf system 50H.

Complement-mediated cytotoxicity was attained by incubating leukemic cells with purified MAbs for 15 minutes followed by addition of baby rabbit C' (Pel Freez, Rogers, AR) to achieve a final dilution of 1:6. Cell viability was assessed after 1 hour by dye exclusion.

Bone marrow harvesting was performed under general anesthesia as described previously (5). An average of 6.1×10^8 cells/kg of body weight was collected. Mononuclear cells were then isolated with a Haemonetics H-30 cell processor at the DHMC and by Ficoll-Hypaque gradient centrifugation at Scripps. A mean number of 1.1×10^8 cells/kg were then treated with MAb plus C'. Monoclonal antibody treatment consisted of two 1-hour exposures of cells to MAbs at 50 μg/ml and rabbit C' at a 1:6 dilution at 22° C as described previously (6), followed by cryopreservation using a controlled-rate freezer and storage in the vapor phase of liquid nitrogen.

The ability of patients' bone marrow cells to form colonies in methylcellulose cultures with appropriate growth factors before and after MAb treatment was measured as described previously (5). After 14 days, granulocyte-macrophage colony-forming units (CFU-GM), erythroid burst-forming units (BFU-E), and human pluripotent hematopoietic stem cells (CFU-GEMM) were counted on an inverted microscope.

Cytoreductive therapy consisted of 60 mg of cyclophosphamide/kg of

body weight on days −5 and −4, followed by total body irradiation (TBI) (a total of 1,200 cGy in twice-daily doses of 200 cGy 6 hours apart on days −3, −2, and −1) delivered with a cobalt-60 source by opposed lateral fields without lung shielding. On day 0, frozen bone marrow was quickly thawed at 37° C and administered through a central venous line. All patients received 12 mg of intrathecal methotrexate before or during ablative therapy.

As Table 1 shows, the patients, nine of them men, ranged in age from 11 to 47 years. Of the two patients who received transplants during early first relapse, one had extramedullary disease and the other showed 9% marrow blasts. Patients 2, 3, 8, and 16 were treated at the SCRF and the rest at the DHMC.

Table 1. Clinical Characteristics of Patients Who Underwent Autologous Bone Marrow Transplantation

			Remission Status at	
Patient	Age/Gender	FAB	Harvest	ABMT
1	44/M	M_3	1st	1st
2	46/M	M_2	1st	1st
3	47/M	M_2	1st	1st
4	28/F	M_1	1st	1st-rel[b]
5	16/F	M_2	1st-rel[a]	1st-rel[a]
6	20/M	M_2	2d	2d
7	36/F	M_2	2d	2d
8	24/M	M_3	2d	2d
9	44/M	M_4	1st	2d
10	32/F	M_4	2d	2d
11	28/M	M_3	2d	2d
12	44/F	M_4	1st	2d
13	44/M	M_2	2d	2d
14	11/F	M_4	2d	2d
15	44/F	M_2	3d	3d
16	27/M	M_2	3d	3d

Abbreviations: FAB, French-American-British classification; ABMT, autologous bone marrow transplantation.

[a]*Bone marrow was harvested from this patient and autologous bone marrow transplantation performed at the time a breast chloroma was diagnosed. The chloroma was treated with external beam radiotherapy.*

[b]*Relapse was diagnosed on the basis of 9% blasts in the bone marrow at the time of autologous bone marrow transplantation.*

RESULTS

The results of surface-marker analysis of leukemic cells are shown in Table 2. All patients studied had some degree of reactivity with the MAb PM-81, consistent with our findings that cells from more than 95% of patients express this antigen (7,12,13). The binding of MAb AML-2-23 was limited to cells of myelomonocytic morphology, consistent with our previous findings that cells with French-American-British (FAB) classification M_4 and M_5 AML express this antigen (15). Of the 16 patients, 9 had FAB M_2 morphology, 3 had FAB M_3, and 4 had FAB M_4. When cells expressed either antigen by flow cytometry, the corresponding MAb was concordantly able to lyse the cells in the presence of C'.

Table 2. Surface-Marker Expression on Leukemic Cells at Diagnosis[a]

Patient	FAB	PM-81 (% Positive)	AML-2-23 (% Positive)
1	M_3	ND	ND
2	M_2	70	5
3	M_2	72	6
4	M_1	21	3
5	M_2	40	2
6	M_2	77	32
7	M_2	91	9
8	M_3	64	5
9	M_4	80	70
10	M_4	ND	ND
11	M_3	80	65
12	M_4	58	24
13	M_2	ND	ND
14	M_4	98	79
15	M_2	83	1
16	M_2	32	6

Abbreviations: FAB, French-American-British classification; ABMT, autologous bone marrow transplantation; ND, no data.

[a]*Leukemia cells were separated from bone marrow by Ficoll-Hypaque gradient centrifugation and analyzed for surface expression of PM-81 and AML-2-23 by indirect immunofluorescence and flow cytometry and by complement-dependent lysis. Results shown represent the percentages of cells labeled with each monoclonal antibody (MAb), as determined by flow cytometry, with background values obtained by subtracting an isotype-matched control MAb. The results of complement-mediated lysis were similar.*

As shown in Table 3, major cell losses (95% on average) occurred during the combination of the cell processing procedure and the MAb treatment, consistent with depletion of the majority of mature myeloid cells as observed on Wright's stained cytospin preparations of posttreatment samples.

The mean time to reach a neutrophil count of 500 cells/μl was 32 days (Table 4). Reticulocytes appeared in the blood, on the average, by day 20 and the hemoglobin levels were sustained at greater than 10 g/dl without transfusion by day 48. Recovery of platelets was slow and variable.

Table 3. Number of Cells Harvested and Remaining After Cytopheresis and MAb Treatment

	Nucleated Cells			
Patient	Harvested[a]	Postcytopheresis[a]	Post-MAb Treatment[b]	CFU-GM[c]
1	8.0	1.5	4.2	1.9
2	2.8	0.6	4.0	ND
3	2.9	0.3	3.4	ND
4	6.4	0.9	2.4	1.1
5	9.0	0.7	2.0	1.4
6	3.5	1.9	2.6	2.0
7	11.5	7.6[d]	3.1	0.6
8	2.5	0.4	2.7	ND
9	5.1	0.4	2.2	0.8
10	4.7	0.7	2.6	0.7
11	3.8	0.4	1.5	0.8
12	5.6	0.4	1.8	2.4
13	7.0	0.6	1.9	1.0
14	14.5	0.9	6.0	3.5
15	6.6	0.5	1.8	1.1
16	3.7	0.7	4.0	ND
Average	6.1	1.1	2.9	1.4

Abbreviations: MAb, monoclonal antibody; CFU-GM, granulocyte-macrophage colony-forming unit; ND, no data.

[a] *Cells × 10^8/kg.*

[b] *Cells × 10^7/kg. This is the actual number of cells infused. Viabilities after thawing were ≥95%.*

[c] *Cells × 10^4/kg.*

[d] *Cells from this patient were collected as a buffy coat by centrifugation. This number was excluded from the average value.*

Table 4. Engraftment, Relapse, and Survival of Patients Who Underwent Autologous Bone Marrow Transplantation

	Days to Reach			Survival (mo)	
Patient	Neutrophils >500/μl	Hemoglobin >10 g/dl	Platelets >50,000/μl	Disease-Free[b]	Total
1	NA[a]	NA	NA	1+	1+
2	46	89	96	7+	7+
3	26	45	53	5+	5+
4	30	45	55	11	14+
5	60	48	NA	6	6
6	17	33	45	21	27+[c]
7	12	62	46	19+	19+
8	32	36	48	15+	15+
9	25	35	55	9	12+[c]
10	45	50	NA	8	8
11	32	60	NA	7+	7+
12	14	35	45	6	7
13	30	35	NA	3	3
14	36	64	NA[a]	3	4
15	33	45	150	22+	22+
16	43	NA[a]	NA	2	3
Average	32	48	66		
Median	32	45	53		

[a] *NA, not achieved (patient 1, too early; patients 14 and 16, early relapse).*
[b] *Patients 4, 6, 9, 12, 14, and 16 relapsed at the indicated times.*
[c] *These patients have undergone second ABMT with MAb-treated marrow and are in remission at 2 and 4 months posttransplantation.*

Nausea, vomiting, and mucositis were mild to moderate. All patients were treated with multiple antibiotics including amphotericin B for proved or suspected fungal infection. Three patients died of toxic effects, two from persistent thrombocytopenia and eventual subarachnoid hemorrhage (3 and 8 months posttransplantation) and one from respiratory infection (6 months posttransplantation). None of these three patients had evidence of leukemia at postmortem examination.

The numbers of CFU-GM remaining after MAb treatment are shown in Table 3. Only minimal losses of CFU-GM occurred in the process of freezing,

and recovery was generally greater than or equal to 90%. An average of 50% of CFUs-GM was lost during MAb treatment. No significant depletion of erythroid or mixed erythroid/myeloid colonies was noted (data not shown).

Disease-free and overall survival data are shown in Table 4 and Figures 1 and 2. Ten of the 16 patients who underwent transplantation are surviving; 7 of the 16 are surviving continuously disease free. Three patients have died in remission, two from subarachnoid hemorrhage secondary to thrombocytopenia (patients 10 and 13) and one from respiratory infection (patient 5). Six of the 13 patients (46%) who underwent transplantation later than in CR1 (patients 4, 6, 9, 12, 14, 16) experienced relapses, as did three of the four patients with FAB M_4 disease (Table 1). In three of the six patients who relapsed, third CR (CR3) was successfully induced with mitoxantrone (two

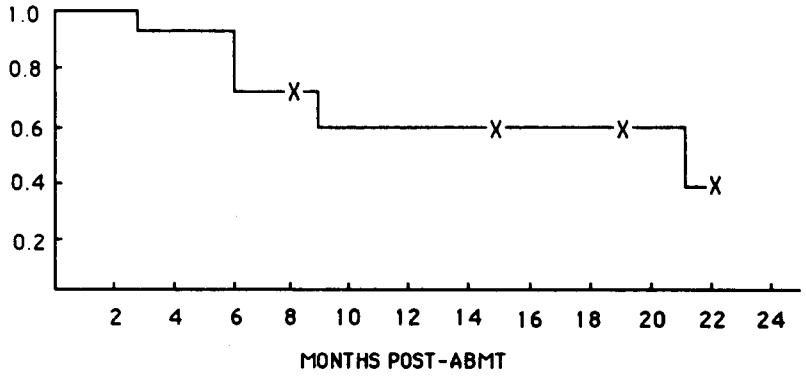

Figure 1. *Probability of relapse-free survival of a combined group of 11 patients who received transplants during CR2 (n = 9) and CR3 (n = 2). Hatch marks indicate patients surviving at the indicated times.*

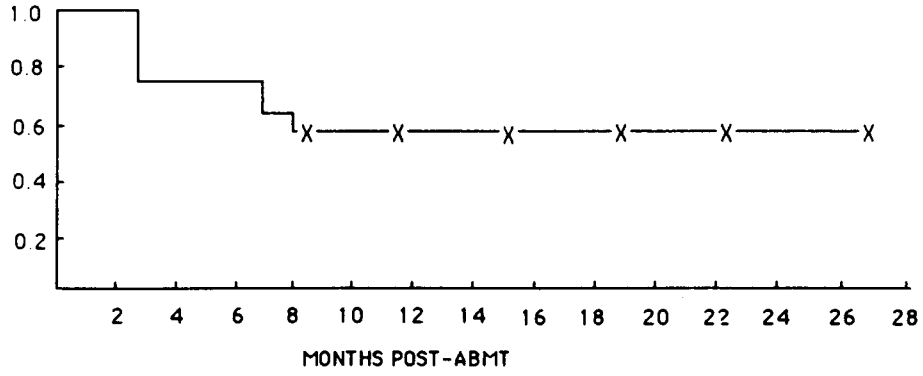

Figure 2. *Probability of survival of a combined group of 11 patients who received transplants during CR2 (n = 9) and CR3 (n = 2). Hatch marks indicate patients surviving at the indicated times.*

patients) or high-dose cytarabine. Two of these patients recently underwent a second ABMT, with busulfan and cyclophosphamide used as induction therapy (16). At the present time, both patients have engrafted and are surviving disease free at 2 and 4 months posttransplantation. The three patients who received transplants during CR1 are all disease free 1, 5, and 7 months later, the latter two with normal blood counts. The first patient is early in the posttransplantation course but is demonstrating engraftment.

Since patients 6 to 16 are a relatively homogeneous group (in CR2 and CR3), their survivals were analyzed separately (17). Survival curves (relapse free and overall) for these patients are shown in Figures 1 and 2. Actuarial median relapse-free survival was 21 months. Overall actuarial median survival has not been attained.

DISCUSSION

High-dose chemotherapy, with or without TBI, with bone marrow rescue offers patients with AML a greater chance of cure than other treatment combinations. Although it has not been proved that autologous bone marrow must be treated in some manner to remove residual leukemic cells, this conclusion is supported by the high relapse rates found in previous studies (18). The results reported here indicate that successful engraftment can be accomplished by using the particular MAbs (plus C') that we selected for marrow treatment. Although 2 of the 16 patients did not demonstrate adequate recovery of platelets, in general, engraftment of the three major hematopoietic lines was within acceptable time limits in most patients. It is difficult in an uncontrolled phase I and II study to draw conclusions regarding the efficacy of the marrow treatment in preventing relapse. The relapse rate in allogeneic BMT during first relapse or CR2 is as high as 50% (4). Our 46% actual relapse rate, albeit with a relatively small sample is consistent with that of other studies. A recent report from the Johns Hopkins Oncology Center about the use of 4-hydroperoxycyclophosphamide to treat marrow from AML patients in CR2 or CR3 showed an actuarial relapse rate of 43% in 25 patients (6). Final conclusions regarding the relative efficacy of ABMT with and without marrow treatment will depend on sample size, length of follow-up, and ultimately on randomized controlled studies that minimize selection bias.

A trial of ABMT in CR1 is attractive because a lower rate of relapse from allogeneic BMT has been documented in CR1, demonstrating that successful eradication of the leukemic clone by the preparative regimen is more effective then. Thus, we are directing our efforts to early institution in CR1 of ABMT with MAb and C' treatment. Again, long follow-up and, ultimately, controlled studies will probably be necessary to prove conclusively the efficacy of this procedure. Cooperative group studies may offer the setting for such studies.

Since we used an immunologic method in our in vitro purging protocol, a question could be raised about the escape of antigen-negative variants of the

leukemic clone from this treatment. Comparison of the blast cell phenotype in pretransplant cells with that of relapse cells has revealed them to be very similar. No firm conclusions can be drawn from this observation other than to exclude the possibility that selection of antigen-negative cells is responsible for the relapse in these patients.

We are currently exploring improved methods for in vitro treatment of bone marrow, such as immunotoxin treatment and chemical modification of antigenic sites on leukemia cells. For example, the removal of sialic acid residues from the cell surface by neuraminidase treatment can increase the binding of PM-81 severalfold (19). We are exploring whether such enzymatic treatment of bone marrow can be accomplished without excessive toxicity to normal hematopoietic stem cells. Thus, it may be possible by this means to increase the degree of killing of bone marrow leukemic cells. Most investigators note, however, that the high endogenous relapse rate in patients who have undergone BMT later than in CR1 requires a considerably improved ability to eradicate leukemic cells from the patient. With this goal in mind, we are considering additional in vivo intervention such as infusion of antibodies tagged with cytotoxic compounds or radioisotopes at the time of ablative therapy to augment the degree of cell kill. The introduction of BMT as a modality of leukemia treatment is an exciting development that seems to offer a new focus of hope for patients with AML as well as several other neoplastic diseases.

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Chemopurging of Autologous Bone Marrow With Mafosfamide in Adult Acute Leukemia

L. Douay, J.-P. Laporte, J.-Y. Mary, M. Lopez, A. Najman, and N.-C. Gorin

Following the initial report on the efficacy of cyclophosphamide (CY) derivatives in eliminating residual marrow leukemic cells in brown Norway rat myelocytic leukemia model (1), we studied the in vitro effect of Asta Z 7557 (International Nonproprietary Name mafosfamide), kindly provided by Professor N. Brock, Asta-Werke, Bielefeld, Federal Republic of Germany on human leukemic and normal hematopoietic stem cells (2). We began a therapeutic trial using autologous bone marrow transplantation (ABMT) with marrow incubated with this drug as consolidation therapy in adult patients who had acute leukemia in remission. The two goals were 1) to treat each patient's marrow with the highest possible dose of mafosfamide for maximum antileukemic activity, which would also spare enough normal stem cells for successful engraftment; and 2) to assess the potential benefit of high-dose consolidation followed by ABMT with marrow cleansed as described above, in terms of duration of remission and disease-free survival.

MATERIALS AND METHODS

In Vitro Studies

Progenitor Cell Culture in Semisolid Media

Blast cells were taken at initial diagnosis from the marrow of nine patients with acute nonlymphoblastic leukemia (ANLL). "Normal" marrow hematopoietic progenitors were derived from 37 patients (14 with acute lymphoblastic leukemia [ALL], 23 with ANLL) in whom the disease was in complete remission (CR). Leukemic cell progenitors from myeloblastic leukemia (CFU-L, leukemic colony-forming units) were cultured in methylcellulose according to the technique of Chang *et al.* (3) and scored on day 7. Granulocyte-macrophage progenitors (CFU-GM) were assayed in semisolid agar by a modification of the technique of Pike and Robinson (4). Erythroid progenitors or erythroid burst-forming units (BFU-E) were cultured in methylcellulose by a modification of the technique of Iscove and Sieber (5). Results were expressed in terms of the number of colonies per milliliter of marrow.

Continuous Liquid Cultures

Long-term liquid cultures were established using a modification of the technique of Dexter *et al.* (6). Bone marrow buffy coat cells (10^6) from normal donors were suspended in 1 ml of alpha medium (GIBCO Biocult) supplemented with 20% fetal calf serum; 5 ml of the suspension was put into Falcon 25-cm^2 tissue culture flasks and incubated at 37°C in a 5% CO_2 atmosphere. Cultures were maintained by weekly demidepopulation with addition of fresh medium. Under those conditions, a uniform adherent layer spread within 3 to 4 weeks. This primary culture was then depopulated of all suspension cells and irradiated at 30 Gy. A new allogeneic inoculum of bone marrow cells was added to the adherent layer. Cultures were maintained by replacing all of the medium each week, without demipopulation. At the same time, suspension cells were assayed for CFU-GM as described above.

Cell Treatment

Bone marrow cells were incubated with increasing doses of mafosfamide for 30 minutes at 37°C in a water bath with frequent agitation. Then they were washed once in Hanks' balanced salt solution and cultured. Buffy coat cells were obtained from bone marrow samples spun at 2,500 rpm for 10 minutes and adjusted to 2×10^7 cells/ml in autologous plasma and TC 199 solution, with a final hematocrit of 5%. The sensitivity of leukemic and normal progenitor cells to mafosfamide was evaluated by the diminution of their number. For the preincubation test (PIT), a 10-ml marrow aspirate was taken 15 days before bone marrow collection. The sensitivity of remission CFU-GM and BFU-E to increasing doses of mafosfamide was studied as described above, and a dose-response curve was established for each patient. The optimal dose for

incubation of the marrow was defined as the dose sparing 5% CFU-GM on the PIT.

Clinical Trial

After analyzing the results of the in vitro preclinical study, we started the clinical trial, performing ABMT in the consolidation mode. From January 1983 to October 1986, 57 adult patients entered the study. The clinical data are reported in another chapter (N. C. Gorin *et al.* "Use of Bone Marrow Incubated With Mafosfamide in Adult Acute Leukemia Patients in Remission: The Experience of the Paris Saint-Antoine Transplant Team") in this volume.

Collection of Marrow, Incubation With Mafosfamide, and Cryopreservation

A total volume of 600-1,300 ml of bone marrow (BM) was collected from the posterior iliac crest of each patient while he or she was under general anesthesia. A volume corresponding to 0.5×10^8 nucleated BM cells/kg was saved and immediately cryopreserved to serve as back-up marrow. The remaining volume was processed for incubation with mafosfamide. The buffy coat was collected on a Haemonetics H-30 cell separator (Haemonetics, Plaisir, France) and adjusted with TC 199 medium to a final cell concentration of 2×10^7 cells/ml with a final hematocrit of 5%. The suspension was finally incubated with mafosfamide (at the concentration previously established from the PIT as sparing 5% CFU-GM) for 30 minutes in a water bath at 37°C, with gentle shaking. After incubation, the BM suspension was immediately cooled and centrifuged at 4°C to block the drug's action abruptly. After two washes, the BM cells were resuspended in irradiated (40 Gy) autologous plasma and TC 199 medium and finally frozen with 10% dimethyl sulfoxide.

High-Dose Consolidation ABMT

The basic heavy consolidation treatment consisted of two doses of 60 mg/kg of CY (along with mesna, 60% of the CY dose), followed by total body irradiation at 10 Gy. Day 0 was defined as the day of marrow infusion.

Analysis of Data and Statistical Methods

The sensitivity of leukemic and normal hematopoietic stem cells to mafosfamide was studied by measuring (assaying, in triplicate, each dose of mafosfamide from 20 to 160 μg/ml in 10-μg increments) the proportion of remaining stem cells in treated compared with untreated marrow. Because of a sigmoid evolution of this proportion in relation to the mafosfamide dosage for each patient's sample, a probit transformation was used to linearize the relationship, taking into account the number of replicates used for each dose. For each curve, the linear slope and the derivation from linearity were tested by classical F test. Each curve was characterized by its linear slope and the doses necessary to achieve a 50% (LD_{50}) and a 95% (LD_{95}) reduction of the initial stem

cell pool. For each marrow sample, the mean and SD of these three parameters were determined. We tested the heterogeneity of the data by using components of variance analysis, using the classical F test to compare the variability of each parameter among patients with the common estimate of this variability within each patient. Comparison between sensitivities of various stem-cell categories was performed with the nonparametric Wilcoxon or paired Wilcoxon test, to avoid disturbance by the nonnormality of the parameters. For continuous liquid-culture data, correlation between the maximum CFU-GM regenerations and the percentage of residual CFU-GM at the time of culture initiation was studied by the Spearman nonparametric test.

RESULTS

In Vitro Studies

Sensitivity of CFU-L, CFU-GM, and BFU-E to In Vitro Treatment With Mafosfamide

The slope of the linear decrease was greater for CFU-L when compared with both CFU-GM ($P < .05$) and BFU-E ($P < .001$) (Table 1). No difference was observed for the LD_{50}. When the LD_{95} was studied, BFU-E appeared less sensitive than CFU-GM ($P < .01$) or CFU-L ($P < .05$). A major observation was the existence of wide variations in sensitivity among patients. These seemed to be greater than the variability of results within individuals, according to both the LD_{50} and the LD_{95} variances ($P < .01$ for CFU-GM with 36 and 270 *df*; $P < .05$ for BFU-E with 10 and 94 *df*). In our view, this observation supported the individual adjustment of the mafosfamide dose.

Table 1. Sensitivity of CFU to Mafosfamide

	CFU-L (n = 9) Median (range)	CFU-GM (n = 37) Median (range)	BFU-E (n = 11) Median (range)
S[a]	0.073	0.046	0.024
	(0.033 - 0.149)	(0.023 - 0.119)	(0.019 - 0.035)
LD_{50}[b]	35.1	39.1	44.4
	(18.8 - 51.5)	(17.9 - 84.6)	(11.8 - 9.24)
LD_{95}[b]	62.6	75.3	122.1
	(47.4 - 93.7)	(37.7 - 132.9)	(66.2 - 179.0)

Abbreviations: CFU-L, leukemic colony-forming units; CFU-GM, granulocyte-macrophage colony-forming units; BFU-E, erythroid burst-forming units.

[a]*Slope (percentage of destruction per microgram of mafosfamide) characterizing the linear decrease in residual stem cells in relation to increase of mafosfamide.*

[b]*Doses (μg of mafosfamide/2 x 10^7 cells) resulting in a 50% (LD_{50}) and a 95% (LD_{95}) destruction of initial stem cells.*

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Determination of the Optimal Mafosfamide Dose

Because of the inability, at least with routine laboratory techniques, to evaluate the risk of injuring pluripotent stem cells when destroying all CFU-GM, we defined the optimal mafosfamide dose for in vitro treatment of the whole BM as the dose that spares 5% of residual CFU-GM. As shown in Figure 1, the LD_{95} definition zone for most patients spreads over 14-30 $\mu g/2 \times 10^7$ buffy coat cells. In our experience, this allowed an accurate and reliable determination of the appropriate dose on a PIT. In a series of 32 marrow-cleansing procedures (for 24 patients in the clinical trial and 8 additional ones), residual CFU-GM were evaluated after incubation of the whole BM with the predetermined dose, and the results were compared with those given by the PIT. In 25 cases, in which all the technical parameters were adequate, the actual CFU-GM recovery measured after treatment of collected marrow was correlated with the expected recovery predicted by the PIT curve ($r = .473$, $P < .03$), and accuracy of the recovery was ± 4%. In the other seven cases, discrepancies were observed that could be retrospectively attributed either to an excessive hematocrit at time of bone marrow incubation, an inappropriately low temperature of the incubator, or the presence of cell aggregates. Figure 1 shows that if we decided to incubate all marrows with a standard dose of mafosfamide, rather than adapt the dose to each patient, the best choice of dose would be 70 $\mu g/2 \times 10^7$ cells, which would fit our objective of leaving 5% residual CFU-GM in 35% of the patients. With this

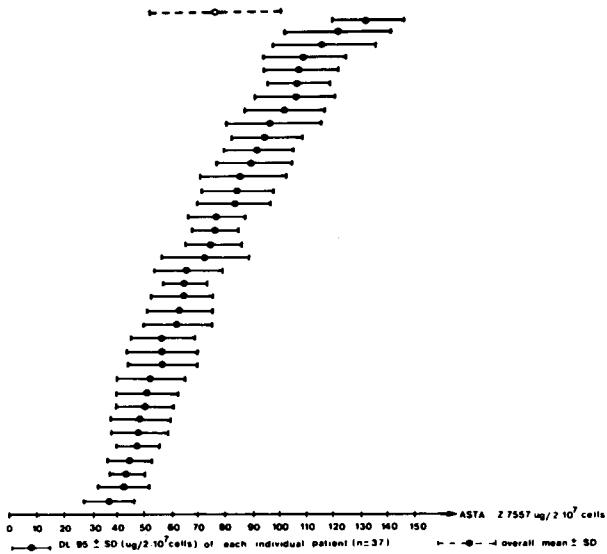

Figure 1. *Individual susceptibility of granulocyte-macrophage colony-forming units to mafosfamide (Asta Z 7557). (Reproduced from* Blood, *vol. 67, 1986, with permission of Grune & Stratton.)*

standard dose, 38% of the patients would have their marrow treated less intensively than is possible and 27% would be overtreated, with possible damage to the normal pluripotent stem cell pool.

Continuous Liquid Culture

Twenty-eight BM samples from patients with acute leukemia in CR were treated with various doses of mafosfamide (ranging from 25 to 140 $\mu g/2 \times 10^7$ buffy coat cells). Three series were defined: series 1 (n = 7) included marrows treated with moderation and still producing a median CFU-GM recovery of 39% (22-58%); series 2 (n = 13) included marrows treated according to our protocol with a median CFU-GM recovery of 3% (1.5-9%); series 3 (n = 8) included heavily treated marrows with no CFU-GM recovery. Three patients of this third group had their marrows treated with mafosfamide doses higher than the dose expected to reduce CFU-GM to undetectable levels. The control series (n = 13) consisted of untreated marrows from the same patients. Marrows of the three series and the control were studied in continuous liquid cultures, over an irradiated allogeneic adherent layer that did not give rise to CFU-GM per se in control experiments. The level of CFU-GM regeneration was assessed every week, and results were expressed as a percentage of CFU-GM recovery per flask compared with the number of CFU-GM detected when the culture was started in the same but untreated marrow. Figure 2 shows timing and level of CFU-GM regeneration in the three series.

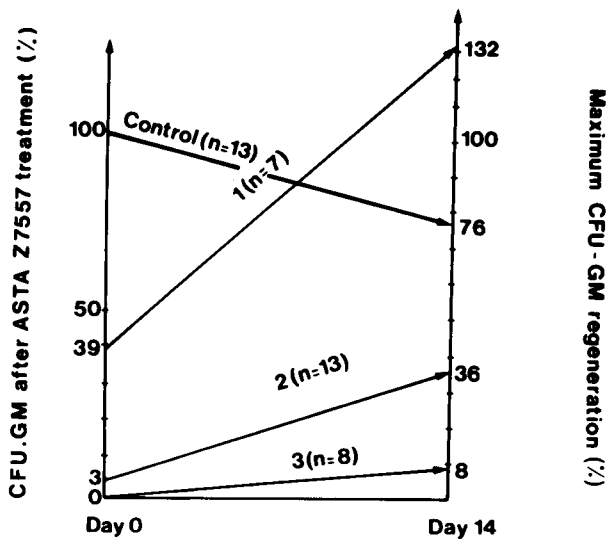

Figure 2. *Continuous liquid cultures after treatment of bone marrow with mafosfamide (Asta Z 7557): series of marrows treated with moderation 1), according to our protocol 2), or heavily 3), with median granulocyte-macrophage colony-forming unit (CFU-GM) recoveries in the input of 39%, 3%, and 0%, respectively. (Reproduced from* Blood, *vol 67, 1986, with permission of Grune & Stratton.)*

Clinical Results

Engraftment

Fifty of 52 patients showed consistent engraftment on reticulocytes and WBCs. The other two patients did not experience engraftment because of inadequate in vitro treatment that left no residual CFU-GM or BFU-E after incubation. We observed a major difference in the patterns of hematopoietic reconstitution between patients with ALL and ANLL on all parameters studied (Table 2), grafting being significantly faster in ALL patients. Moreover, platelets support had to be given for more than a year in three patients with ANLL.

Antileukemic Efficacy and Survival

These data are detailed in the chapter by N. C. Gorin *et al.*

DISCUSSION

Our preliminary in vitro studies demonstrated that CFU-L were more sensitive to mafosfamide than BFU-E, but not more than CFU-GM, which confirmed our previous observations with 4-hydroperoxycyclophosphamide (2). These results proved to us, however, that sensitivity to mafosfamide depended on technical parameters such as RBC contamination. This was one reason we elected to have all in vitro tests on buffy coat constantly adjusted to a final hematocrit of 5%; the other reason was that, when we used pure mononuclear fractions from Ficoll-Hypaque gradient-separated marrows, the

Table 2. Comparative Hematopoietic Recoveries in ALL and ANLL Patients After Autologous Bone Marrow Transplantation With Marrow Treated In Vitro by Mafosfamide

	Median No. of Days to Recovery (range)		
Hematopoietic Values	ALL (n = 22)	ANLL (n = 30)	*P* Value [a]
0.1% reticulocytes	15 (11–28)	20.5 (14–32)	<.01
1.0 x 10^9 leukocytes/l	19 (15–30)	33.5 (18–45)	<.001
0.5 x 10^9 neutrophils/l	20.5 (15–30)	35 (19–60)	<.01
50 x 10^9 platelets/l	50 (23–90)	110+ (45–480+)	<.01

Abbreviations: ALL, acute lymphoblastic leukemia; ANLL, acute nonlymphoblastic leukemia.

[a] *McNemar test.*

dose-response curve was too steep to select appropriate drug doses to work with. Others have made similar observations, and the inverse relation between mafosfamide cytotoxicity to progenitor cells and the RBC contamination responsible for nonspecific binding and diversion of the drug from the primary target is now well established.

Our preliminary studies also showed a wide range of sensitivity among patients; further, a few patients tested sequentially (data not shown) revealed an increasing resistance to the drug that developed in parallel with disease progression. When we started the clinical trial, these observations prompted us to study each patient's sensitivity to mafosfamide immediately before marrow collection to select the incubation dosage rather than using a constant amount—hence, we used the PIT. As in our preliminary studies, the PIT showed considerable variations among patients in response to identical drug concentrations, which suggested that adjusting the dose for individuals might be appropriate. Because we could not directly evaluate the tumor log-cell kill and because the biological importance of cells detected in the CFU-L assay is unknown, we decided to define the incubation dose of mafosfamide for marrow as the highest possible dose that would spare enough normal stem cells to ensure consistent engraftment. A residual amount of 5% ± 5% CFU-GM in the cleansed marrow was considered a safe margin beyond which further cytotoxicity would not be measurable, at least by conventional laboratory means. With this definition, doses of mafosfamide ranging from 50 μg to 140 $\mu g/2 \times 10^7$ buffy coat cells were necessary.

Since we initiated our program, other investigators have reported similar individual variations in sensitivity to mafosfamide both in animal progenitor cell populations (8) and in human leukemic and lymphoid cells (9). Our long-term culture studies indicated that even marrows totally depleted of CFU-GM still retained a self-regenerating ability. Although they confirmed the higher resistance of early progenitors to CY derivatives, as suggested by initial preliminary reports of successful autologous engraftment in humans with marrow deprived of CFU-GM (10), these studies also showed that the self-renewal capacity was proportional to the surviving fraction of CFU-GM in the sample initially placed in culture, which reassured us in our intention not to go below the threshold of 5% residual CFU-GM in the cleansed marrow. Fifty of 52 patients in our study experienced engraftment, although they received, as predicted, very low numbers of CFU-GM.

We can draw some conclusions. First, mafosfamide can be used to cleanse the marrow of patients with acute leukemia. Because patients show a wide range of sensitivity to the drug, we believe that individual dosage adjustment is an option, the clinical relevance of which must be tested further. Second, the differences in kinetics of hematopoietic recovery suggest that the engraftment potential has been altered more severely in ANLL than in ALL, which may reflect both the intensity of the in vitro treatment and the intrinsic fragility of the stem cell pool in ANLL.

ACKNOWLEDGMENT

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Autologous Bone Marrow Transplantation for Acute Lymphoblastic Leukemia of Childhood During Second Remission

E. Plouvier, D. Amsallem, A. Noir, and P. Herve

In acute lymphoblastic leukemia (ALL) of childhood, the results of new chemotherapy protocols are promising, since 50-70% of children remain in first complete remission (CR) 5 years after diagnosis (1,2). After a relapse, a second remission is usually induced by chemotherapy but is of short duration in most cases. The prognosis for ALL in second remission therefore remains poor (3,4).

For children who do not have a sibling whose leukocyte antigen is identical, autologous bone marrow transplantation (ABMT) after intensive therapy represents a new approach to treating ALL in second CR (5).

We report here our experience with 19 children with ALL in second CR treated with intensive therapy followed by ABMT.

PATIENTS AND METHODS

Since 1979, 19 children with ALL in second CR, 5 girls and 14 boys, underwent ABMT. The children's mean age was 8 years, with a range of 2-14 years.

Twelve children had L_1 and seven children had L_2 type of ALL, according to the French-American-British classification.

Immunologic typing, done for 14 children, showed the common acute lymphoblastic leukemia antigen (CALLA)-positive phenotype in 9, the T phenotype in 3, and resulted in null findings in 2.

Sites of relapse were: 13 in bone marrow, 4 in testis, and 2 in central nervous system (CNS). Nine children relapsed while in maintenance treatment or during the first 6 months after stopping therapy, and 10 children relapsed while out of therapy.

Marrow cells were harvested during the first CR of eight children who had high-risk ALL and during second CR for the others.

Only one bone marrow specimen was nonpurged, and nine bone marrows were treated by chemotherapy with mafosfamide (Asta Z 7557).

Nine children received bone marrow treated by immunologic methods according to the lymphoblasts' initial immunophenotype. Two children with T-ALL were treated with a combination of three monoclonal antibodies (MAb), CD2/D66, CD5/A50, and CD7/I21, and seven children were treated with anti-CALLA antibodies, CD10 and J5 or AL B2; in both cocktails, two rounds of rabbit complement were used.

Median interval between second CR and ABMT was 4 months (range, 2-12 months). After autografting, no maintenance treatment was given.

Conditioning regimens before ABMT were total body irradiation (TBI) with 10-13.2 Gy and 60 mg/kg/day of cyclophosphamide over 2 days for 15 children, TBI and 140 mg/m^2 of melphalan for 3 children, and TBI and high doses of cytarabine and melphalan for 1 child.

All children underwent total gut decontamination; 5 were treated in a plastic isolator and 14 in single rooms with careful protection against contamination.

RESULTS

Engraftment occurred in all children. Peripheral blood neutrophils reached 0.5×10^9/l at a median of 27.4 days (range, 18-58 days). Only one child never recovered a normal platelet count and died on day 104 of CNS hemorrhage while in CR. In the other children, platelet count reached 50×10^9/l at a median of 38 days (range, 19-68 days).

During the first 3 months after the graft, we observed six cases of bacterial sepsis, three of viral infections (herpes), and one of interstitial pneumonitis (cytomegalovirus, CMV). The children tolerated the conditioning regimen well, but the child with CMV pneumonitis died early, 34 days after ABMT.

Late complications, uncommon in this group of children, included one mild case of cataract, two of decreased growth, and one death of CNS hemorrhage. One year after ABMT, we do not see any significant long-term change in the surviving children's intellectual or other specific neuropsychological functions.

In 17 children, the antileukemic effect of the conditioning regimen,

followed by ABMT, was evaluable. Twelve of these children are still in unmaintained CR with a median follow-up of 28.7 months (range, 6+-68+ months); in 10 children, CR has lasted longer than 12 months (Fig 1). Five children relapsed between 4 and 10 months, and three died soon after this second relapse.

Among nine children who relapsed while in therapy, four relapsed post-ABMT, four are in second continuous CR (CCR), and one, as explained, died of CMV pneumonitis.

Among 10 children who relapsed off therapy, only 1 child relapsed post-ABMT, 1 died of CNS hemorrhage, and 8 are in second CCR with a median follow-up of 30.1 months (range, 6-68 months).

DISCUSSION

In our study, the incidence of relapse was lower than in others concerning ABMT for childhood ALL (6,7). In the near future, we shall have to compare the clinical results of ABMT in terms of event-free survival with the best chemotherapy and with allogeneic bone marrow transplantation.

In few studies (8,9) allogeneic bone marrow transplantation was compared with normal-dose chemotherapy in the management of children with ALL after

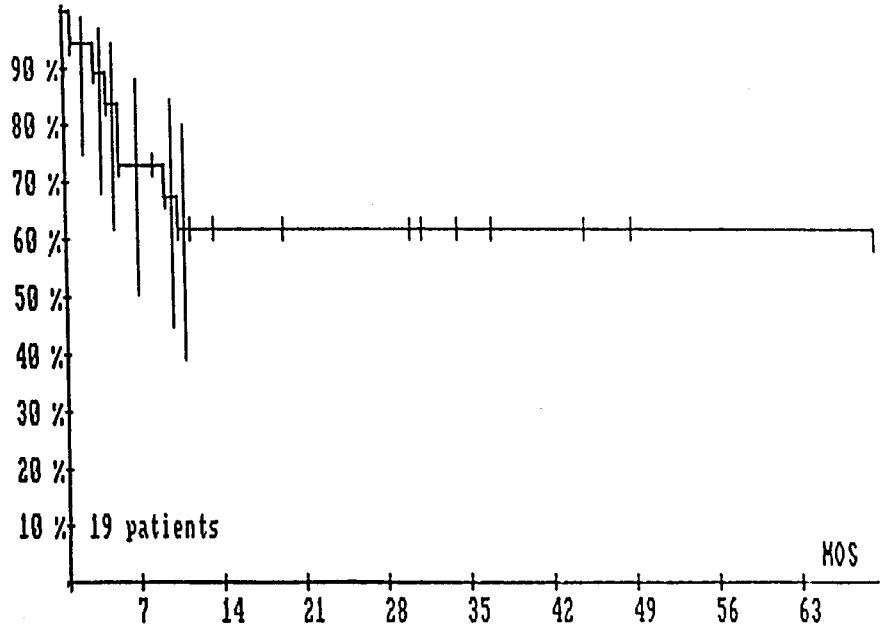

Figure 1. *Event-free survival of 19 children with acute lymphoblastic leukemia autografted during second complete remission.*

first relapse. These studies showed a survival advantage for children undergoing allogeneic bone marrow transplantation. For children who relapsed off therapy, the results of allogeneic bone marrow transplantation and ABMT were satisfactory; the authors (8,9) found that TBI may be fractionated and the total dose increased to 12 Gy without major toxicities when TBI is associated with cyclophosphamide administration alone.

For children who relapsed while in therapy, the relapse rate after transplantation was high. It will be necessary to increase dosages of the conditioning regimen, adding another chemotherapeutic agent such as high-dose cytarabine or melphalan.

The clinical results of ABMT in ALL of childhood should be evaluated according to the course of the disease—whether relapse occurs during or after therapy. We still do not know whether chemical agents such as mafosfamide and VP-16-213 (etoposide), MAbs such as CD10 plus CD19 or a pan-T MAb cocktail provide the best methods of cleansing bone marrow (10). The best way to cleanse marrow cells might be to combine chemoseparation and immuno-separation (paper submitted for publication).

Our results seem to favor ABMT during second remission, but prospective, controlled trials must be done before any conclusion can be reached.

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Ex Vivo Use of Monoclonal Antibodies With Complement and Mafosfamide

P. Herve, E. Plouvier, D. Amsallem, E. Racadot, and A. Noir

Autologous bone marrow transplantation (ABMT) following intensive therapy represents a new approach in treating children with acute lymphoblastic leukemia (ALL) in second or subsequent complete remission (1), but the question of which methods are most suitable for removing contaminating leukemic cells from remission bone marrow is unresolved.

Among the most extensively studied techniques are those using monoclonal antibodies (MAbs) (in complement-mediated cytolysis, as immunotoxins, and in immunophysical separation) and those using chemotherapeutic agents (cyclophosphamide derivatives and etoposide) (2-5). Until now no clinical studies have compared the relative efficiency of drugs and MAb treatments (in terms of relapse rate after ABMT) used to eliminate leukemic cells from autologous inoculum. However, two recent studies have reported the efficiency of combining MAbs with complement and a cyclophosphamide derivative such as 4-hydroperoxycyclophosphamide or mafosfamide (sometimes called Asta Z 7557) (6,7).

In our laboratory we compared the efficiency of combining MAb clusters of differentiation 10 and 19 (CD 10 and CD 19) with rabbit complement and mafosfamide to that of a single treatment with either MAbs and complement alone or mafosfamide alone for the treatment of marrow cells and the Reh6 clonogenic cell mixture. Here we describe the clinical course of a child grafted

with marrow treated ex vivo with a combination of MAbs and complement with mafosfamide.

PRECLINICAL STUDIES

Materials and Methods

Normal bone marrow cells were procured from the transplant donor. The Reh6 leukemic cell line was continuously cultured in Iscove's medium with 20% heat-inactivated fetal calf serum. The cell concentration was kept at 0.6-1.5 × 10^6 cells/ml. The cells were incubated at 37°C and 5% Co_2 in air. Before treatment, equal volumes of bone marrow cells (3.8 × 10^7 cells/ml) and the Reh6 cell line (2 × 10^6 cells/ml) were mixed to obtain a final concentration of 5% Reh6 cells. The mixed cell suspension was adjusted to a concentration of 2 × 10^7 cells/ml.

The MAbs CD10/ALB2 and CD19/AB1 (Immunotech, Marseilles, France) are IgG2a and IgM immunoglobulins, respectively, and have been previously described (8,9). Complement from baby white rabbits (age, 23-28 days) was prepared in our institution and stored in the gas phase of liquid nitrogen (−150°C). The *cis*-4-sulfoethylthiocyclophosphamide or mafosfamide-lysine (provided by Asta-Werke, Bielefeld, Federal Republic of Germany) is a new type of oxazaphosphorine (3). It is a white crystalline powder that in aqueous solution is stable up to several hours at pH 4 but only 15-20 minutes at pH 7. Its molecular weight is 548 daltons.

Normal human bone marrow or 5% of the Reh6 leukemia cell line mixed with irradiated marrow cells was incubated with MAbs for 30 minutes at 4°C. Rabbit complement was then added to a final concentration of 1:1 for 30 minutes at 25°C. Cells were pelleted, and the complement procedure was repeated for a total of two cycles. After two washings, the cells were exposed to 30 μg/ml mafosfamide for 30 minutes at 37°C. After incubation, cells were washed one time and were resuspended in Iscove's modification of Dulbecco's medium (IMDM) for leukemic cell line clonogenic assay and granulocyte-macrophage colony-forming unit (CFU-GM) assay (7 and 21 days) as previously described (3).

Results

We summarize in Table 1 the results of a series of experiments that indicate that a kill of greater than 4 logs of the Reh6 leukemic cell line can be achieved by combining purging protocol with a chemical agent of acceptable toxicity for myeloid progenitor cells detected in a middle-term liquid culture (21 days). Combining CD 10, CD 19, and complement with mafosfamide is superior in terms of log kill to using either the MAbs or the chemical agent alone.

Table 1. Effect of Protocol on CFU-GM Recovery and Reh6 Cell Line Growth

Protocol	CFU-GM Recovery (%) Day 7 (n = 4)	CFU-GM Recovery (%) Day 21 (n = 4)	Reh6 Cell Target Log Cell Kill (n = 4)
Complement	77.4 ± 4.8	91.5 ± 12	0.16 ± 0.02
CD 10 + CD 19 + C1C2	79.8 ± 11.9	100	3.4 ± 0.1
Mafosfamide (30 μg/ml)	12.8 ± 9.1	51 ± 35	2.9 ± 0.5
CD 10 + CD 19 + C1C2 + mafosfamide	11 ± 8	45.5 ± 38	4.7 ± 0.2

Abbreviation: C1C2, two cycles of complement.

REPORT OF A CASE

We examined the feasibility of ABMT using marrow treated with CD 10 and CD 19 MAbs with two cycles of complement and mafosfamide before cryopreservation. The patient was a child with non-T, non-B ALL in fourth remission.

The 1-year-old boy was first admitted to our hospital in October 1984. An ALL diagnosis was established—he was considered pre-B ALL, his test results were positive for the common acute lymphoblastic leukemia antigen (CALLA), and he was classed as L_1 according to the French-American-British classification system. The disease was induced into remission in November 1984 with the Berlin-Frankfurt-Munich protocol (10). In January 1985 the first marrow relapse occurred on therapy. When three courses of chemotherapy failed to reach a durable remission, the patient was put into fourth remission by one course of high-dose cytarabine and amsacrine. Given that no donor was available for allogeneic bone marrow transplantation, autologous marrow was harvested during the fourth remission. We harvested 8×10^8 nucleated cells/kg and 1×10^5 CFU-GM/kg. To eliminate leukemic cells, we incubated the cells in CD 10 and CD 19 and added baby rabbit complement for two cycles and 30 μg/ml mafosfamide for 30 minutes at 37° C (Fig 1). The CFU-GM recovery was 0.1%; continuous liquid culture (21 days) produced a CFU-GM recovery of 13%. Before treatment, cytofluorometry revealed 17% CALLA-positive cells in the inoculum, but after the complement-mediated cytolysis, only 2 CALLA-positive cells (ethidium bromide negative) were detected in 500 marrow cells.

The ablative regimen consisted of a fractionated total body irradiation during 3 days at 2 Gy/fraction up to a total of 12 Gy, followed by high-dose cytarabine (3 g/m^2 every 12 hours for eight doses) and high-dose melphalan (140 mg/m^2). This protocol was well tolerated by our patient. The hematopoiesis recovery occurred slowly (1×10^9 leukocytes/l on day 24, 0.5×10^9 granulocytes/l on day 26, and 50×10^9 platelets/l on day 37) (Fig 2).

The patient has not received additional chemotherapy following the ABMT procedure. He is alive and well in complete remission 8 months after ABMT.

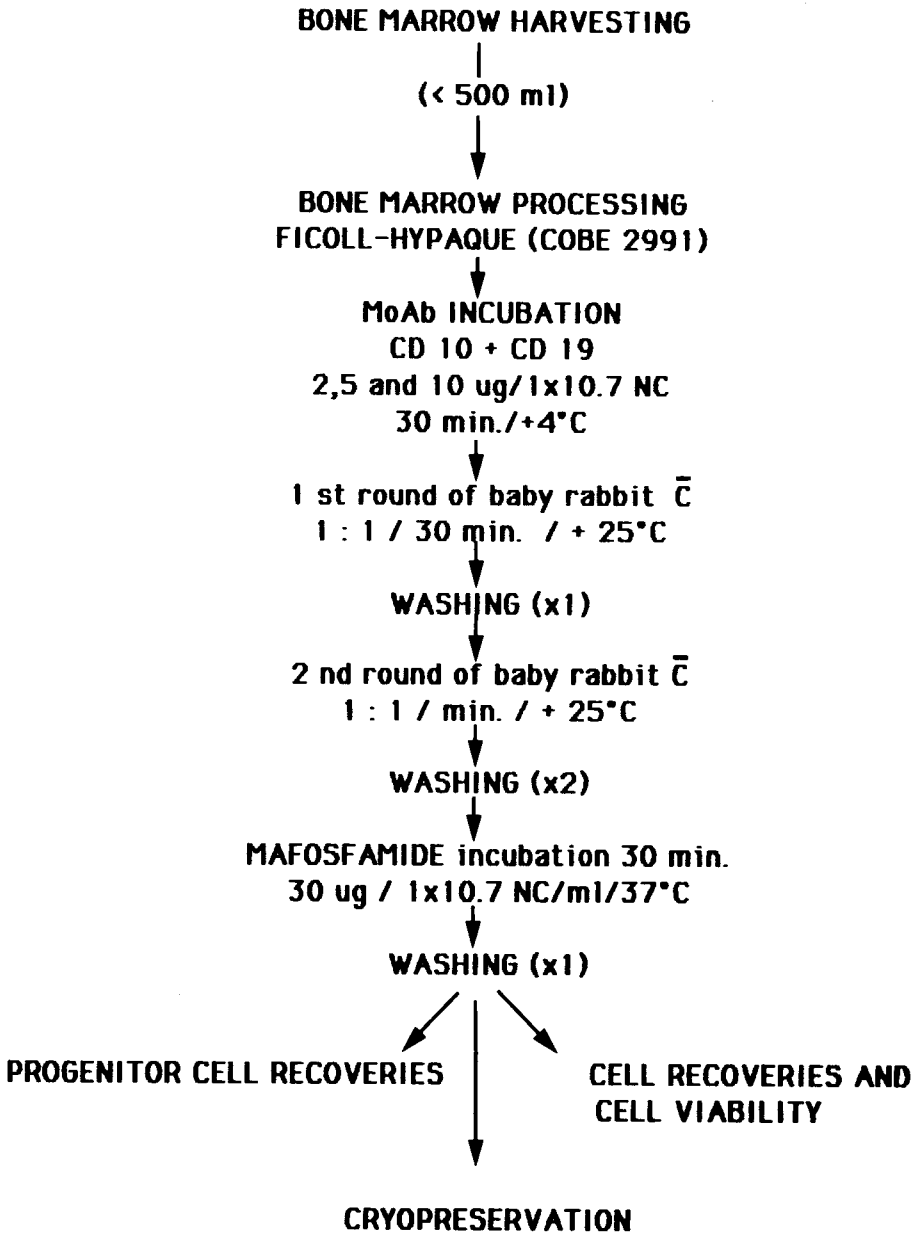

Figure 1. *Bone marrow processing and ex vivo treatment protocol (MoAb, monoclonal antibody; CD, cluster of differentiation; NC, nucleated cells; C, complement).*

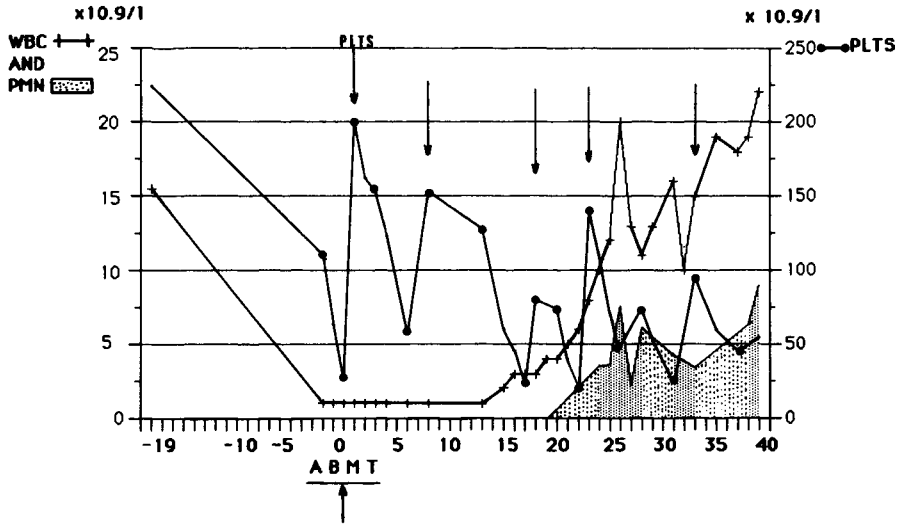

Figure 2. *Hematologic recovery after autologous bone marrow transplantation with marrow cleansed by monoclonal antibodies and mafosfamide (PMN, polymorphonuclear neutrophil leukocytes; PLTS, platelets).*

DISCUSSION

It has been shown that with rabbit complement and sometimes with human complement most of the MAbs belonging to the CD 10 and CD 19 groups are strongly lytic against common ALL cell lines and fresh human leukemic blasts (4,8,11). The MAbs CD10/ALB2 and CD19/AB1 are efficient candidates for immunoseparation of the autologous marrow (8,9). Preclinical studies have established the highly efficient tumor cell killing ability of cyclophosphamide derivatives, and clinical trials have demonstrated that in vitro chemotherapy of autologous inoculum could ensure successful engraftment (3,12).

In other preclinical studies, we have shown that by combining the techniques of chemoseparation and immunoseparation, it was possible to obtain more effective removal of leukemic cells from autologous marrow. Using a clonogenic assay, we could define optimal conditions for eliminating the Reh6 cell line from an excess of normal marrow cells. The present in vitro study demonstrates that MAbs and complement followed by 30 μg/ml mafosfamide allowed a 40-50% recovery of CFUs-GM after 21 days in liquid culture.

Middle-term liquid culture might be more correlative in predicting hematopoietic reconstitution kinetics. In previous clinical studies we observed that marrow treatment with higher doses of mafosfamide (50 μg/ml/l $\times$ 10^7 nucleated cells) totally inhibits 7 days' CFU-GM growth but permitted CFU-GM generation after middle-term liquid culture.

The Reh6 cell line represents an experimental model system that does not

reflect the leukemic cells that contaminate patients' bone marrow. In the future it would be desirable to use the patient's leukemic cells to appraise the efficiency of such combined treatment, especially inasmuch as promising results are emerging for growing leukemic lymphoid cells (13,14).

Development of immunopharmacological strategies for ex vivo treatment of marrow may lead to possible eradication of the residual disease in high-risk ALL. This first case report supports the view that this approach is feasible. To further explore its merits, we have undertaken a pilot study in our institution.

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Conditioning Regimens Before Bone Marrow Transplantation in Acute Myelocytic Leukemia

Anton Hagenbeek and Anton C. M. Martens

Given the significant relapse rate after both allogeneic and autologous bone marrow transplantations in patients with acute myelocytic leukemia (AML), much emphasis has been placed in recent years on improving ablative conditioning regimens before bone marrow transplantation. Figure 1 shows that at present a number of different ablative conditioning regimens are being applied during that period.

The general characteristics of ideal conditioning agents are: 1) a steep dose-response curve for leukemic cells; 2) myelotoxicity as the major dose-limiting factor; 3) a low degree of nonmyeloid toxicity to gut, heart, lungs, bladder, CNS, and liver; and 4) appropriate pharmacokinetics. Gale and Champlin (1) recently surveyed the clinical data concerning relapses of patients after allogeneic bone marrow transplantation (BMT) performed during first remission. They found the following relapse percentages after a variety of conditioning regimens were used: cyclophosphamide (CY) and total body irradiation (TBI), 25%; high-dose cytosine arabinoside and fractionated TBI, 20%; fractionated TBI and CY, 10%; busulphan (Bu) and CY, 0%.

In autologous bone marrow transplantation (ABMT), the major factor responsible for a leukemia relapse seems to be residual leukemic cells' survival

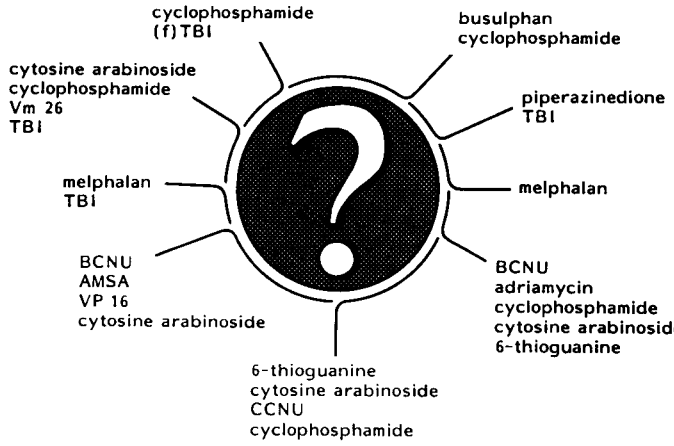

Figure 1. *Ablative conditioning regimens preceding bone marrow transplantation in patients with acute leukemia.*

of ablative chemo- and radiotherapy, and not the presence of reinfused leukemic cells in the autologous marrow graft. Arguments for this statement are: 1) Similar relapse rates occur after autologous and isologous BMT. 2) Leukemia relapses occur at about the same time after autologous, isologous, and allogeneic BMT, median of 9 months. 3) The ED_{50} for human AML is 10^3-10^4 clonogenic cells (extrapolated from preclinical data in the brown Norway rat AML (*Leuk Res* 1985;9:1389-1395). 4) So far in vitro "purging" has had no beneficial effect.

The differences in efficacy of different conditioning regimens in eradicating residual leukemia in the host lack a quantitative basis in terms of log leukemic cell kill. In parallel to the clinical studies, therefore, the antileukemic effect of the components of two conditioning regimens were evaluated in AML in the brown Norway rat as a relevant model for AML: CY with TBI, and CY with Bu. All experiments were initiated on day 13 after inoculation with 10^7 brown Norway rat myelocytic leukemia (BNML) cells. At that particular stage the total leukemic cell load amounts to 5×10^9-10^{10} cells.

MATERIALS AND METHODS

Rats

The experiments were performed with the inbred brown Norway rat strain produced in the Rijswijk colony. Male rats between 13 and 16 weeks of age and a mean body weight of 200 g were used.

The Rat Leukemia Model

The origin, classification, transplantation procedure, and growth characteristics of the BNML model have been described in detail elsewhere (2,3).

Leukemia was induced in a female brown Norway rat by 9,10-dimethyl-1,2-benzanthracene. The disease shows a reproducible growth pattern upon intravenous cellular transfer within the brown Norway rat strain, and it is cytologically and cytochemically similar to human acute promyelocytic leukemia. Further analogies with the human disease are: 1) slow growth rate (10^7 BNML cell kill after 18-23 days; growth fraction, 0.60-0.40); 2) severe suppression of normal hematopoiesis because of an absolute numerical decrease in the number of normal hematopoietic stem cells; 3) diffuse intravascular coagulation; 4) prolonged blood transit time of leukemic cells (34-36 hours); 5) response to chemotherapy as in human AML; 6) presence of clonogenic leukemic cells (including in vitro colony formation; TD_{50}, 25 cells); 7) low antigenicity; and 8) no evidence for a virus as an etiologic agent.

Cytostatic Agents

Cyclophosphamide was kindly provided by Asta-Werke, Bielefeld, Federal Republic of Germany. The drug was dissolved in 0.9% saline and injected intraperitoneally. Busulphan was a gift from the Wellcome Company, Beckenham, England. It was dissolved in carboxymethylcellulose and injected intraperitoneally. Cytosine arabinoside (cytarabine), provided by The Upjohn Co., Kalamazoo, MI, USA, was dissolved in 0.9% saline and injected intravenously into the tail vein.

Total Body Irradiation

The procedure for TBI has been described extensively (4). In short, TBI was carried out with a γ-radiation source (Gammacel 220, ^{137}Cs, Atomic Energy of Canada, Ltd.) yielding a dose rate of 1.15 Gy/minute. The D_0 for γ-rays in vivo for BNML cells is 0.85 Gy, with an extrapolation number of 3.7.

Determination of Log Leukemic Cell Kill by Different Conditioning Regimens

Given the linear dose-effect relationship between the number of (injected) leukemic cells and the survival time (2), the log leukemic cell kill induced by any given treatment regimen is deduced from the increase in life span as compared with that of nontreated leukemic control rats, according to the rule that a 4-day prolongation of life span corresponds to a 1-log cell kill. All experiments were started on day 13 after inoculation with 10^7 BNML cells (i.e., at a total body tumor burden of approximately 5×10^9-10^{10} cells).

RESULTS AND DISCUSSION

Cyclophosphamide and Total Body Irradiation

At a dose of 100 mg of CY/kg, a 5-log leukemic cell kill was induced, the LD_{50} for CY being 164 mg/kg (5). Higher dosages, in combination with

Table 1. Summary: Efficacy of Ablative Conditioning Regimens Before Bone Marrow Transplantation in Acute Myelocytic Leukemia

Regimen	Log Leukemic Cell Kill in Rat (BNML)	% Relapse After Allogeneic BMT in First-Remission AML
CY + TBI	8 - 9	25
TBI + CY	9 - 10[a]	10
CY + Bu	10	—
HDAC + CY + TBI	>10[b]	5 - 10
Bu + CY	>10	0

Abbreviations: CY, cyclophosphamide; TBI, total body irradiation; Bu, busulphan; HDAC, high-dose cytosine arabinoside.

[a] *20% treatment-related mortality.*

[b] *25% treatment-related mortality.*

isologous bone marrow transplantation, resulted in an increasing percentage of toxicity-induced animal deaths and were thus abolished.

Although TBI alone appeared to be most effective if given as a single dose (9.0 Gy: 4-log cell kill) compared with fractionated regimens (e.g., 2.25 Gy daily × 4; 2.0 Gy twice daily × 4; 3.0 Gy twice daily × 2; 4.5 Gy daily × 3: 2- to 3-log cell kill; [4]), a combination of 100 mg/kg CY with either one 9.0-Gy TBI fraction or fractionated TBI (2.0 Gy twice daily × 3.5, or 4.5 Gy TBI daily × 3) resulted in a 9-log leukemic cell kill. With the reverse order—i.e., 9.0-Gy TBI followed by 100 mg/kg CY—a 9- to 10-log leukemic cell kill was achieved at the expense of 20% treatment-related mortality. At present, the underlying mechanism of the increased efficacy and toxicity of TBI/CY compared with CY/TBI is unknown.

If, preceding CY/TBI treatment (100 mg/kg CY; 9.0-Gy TBI), the total body tumor load was reduced by cytarabine in a regimen of 200 mg/kg every 12 hours × 6, 75% cures were achieved (i.e., more than 10-log leukemic cell kill). However, 25% toxic deaths were observed after this intense treatment regimen (6).

Busulphan and Cyclophosphamide

Busulphan alone in a dose of 30 mg/kg (LD_{50}, 56 mg/kg) induced a 6-log cell kill and was thus more effective than CY alone, given comparable normal tissue toxicity. Cyclophosphamide (100 mg/kg) followed by Bu (30 mg/kg) yielded a 10-log cell kill with 100% late relapses. The reverse order—similar dosages of Bu followed by CY—resulted in 25% cures (more than 10-log leukemic cell kill) and 75% relapse deaths.

In summary, as indicated in Table 1, the efficacies of the various

conditioning regimens explored in the BNML model were: CY followed by TBI, 8- to 9-log cell kill; TBI followed by CY, 9- to 10-log cell kill but 20% toxic deaths; CY followed by Bu, 10-log cell kill; cytarabine preceding CY/TBI, more than 10-log cell kill but 25% toxic deaths; and Bu followed by CY, more than 10-log cell kill.

In extrapolating to human AML (tumor load in remission equal or less than 10^{10} cells), the difference between an 8- and a 10-log cell-kill conditioning regimen might be of crucial importance in preventing patients from developing a leukemia relapse after bone marrow transplantation from "minimal residual disease." The results obtained with the BNML model are in line with those recently summarized for humans ([1], see Table 1).

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Double Unpurged Autologous Bone Marrow Transplantation in 41 Patients With Acute Leukemias

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Following the results obtained by allogeneic bone marrow transplantation in the treatment of acute leukemias, autologous bone marrow transplantation (ABMT) became a therapeutic approach, alternative to similarly intensive radiochemotherapies, for patients who had no marrow donors.

The limits of such methods could be the inability of the conditioning regimen to kill the last leukemic cell, the reinfusion of leukemic cells with the marrow transplant, and the absence of an allogeneic graft-versus-leukemia reaction. Because of melphalan's good record of tolerance and antitumoral effects, we used it as a conditioning regimen for several malignant conditions (1-3). After preliminary experience with one ABMT (4), we considered that a second course of high-dose chemotherapy followed by another ABMT could yield a superconsolidation useful for some patients with leukemia. Further, by collecting the marrow after the first transplantation, we could presume that the leukemic contamination was minimal at that time; we decided, therefore, to use this second marrow transplantation after a new conditioning regimen which was given to the recipient as a last therapeutic intensification. We report here

our experience with 41 leukemic patients who completed this double unpurged ABMT program.

PATIENTS AND METHODS

Patients

Among the 41 patients who entered the study (Table 1), 12 had acute lymphoblastic leukemia (ALL); 6 of these were in more than second relapse at the time of ABMT, and 6 were in complete remission (CR) at that time. Two of the patients in CR were adults who had received autografts during their first CR, and four were children who were at the end of their first CR at the time of ABMT.

Of the 29 patients who had acute myeloid leukemia (AML), 16 received ABMT at the time of relapse, and 13 did so to consolidate the first CR.

Conditioning Regimens

Acute Lymphoblastic Leukemia

Before their first ABMT, the patients received 140 mg/m^2 of melphalan (1), and afterward 10 mg/m^2 of methotrexate was administered intravenously on days +1, +3, +6, and +11; then, weekly, from day +32 to day +102 post-ABMT, methotrexate was given alternately in intravenous and intrathecal administration according to the Seattle schedule of allogeneic transplantation. Before the second ABMT, the patients were conditioned with the CBV protocol as described previously (5): cyclophosphamide, 1.5 g/m^2/day for 4 days; BCNU (carmustine), 300 mg/m^2/day for 1 day; VP-16-213 (etoposide), 125 mg/m^2/day for 4 days.

Acute Myeloid Leukemia

Before each ABMT, the patients received 140 mg/m^2 of melphalan given as an intravenous bolus during hyperhydration (1). After the second marrow collection the patients received one to three cycles of chemotherapy with 100 mg/m^2/day of etoposide for 2 days and 100 mg/m^2/day of cytarabine for 5 days before the second bone marrow transplantation.

Table 1. Clinical Data of Patients

Status at ABMT	n	M/F	Median Age (range)	Median Follow-up Post-ABMT (mo)
ALL relapse	6	2/4	11 (4–50)	>24
ALL remission	6	2/4	14 (8–25)	24 (23–36)
AML relapse	16	6/10	42 (15–66)	18 (2–62)
AML first remission	13	7/6	16 (2–50)	15 (5–42)

Abbreviations: ABMT, autologous bone marrow transplantation; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia.

Marrow Procedures

Marrow was aspirated during CR up to collection of a minimum of 2×10^8 medullary cells/kg. Marrow was stored in liquid nitrogen after cryopreservation with dimethyl sulfoxide until clinical use. After the first ABMT, when granulocytes numbered more than 1000/mm^3 and platelets more than 100,000/mm^3, a second marrow collection was done and the marrow cryopreserved. The second marrow was used for the second transplantation; no in vitro purge was used.

Supportive Care

Patients were managed in single rooms with reverse isolation. All had had a right atrial indwelling catheter inserted before admission. They received parenteral nutrition if needed and broad-spectrum intravenously administered antibiotics if they became febrile. Blood transfusions were done to maintain hematocrit at more than 30% and platelets higher than 20,000/mm^3. All blood products, except marrow, were irradiated at 15 Gy before transfusion.

RESULTS (Table 2, Fig 1)

Acute Lymphoblastic Leukemia

Five of the six patients (83%) who were in relapse at the time of the first ABMT achieved a CR after melphalan administration. One died of pneumonia 1 month after the procedure while in CR. Two patients had relapses early at 1 and 3 months and did not receive a second ABMT. Two underwent a second procedure after a conditioning regimen by CBV; both relapsed subsequently, one 9 and the other 17 months post-ABMT. Of the six patients who were grafted

Table 2. Antileukemic Response, Relapse, and Survival After Autologous Bone Marrow Transplantation

Status	n	CR	Death in CR	Relapse	CCR	Alive
AML in relapse	16	11	3	6	2	3 (52, 62, 63+)
ALL in relapse	6	5	1	4	—	—
AML in first CR	13	—	1	6	6	9 (5, 6, 9, 12, 15, 15, 20, 22, 42+)
ALL in CR	6	—	—	3	3	3 (23, 29, 36+)

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; CR, complete remission; CCR, continuous complete remission; ( ), follow-up in months post-ABMT.

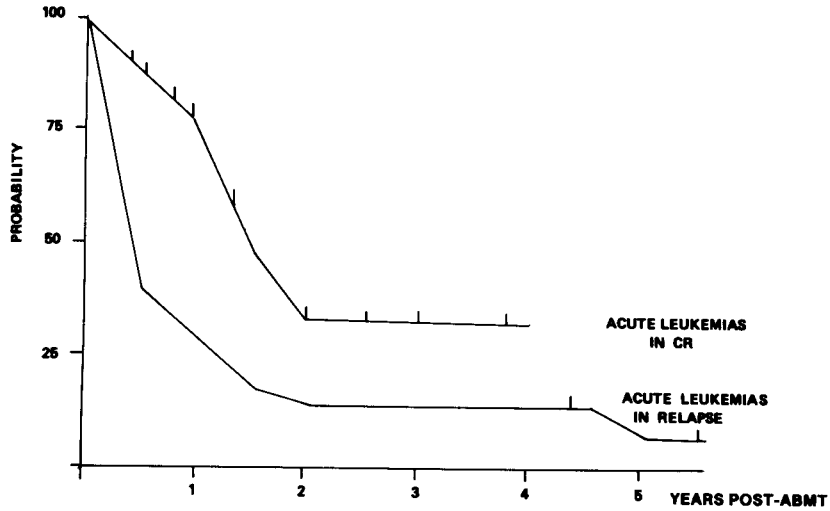

Figure 1. *Disease-free survival after ABMT.*

during a CR, two relapsed at 3 and 4 months and could not receive a second ABMT. Of four patients who received two transplantations, one relapsed at 12 months, and three are in continuous complete remission (CCR) at 23+, 29+, and 36+ months post-ABMT. Two of these three patients are adults who received autografts as a consolidation of a first CR; one is a child who received an autograft to consolidate a third CR.

Acute Myeloid Leukemia

Of 16 patients in relapse at the time of the first ABMT, 11 (69%) obtained a complete response after melphalan administration. One died in CR 2 months later of fulminant hepatitis, and two patients relapsed early at 2 months. One patient refused a second ABMT and remains in CCR at 52+ months post-ABMT. Seven patients received a second ABMT. Two died of infection while in second CR, one of fulminant gram-negative pneumonia (at 4 months) and one of extensive aspergillosis (at 20 months). Four patients relapsed at 8, 11, 12, and even 60 months post-ABMT. One patient remains in CCR 62 months+ post-ABMT.

Of 13 AML patients who received autografts as a consolidation of a first CR (median duration of the first CR, 4.5 months; range, 2-10 months), two are disease free 6 and 9 months later, with fewer than 100,000 platelets/mm^3; they did not receive a second ABMT. Eleven patients received two courses of melphalan and two ABMTs; one died while in CR of human immunodeficiency virus infection induced by unscreened blood transfusions 17 months after ABMT; six relapsed between 8 and 20 months (median, 13 months) after ABMT; and four are in unmaintained CCR between 5+ and 42+ months post-ABMT (median, 13 months).

Engraftment (Table 3)

All patients who achieved or were in CR after the first ABMT had hematologic recovery, although full hematologic recovery was not achieved by two AML patients 6 and 9 months, respectively, after they received ABMT during their first CR. In the other patients, a second bone marrow aspiration was done within 3 months (range, 1-12 months) after the first ABMT and of those, 24 patients received a second ABMT after 5 months (range, 1-13 months) following the first autograft. A comparison of the hematologic recovery of these 24 patients between the two grafts shows that the duration of aplasia was generally longer after the second ABMT than after the first.

Transplantation-Related Complications

Overall, morbidity and mortality of transplantation were more severe in the patients who received transplants for relapsed leukemia rather than for leukemia in CR. Among the 22 patients who received transplants during relapse, four died of opportunistic infections while they were in CR, compared with only one death in CR (from human immunodeficiency virus infection) among 19 patients who underwent transplantations during CR.

DISCUSSION

These results confirmed that melphalan given at high dose (>140 mg/m^2) is able to induce a high remission rate in leukemic patients in relapse (1,4). With this single alkylating agent, the 73% CR rate is similar to that possible to obtain with the "best" combinations of drugs, with or without total body irradiation (TBI) (5-8). Despite this marked antileukemic response, however, the duration of the remissions was short in the patients who relapsed: of 16 patients who achieved a CR after a first dose of melphalan, four relapsed before 3 months and could not have a new marrow aspiration and a second ABMT. Although all

Table 3. Comparison of Hematologic Recovery Between First and Second Autologous Bone Marrow Transplantations of 24 Patients Who Received Two Autografts

Diagnosis	Days to Recovery of	First ABMT median value (range)	Second ABMT median value (range)
ALL	Nucleated cells/kg × 10^8	1.8(1.2-2.4)	1.85(1.8-2.8)
n = 6	Granulocytes > 500/mm^3	17(12-23)	17(14-28)
	Platelets > 50,000/mm^3	23(15- >50)	35(12-42)
AML	Nucleated cells/kg × 10^8	2.5(0.6-5)	1.9(1-6.1)
n = 18	Granulocytes > 500/mm^3	12(5-48)	15(9-102)
	Platelets > 50,000/mm^3	21(10-96)	35(14- >240)

Abbreviations: ALL, acute lymphoid leukemia; ABMT, autologous bone marrow transplantation; AML, acute myeloid leukemia.

the ALL patients who received autografts during relapse had a subsequent relapse and died, some patients with AML in relapse (3 of 16) remained disease free more than 4 years after ABMT, results close to those obtained with allogeneic bone marrow transplantation under the same conditions (9). The occurrence of a late relapse at 5 years suggested, however, that a very long follow-up is necessary to assess a possible rate of "cure" in these patients.

When the patients have undergone transplantation during CR, both the rates of transplant complications and of leukemic relapses were lower. In this study, for which the follow-up is still short, it is impossible to assess with certainty the rate of long-term, disease-free survival after ABMT for leukemia in remission. At the present time, our results are comparable to those of other investigators who are using more aggressive conditioning regimens containing TBI for autologous or even syngeneic transplantation (7,10,11). The value of "in vitro purging" for ABMT in leukemias remains to be demonstrated (7,10). The double ABMT could be an approach to diluting the residual leukemia in the marrow transplant indirectly by an in vivo rather than an in vitro procedure.

The place of ABMT for patients with acute leukemias remains to be determined. If our results suggest that some patients may have relatively long-term, disease-free survival after such procedures, prospective studies are needed to compare the outcome of similar patients after intensive chemotherapy or allogeneic bone marrow transplantation.

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Double Autografting as Potential Curative Therapy for Acute Leukemia

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We applied the same chemotherapeutic treatment protocol to 62 patients with acute leukemia, using combination chemotherapy for bone marrow ablation. The response to high-dose chemotherapy and autologous bone marrow transplantation (ABMT) and its associated morbidity and mortality were compared in 35 patients with acute myeloblastic leukemia (AML) and 27 patients with acute lymphoblastic leukemia (ALL).

Eleven AML patients and seven ALL patients received second autografts during first remission, whereas two AML and six ALL patients underwent second autografts after that period.

PATIENTS AND TREATMENT PROTOCOLS

Induction remission for AML is based on Medical Research Council protocols, Medical Research Council 9 for AML and UKALL IX and UKALL X for ALL.

Marrow remission was established in each patient by morphologic light-microscopic examination of aspirate taken within 5 days of the harvest. Bone marrow was harvested, processed, and cryopreserved in liquid nitrogen as described previously (1). No ex vivo manipulation of marrow was performed in an attempt to eliminate minimal residual disease.

The high-dose chemotherapeutic regimen for ablation used in all cases was the UCH (University College Hospital) 1 protocol: cyclophosphamide 1.5 g/m^2/day i.v. on days -5, -4, and -3 (with mesna administration); doxorubicin, 50 mg/m^2 i.v. on day -5; 1,3,bis-(2 chloroethyl)-1-nitrosourea, 300 mg/m^2 i.v. on day -5; cytarabine, 100 mg/m^2 twice daily i.v.; and thioguanine, 100 mg/m^2 twice daily orally on days -5, -4, -3, and -2. The autologous bone marrow was thawed and reinfused through a Hickman central venous catheter. The mean number of nucleated cells frozen was 1.9×10^8 cells/kg of the recipient's weight.

All patients in the study were considered eligible for two sequential autografts, marrow for ABMT II being harvested as soon as the patient's hematologic system recovered. Only 26 of the 62 patients (42%) proceeded to ABMT II. The reasons for the remaining patients not having a second ABMT are outlined below for each patient group.

The rationale for two autografts was to increase the tumoricidal dosage of drugs and to effect a form of "in vivo purging," as reported previously (2).

RESULTS

First-Remission AML

Of the 27 patients treated during first remission, 19 (70%) remain in treatment-free remission at median follow-up of 26 months (range, 3-70 months). Eleven patients had two grafts and all remain in treatment-free remission at median follow-up of 29 months (range, 4-70 months) after transplantation. Sixteen patients received a single graft and 8 (50%) are in treatment-free remission at a median follow-up of 15 months (range, 2-70 months) after ABMT. Of the 27 patients, 1 patient (3.7%) died of cerebral hemorrhage during the thrombocytopenic phase posttransplant. No other procedure-related mortality occurred in this group of patients. Seven patients have relapsed following a single transplant at 4, 4, 5, 6, 8, 10, and 11 months after ABMT. Of these patients, three had secondary leukemia and relapsed at 4, 4, and 5 months, respectively. The reasons for not progressing to ABMT II are that five patients refused to proceed, three patients wish to proceed to ABMT II but have not yet achieved sufficient hematologic recovery, three patients relapsed before the second marrow harvest could be taken, one patient died during ABMT I of the procedure, and four patients had delayed hematopoietic recovery. Figure 1 shows the predicted disease-free survival of all patients transplanted in first complete remission (CR) with AML. Figure 2 shows a comparison of patients who received one ABMT with those who received two.

First-Remission ALL

Twelve patients with ALL received autografts during first CR, and only three patients remain in remission after ABMT at 12, 13, and 33 months, respectively.

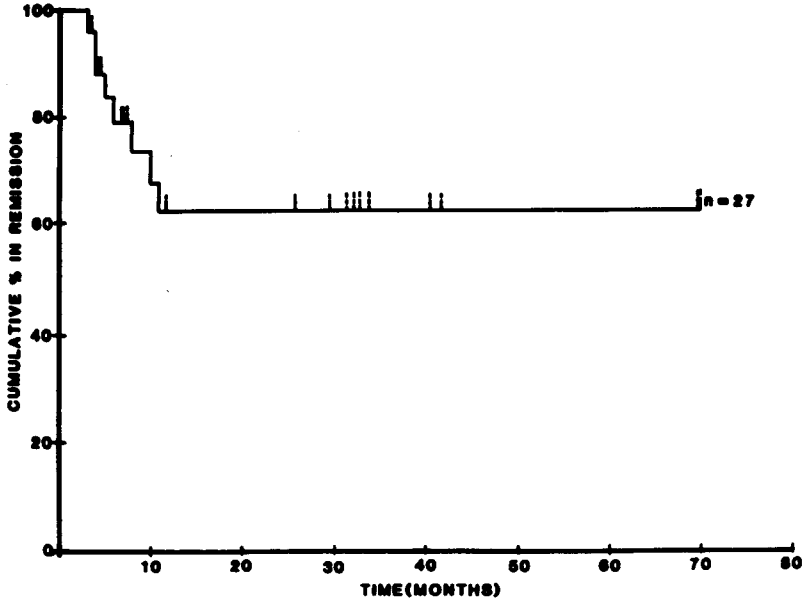

Figure 1. *Disease-free survival of patients (n = 27) with acute myeloblastic leukemia who received autografts during first complete remission.*

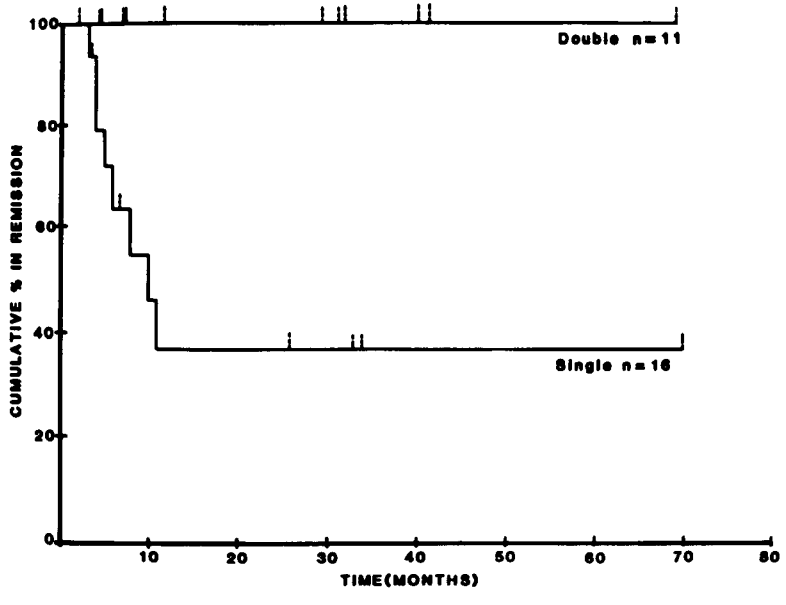

Figure 2. *Disease-free survival of patients (n = 11 vs. n = 16) with acute myeloblastic leukemia who received autografts during first complete remission: double vs. single grafts.*

All three patients had T-cell ALL. Seven of the 12 patients have had two grafts; three of these remain alive at 12 and 13 months after ABMT. Two other patients relapsed late, at 32 and 33 months after ABMT. One patient died of procedure-related complications during the first autograft, and three patients died during the second, making the total procedure-related mortality 25% in this group. Two patients refused the second procedure, and two patients relapsed before ABMT II could be carried out. Figure 3 shows the estimated disease-free survival for this group.

After First Remission

Of the eight patients who had AML beyond first remission, all have died. Two of these patients had two grafts and relapsed at 5 and 8 months after ABMT I. Six patients had one graft; one patient died of sepsis during the neutropenic phase, and the others relapsed at 2, 2, 3, 5, and 7 months, respectively. Figure 4 illustrates the poor disease-free survival of this group.

Fifteen patients who had ALL received transplants after the first CR, and six of these patients had two grafts. Two patients remain in remission at 31 and 56 months after transplantation, the first having had one and the other two grafts. One patient died of nonengraftment on day 124; the remaining 12 patients died of leukemia at a median of 4 months after transplantation (range, 2-10 months). Figure 5 shows this group's estimated disease-free survival.

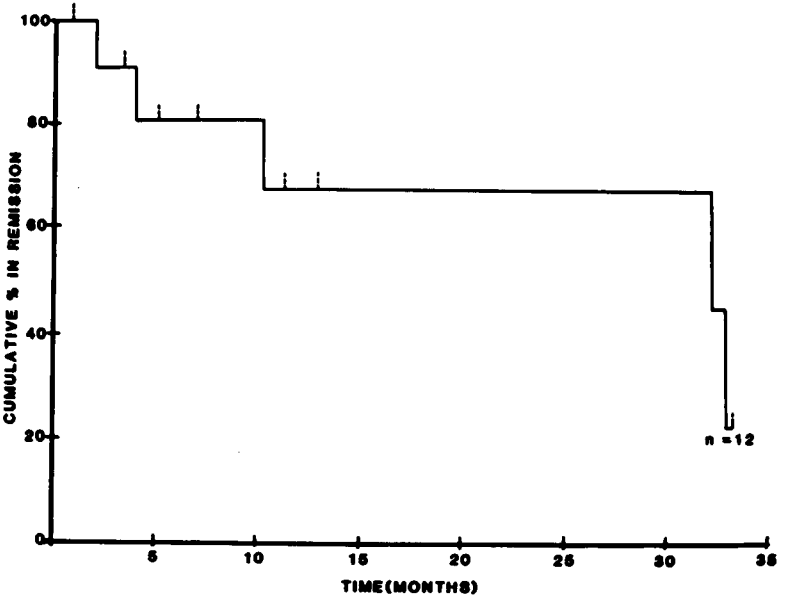

Figure 3. *Disease-free survival of acute lymphoblastic leukemia patients (n = 12) who received autografts during first complete remission.*

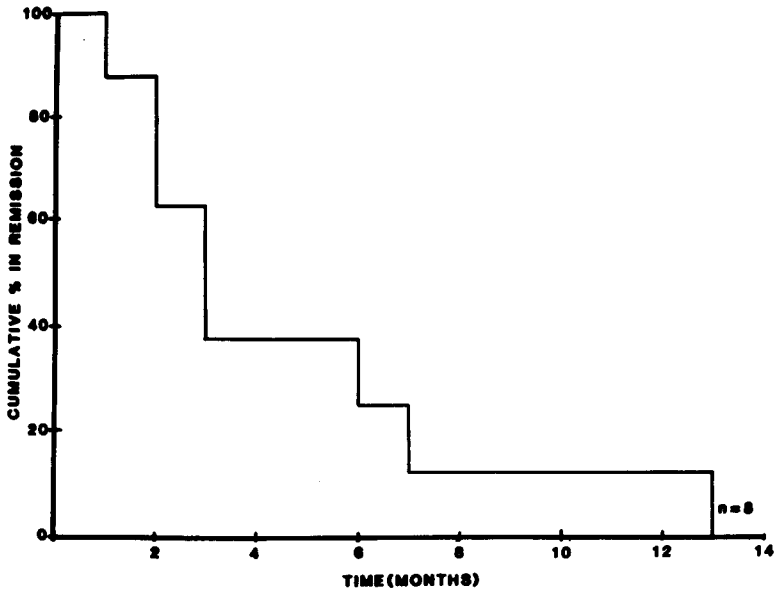

Figure 4. *Disease-free survival of patients (n = 8) with acute myeloblastic leukemia who received autografts during second or later remission.*

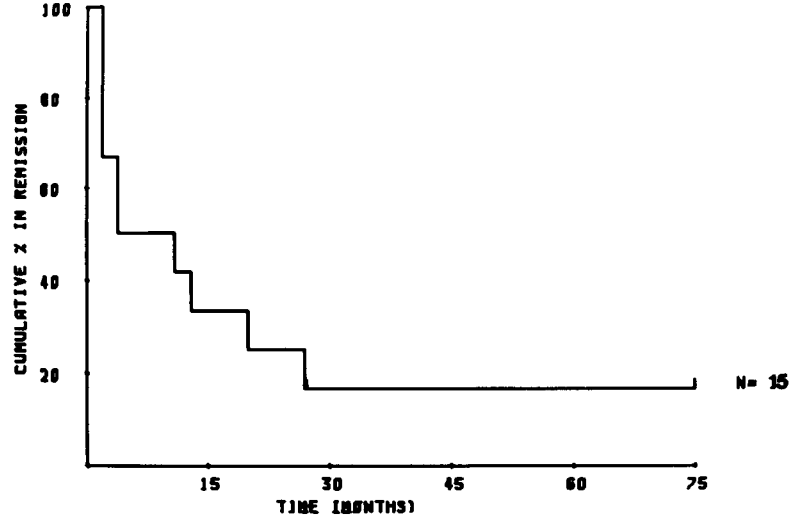

Figure 5. *Disease-free survival of patients (n = 15) with acute lymphoblastic leukemia who received autografts during second or later remission.*

Hematologic Recovery

The hematologic recoveries are shown in Table 1. Assays of granulocyte-macrophage colony-forming unit (CFU-GM) did not predict speed of hematologic recovery in this study (H. M. Jones from Joint Medical School of University College & Middlesex Hospitals, London, England, personal communication).

Support Requirements

Duration of inpatient care was similar for patients with AML and ALL. Median inpatient stay during ABMT I was 30 days (range, 20-64 days), and for ABMT II it was 43 days (range, 26-74 days).

Blood product support was also similar for AML and ALL patients, the mean number of units of RBCs and platelets required being 8 units and 74 units, respectively, during ABMT I and 12 units and 93 units, respectively, during the second procedure. Normal donor granulocyte transfusions were given to 12 patients because of severe sepsis.

Early Complications

All except five patients became pyrexial and required broad-spectrum antibiotic administration. One patient developed an aspergilloma during ABMT II. Gram-negative septicemia was documented in 19 episodes and was more common in patients with AML (16 episodes) than in those with ALL (3 episodes). Gram-positive infections were documented in 28 episodes with no statistically significant difference between patients with ALL or AML.

Table 1. Hematologic Recovery After Autologous Bone Marrow Transplantation

		Days After Marrow Infusion	
		Median	Range
ABMT I (n = 54)[a]			
Leukocytes	>1 x 10^9/l	19	(12-40)
Neutrophils	>0.5 x 10^9/l	21	(12-52)
Platelets	>50 x 10^9/l	32	(13-228)
ABMT II (n = 22)			
Leukocytes	>1 x 10^9/l	26	(12-56)
Neutrophils	>0.5 x 10^9/l	28	(14-56)
Platelets	>50 x 10^9/l	42	(22-94)

Abbreviation: ABMT, autologous bone marrow transplantation.

[a]*Patients who died during the aplastic phase excluded from analysis.*

Sepsis during the aplastic phase was the primary cause of death in five patients.

Five of 62 patients (7.1%) had significant hemorrhagic complications and two patients died of intracerebral hemorrhage despite platelet support. During ABMT II, one patient with ALL in first CR died of cardiac arrest, for which no cause could be identified at postmortem examination.

DISCUSSION

Double ABMT may be considered as an in vivo variant of the ex vivo purging of marrow that is under investigation in various centers (3,4). The potential role of ex vivo manipulation of bone marrow to eliminate residual disease remains unclear (5).

Our results, which showed the better disease-free survival from AML in first remission for patients who had two grafts over those who had one, must be interpreted with caution. The patients proceeding to ABMT II were the fittest patients in whom engraftment was quick and who did not relapse early after ABMT I. It may be that rapid hematologic recovery is a manifestation of a "healthy" marrow uncontaminated by residual disease. Moreover, ABMT II was carried out between 50 and 100 days after infusion of the first marrow, as soon as the patient had a neutrophil blood count higher than 1.5×10^9/l and a self-sustaining platelet count of more than 100×10^9/l. The patient's general fitness was also taken into account before reharvesting. This means that most patients going through ABMT II had been in remission about 8 months, making them an even more select group in terms of survival. Survival of this group should therefore be considered in comparison with that of chemotherapy patients who have already had 8 months of disease-free survival after remission.

The practicalities of double ABMT for patients with AML in first remission are such that only 41% of patients have achieved a second graft. Nonetheless, the few patients in this group who are able to have ABMT II seem to do well. Hematologic recovery, particularly platelet recovery, after ABMT II is delayed.

The disappointing results of this chemotherapeutic approach for first-remission ALL patients, using either a single or a double graft, may be interpreted in various ways. The high procedure-related mortality occurring in this group, as opposed to that of AML first-CR patients, makes interpretation of this small group's results even more difficult. Nonetheless, the patients seem not to have benefited. First, induction with UKALL IX may leave too high a yield of minimal residual disease to be ablated by high-dose chemotherapy and ABMT. Second, chemotherapy ablation for ABMT in ALL appears less effective than total body irradiation, considering the European Bone Marrow Transplant Group results (5).

For patients beyond first remission who have either AML or ALL, this chemotherapy protocol with either a single or a double ABMT appears unsuitable. The ablative procedure is clearly inadequate to remove underlying

resistant leukemia. More encouraging results have emerged from the Baltimore group in these patients (4).

We have abandoned this ablative protocol for all groups other than patients with AML in first remission.

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Hematopoietic Recovery After Bone Marrow Transplantation Using Marrow Purged With 4-Hydroperoxycyclophosphamide

Craig S. Rosenfeld, Kenneth F. Mangan, Richard K. Shadduck, and O. Michael Colvin

The prognosis for patients with acute myelogenous leukemia (AML) in relapse is poor, median survival after relapse being about 6 months with conventional chemotherapy (1). To improve this outcome, allogeneic bone marrow transplantation has been used (2,3), but few patients can obtain such a transplant because of age or lack of a major histocompatibility complex (MHC) identical donor.

Results of syngeneic bone marrow transplants (those from identical twins) suggest that the relapse rate is approximately 50% (2). With syngeneic transplantation, though, the failure to relapse can be attributed to the antileukemic effect of the conditioning regimen and not a graft-versus-leukemic effect (4). This observation suggests that autologous bone marrow transplantation (ABMT) may be a successful therapy for leukemia, provided the bone marrow can be purged of residual leukemic cells. Experience in a rat model of AML indicates that 4-hydroperoxycyclophosphamide (4-HC), a metabolite of cyclophosphamide, is capable of killing all residual tumor in marrow suspensions intentionally contaminated with leukemic cells (5), and clinical studies with treated marrows have produced promising results (6,7). Because

during these clinical trials many patients had delayed bone marrow recovery, we carried out a study to determine if the number of granulocyte-macrophage colony-forming units, or progenitor cells (CFUs-GM), after 4-HC treatment correlated with the kinetics of bone marrow recovery.

METHODS

All protocols for the high-dose preparative regimen and ex vivo marrow treatment were reviewed and approved by the Protection of Human Subjects Committee of Montefiore Hospital. Informed consent was obtained from all patients and from the parents of children who were patients.

Thirteen patients were included in this study (Table 1). Patients with acute lymphocytic leukemia (ALL) (median age, 15 years) were significantly younger than patients with AML (median age, 25 years). All patients had at least one documented hematologic relapse of leukemia. The disease status of the patients at the time of bone marrow transplantation is shown in Table 1.

The procedures for collecting and processing marrow have been described in detail previously (6). In brief, 8×10^8 nucleated marrow cells per kilogram of body weight were collected according to the method of Thomas and Storb (8). Approximately one third of the collected marrow was treated ex vivo with 4-HC at a concentration of 60 μg/ml, and two thirds was treated with a concentration of 100 μg/ml (for ten patients) or 120 μg/ml (for three patients). The 4-HC was produced at the Johns Hopkins Oncology Center in Baltimore. The marrow treated with the lower dose of 4-HC was reserved for infusion if engraftment with the more heavily treated marrow failed. The treated marrows were frozen in a controlled-rate liquid nitrogen freezer and then transferred to the liquid phase of liquid nitrogen in a freezer storage cabinet. The day of the infusion of the autologous bone marrow was designated day 0.

The CFUs-GM were measured by an agar gel technique (9), which was modified in that cells were separated by Ficoll-Hypaque. Cells were mixed with

Table 1. Patient Characteristics

Characteristic	ALL	AML
Number	6	7
Age (yr)		
Median	15[a]	25[a]
Range	13–41	10–52
Male : Female	1 : 1	6 : 1
Early relapse	2	3
Isolated CNS relapse	1	1
2 Complete remissions	3	1
>2 Complete remissions	0	2

Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia.
[a]*Significant difference between median ages (P < .05).*

McCoy's medium containing 15% fetal calf serum in 0.3% agar. Pretreatment marrow samples were plated at 1×10^5 cells/ml. Because of the reduction in CFUs-GM after incubation with 4-HC, treated cells were plated at 1×10^6 cells/ml. Human fibroblast-conditioned medium served as a standard source of colony-stimulating factor (10). Cultures were incubated for 7 days at 37°C in a humidified atmosphere of 7.5% CO_2 in air. Colonies of more than 25 cells were counted with the aid of a dissecting microscope.

Patients with AML received busulfan (1 mg/kg of body weight/dose orally every 6 hours for 16 doses) on days −9, −8, −7, and −6, followed by cyclophosphamide (50 mg/kg/dose i.v.) on days −5, −4, −3, −2. The bone marrow was infused on day 0. Patients with ALL or AML with evidence of CNS disease received cyclophosphamide (50 mg/kg i.v.) on days −8, −7, −6, and −5 and total body irradiation (3 Gy/day, with the lungs shielded after 9 Gy) on days −4, −3, −2, and −1 with bone marrow rescue on day 0.

Patients were cared for in single rooms using simple isolation. Before transplantation, a right atrial catheter was placed in each patient. All patients at risk for recurrence of herpes simplex infection received prophylactic intravenous acyclovir. Broad-spectrum antibiotics were given for fever during aplasia, and amphotericin was added for persistent fever. Antibiotics and isolation were discontinued when the neutrophil count exceeded 0.5×10^9/l. All blood products were exposed to 2.5 Gy before infusion.

RESULTS

Time to hematologic recovery is shown in Table 2. Patients with ALL had a significantly more rapid recovery than did patients with AML in neutrophil (P = .023) and leukocyte counts (P = .025) but not in reticulocyte count (P = .21). The slower rate of recovery in AML is also reflected in the number of patients who required the back-up marrow. Although the differences are not significant by chi-square analysis (P = .07), the reserve marrow was required by three of seven patients with AML but none with ALL.

There was a marked reduction in CFUs-GM after a 30-minute exposure of marrow cells to 4-HC (Table 3). Approximately 1% of the CFUs-GM remained after incubation with this agent.

The relationship between the number of CFUs-GM infused and hematologic recovery was examined for both ALL and AML. The log of the number of

Table 2. Median Days to Recovery With Autologous Bone Marrow Transplantation

Group	500 Neutrophils/μl[a]	1,000 WBCs/μl[a]	1% Reticulocytes
AML	42 (22–59)	33 (24–60)	49 (29–185)
ALL	19.5 (17–35)	19.5 (14–33)	29 (17–40)

[a]*Significant difference between median values (P < .05).*

Table 3. CFUs-GM Before and After 4-HC Treatment

Group	Cells/kg × 10^{-8}	CFUs-GM/kg × 10^{-4}	
		Before 4-HC	After 4-HC
AML	3.8 ± 0.2	20.6 ± 5.5	0.29 (1.5%)
ALL	4.0 ± 0.3	25.0 ± 15.4	0.19 (0.8%)

Abbreviations: CFUs-GM, granulocyte-macrophage colony-forming units; 4-HC, 4-hydroperoxycyclophosphamide; AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia.

CFUs-GM infused was compared with the number of days to achieve a WBC count of 1,000/μl, neutrophil count of 500/μl, and reticulocyte count of 1% (uncorrected). In ALL there was no correlation (Pearson coefficient) for the neutrophil count (r = .795) or reticulocyte count (r = .640). Likewise, no relationship was found in AML between the CFU-GM dose and WBC count (r = −.574), neutrophil count (r = −.534), or reticulocyte count (r = .159).

DISCUSSION

Autologous bone marrow transplantation is a promising therapy for acute leukemia (6,7,11), but its results may be improved if residual leukemic cells can be purged from the marrow. In this study, 4-HC was used as a purging agent, but we found hematologic reconstitution was slow in this group of patients. The median time to achieve a neutrophil count of 500/μl in patients with AML was 42 days. This compares with 29 days in another series in which 4-HC was used to purge marrow of patients with AML (7). This delayed engraftment may be attributed in part to damage of the progenitor cell by 4-HC. Approximately 1% of harvested CFUs-GM remain after treatment with 4-HC. Other investigators have had similar experiences (12,13).

In our series, the marrow of patients with AML recovered much more slowly than did the marrow of patients with ALL. This delay did not appear to result from a difference in disease status, harvested number of CFUs-GM, or number of CFUs-GM infused. These findings suggest that patients with AML may have greater damage to their stem cell compartment from their disease or prior therapy. It is possible that such stem cells may be more susceptible to injury from exposure to 4-HC.

Our data also suggest that no relation exists between the number of CFUs-GM infused and bone marrow recovery for patients with ALL or AML. Clearly, the toxicity of 4-HC to CFUs-GM is not solely responsible for delayed recovery because patients with ALL had fewer residual CFUs-GM yet they showed more rapid engraftment. Perhaps 4-HC is acting on an earlier progenitor cell than the CFUs-GM. Others' results indicate a reduction in certain human pluripotent hematopoietic colony-forming cells (13) but only limited effects on primitive blast cell colonies after exposure to 4-HC (14).

Other studies addressing the predictive value of the CFU-GM assay on recovery of purged marrows have yielded conflicting results. Gorin and coinvestigators have purged marrows with a dose of mafosfamide that retains 5% of CFUs-GM (15). Our analysis of their data from patients with AML indicates no correlation between the number of CFUs-GM infused and the number of days to a leukocyte count of 1,000/μl. In contrast, Rowley *et al.* have reported a correlation between the log of CFU-GM content and the time to recovery (1,000 leukocytes/μl and 500 granulocytes/μl) (16); however, their observations were based on a human pluripotent stem cell assay or mixed assay, so it is possible that the two assays have different predictive values.

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Autologous Bone Marrow Transplantation in Second and Subsequent Remissions

P. Herve and K. A. Dicke, Chairmen

DR. K. DICKE: Dr. Yeager, can you tell me the frequency of remission inversion in your patients who are relevant?

DR. A. YEAGER: In terms of patients who are survivors, 13 are in remission, 7 of which have had transplantation remissions that exceeded first remissions. Of the 15 patients who had relapses, 1 had a posttransplant complete remission (CR) that exceeded the duration of first remission. And that was 6 months after the second remission to relapse and 3 months to first remission.

DR. DICKE: So basically, in your entire patient population of 40 patients, at this moment, 7 have had transplantation remissions longer than 12 months and exceeded the duration of the preceding remission.

DR. YEAGER: Eight patients have had inversions; seven of those eight are still in CR. One patient had a transplantation remission that lasted less than 12 months.

DR. DICKE: Right, according to our criteria, the inversion rate is 7/40:17.5%. What is the inversion rate in your series, Ted (Dr. E. Ball)?

DR. E. BALL: We have not really looked at that. We have, in the homogeneous group of second and third CR patients, four event-free survivors, and I know two of them have definitely had inversions. I don't know the first-remission data on the other two.

DR. DICKE: Do the participants agree with me that we have to look at inversions? If this is done, the patient selection can be avoided on the basis of long-term first remissions.

DR. G. SPITZER: How many patients have had classical Adriamycin (doxorubicin) therapy with a first-remission duration of 12 months or less and are long-term disease-free survivors in your and Dr. Ball's study?

DR. YEAGER: The median duration in both those that relapsed and those that have been in CR is approximately 15 months and the range is quite wide. We have had some that were in both groups 2 and 3 months. The medians are comparable.

DR. B. VAUGHAN: I think this inversion concept is really very, very important because in these inversions, the patient is, of course, his own control and the prognostic factors become less arguable. In our experience with normal-dose chemotherapy and unmaintained remission of adult myelocytic leukemia, we studied reinductions with the same regimen and then no maintenance in second remission. There are inversions, something like 5 out of 10 inversions in patients whose first CR was greater than 18 months, and no inversions in patients whose first CR, even unmaintained, was less than 18 months.

DR. DICKE: I think that is a very important statement.

DR. VAUGHAN: And so this is a way in which these patients can be controlled by themselves and compared with other experience. I don't think that, at this point, randomized trials, with their tremendous expense and organization, are appropriate for this type of developmental work.

Andy (Dr. Yeager), do you want to talk about inversions in patients whose first remission was less than 18 months? Was your answer to Gary (Dr. Spitzer) that inversions did occur in that group of patients?

DR. YEAGER: I have my cheat sheet in front of me here. Of the patients who have shown inversions, four of the seven have had CRs of actually less than 15 months. The exact times were 14, 2, 5, and 4 months.

DR. A. BURNETT: Just a comment. Karel (Dr. Dicke), since you are about to make the point that Andy's very interesting data do not supply any evidence to support that the purging has had this effect, because they themselves say that the busulfan plus Cytoxan regimen is the greatest thing since sliced bread, which we might believe, we have patients who we have treated with either purged or unpurged marrow in second remission. There is no difference in the outcome. Maybe Dr. Yeager has found the answer, a good cytoreductive protocol. But there is no evidence that purging has had an effect.

DR. DICKE: Thank you. Dr. Ramsay, do you agree that the inversion rate in your patients is 27%? In my opinion, that is positive. I think that is higher than in the Johns Hopkins studies. I realize childhood acute lymphocytic leukemia is a

different disease. You conclude that you want to improve your purging method, but you know when you look at your data and when you compare the results with the allogeneic data, the long-term disease-free survival is about the same. So how do you think that with improved purging you can increase your disease-free survival significantly?

DR. N. RAMSAY: Well, I think that is a good point. I think that not only the purging needs to be improved, but also the conditioning regimen. I agree with you. In our allogeneic patients, the relapse rate is approximately 50%. So we know that at least that much is from presumably inadequate preparation of the patient. But there is still about a 25% difference between the relapse rate in the allogeneic patient population and in the autologous patient group. If we change two things at once, we are not going to know what we are doing. We want to continue to treat our allogeneic patients for another year and one-half or so with our current conditioning regimen because the initial results look encouraging. And so we are changing the purging methodology and will accrue patients over the next year and one-half with the same conditioning regimen and two different purging methods. Hopefully, we can get at least some hint as to whether there is any change.

The only other thing I wanted to say, Karel, about the inversion rate in the ALL patients, is that the relapse after transplant is so early that the remission posttransplant in any of the patients that have a relapse, which is about three-fourths of them, is so short that virtually the only patients in which there is an inversion are the ones who are surviving relapse free.

DR. DICKE: So then, what is your conclusion?

DR. RAMSAY: If the patients survive disease free, then those patients are in the positive, or whatever you call it—they've had inversions. But if they have a relapse, which most of them do very early, then they have not had an inversion.

DR. SPITZER: Norma (Dr. Ramsay), is the similar biology going on in the ALLs as in the acute myelogenous leukemias, which Dr. Vaughan alluded to, that the patients who are surviving relapse free are really all your patients who have first CRs or even second CRs of several years duration? Your best outcome was in patients in third CRs who received a transplant many years after diagnosis.

DR. RAMSAY: I think that what you are asking is good. We have tried to look at this group of patients for some prognostic factors, such as initial WBC count and which remission they were in (second versus third), and have not been able to show that any of these factors are advantageous to these patients, including second versus third remission. Part of that may be just that the long-term survival rate is so low, which makes it difficult to pull out any prognostic factors. We did look at the length of initial remission in these patients and tried to determine whether there was an advantage for patients who had an initial remission greater than 18 months versus those who were in remission less than

18 months. Actually, the disease-free survival was even slightly better but really no different in the patients who had the shorter initial remission. We could not predict, at least even show, any relationship between the patients surviving long-term and the initial remission length.

DR. SPITZER: So the plateau is not patients with long-term CRs?

DR. RAMSAY: No it is not. I mean it is variable.

DR. D. BUCKNER: Dr. Ball, do you have long-term marrow culture data with your antibody PM-81?

DR. BALL: No, we don't have long-term marrow culture data for that antibody because we don't get a decrease of colony-forming unit mix after treating that antibody, so it was not particularly an issue. We have data for the AML 199 antibody, which is somewhat premature, but it looks like there is recovery of colony-forming units in liquid culture after an initial depletion.

DR. BUCKNER: This is in leukemic patients, not in hematologically normal individuals.

DR. BALL: No, this is in hematologically normal individuals. Is that your question?

DR. BUCKNER: Yes, I just wanted to know because we have an experience with a similar antibody—Herb Bernstein's. The interesting thing is that cells from hematologically normal individuals grow well after treatment but, in two thirds of AMLs in remission, it definitely affects the long-term marrow cultures.

DR. C. READING: We have looked at the undifferentiated blast colony assay after magnetic separation and didn't see any loss of those colonies that were thought to be a very primitive cell, even though it hits maybe half of the more mature granulocyte-macrophages.

DR. DICKE: Are there any questions for Dr. Herve? I think the point Dr. Herve was making about the cryopreservation, the damage of cryopreservation of the cells that are already treated in vitro is a very important one. Is there anybody in the audience who has, as we do, difficulties with infusing these cells after thawing, when they are being treated in vitro with 4-hydroperoxycyclophosphamide? We have to wash the cells before infusion because of clumping. I would like to continue with Ton (Anton) Hagenbeek. I think you can conclude from your data that the busulfan plus cyclophosphamide regimen is the best one. Now what dose of cyclophosphamide are you using? Is that the cyclophosphamide of the old Santos regimen or the new one, as advocated by Tutschka?

DR. A. HAGENBEEK: The cyclophosphamide dose that we give in this animal model is 100 mg/kg.

DR. DICKE: You can cure this rat model with Cytoxan, right?

DR. HAGENBEEK: That is right. If you go to very high dosages, at a different

tumor load, especially when the tumor load is not too large, you can cure them. But, on the other hand, we have shown that in regimens that contain cyclophosphamide one regimen is superior to the other.

DR. E. FREI: I found Dr. Hagenbeek's presentation very interesting because, given the heterogeneity of the conditioning regimens, we certainly need experimental models to help us put these things together, and they are certainly complex in terms of dose combinations, etc. Case numbers, as illustrated this morning, are not going to allow for quantitative studies in that area, so anything that relates to models is very important. The one comment I would like to make is that the assumption of following cell kill with alkylating agents, let's say the recovery of the remaining cells, is kinetically normal. And I think there is a risk there because it is very clear that cells resistant, for example, to alkylating agents are kinetically slow growing and it could be that your cell kill is substantially overestimated to the extent that that is a variable.

DR. HAGENBEEK: I agree completely. The way in which we try to solve this pressing problem is by means of computer simulation. And with all the data we have, our computer analyst tried to establish what happens in the area of residual disease. As a matter of fact, we found that if the tumor load is reduced to a very low number of cells, you enter a sort of inert phase with the cells resting. Then they come back into cycle again and start to regrow.

DR. FREI: What is the tumor burden in the rats at the time of treatment?

DR. HAGENBEEK: 5×10^9 cells.

DR. FREI: So that's ascites or subcutaneous nodules?

DR. HAGENBEEK: Just like a human acute myelogenous leukemia, these are cells in the bone marrow. The spleen may be enlarged and the liver might be infiltrated.

DR. DICKE: In conclusion, I think the data of purging look hopeful—maybe the combination of purging methods might be advantageous and will be a lead for continuous investigation. Thank you very much for this session.

IC. Leukemia Purging/Detection of Minimal Residual Disease

Magnetic Affinity Colloid Elimination of Specific Cell Populations From Bone Marrow

Christopher L. Reading, Weiben Yong, Edward D. Ball, and Karel A. Dicke

We have previously described the use of a colloidal magnetic fluid for removing monoclonal antibody-defined cell populations from marrow aspirates used for autologous transplantation (1-4). We describe here the development of an avidin-based magnetic affinity colloid (MAC) capable of eliminating 4-5 logs of specific cell populations reactive with monoclonal antibodies (MAbs) and lectins. We have also phenotyped a number of cell samples from patients with acute leukemia and defined cocktails of MAbs and soybean agglutinin that are highly reactive with fresh blasts. In addition to eliminating clonogenic cells from experimental mixtures with normal bone marrows, we have also removed clonogenic leukemia cells from fresh peripheral blood or marrow samples from patients with acute leukemia who were in relapse.

MATERIALS AND METHODS

Reducing cobaltous chloride with sodium borohydride to form a colloidal suspension has been described before (1,2,4), and the details of MAC production are the subject of another communication (Christopher L. Reading *et al.*, unpublished manuscript). In brief, a solution containing a cobalt-citrate complex is added to a solution containing sodium borohydride to form nuclei of

solid Co_2B. A second addition of the cobalt-citrate complex results in the deposition of additional Co_2B onto these nuclei to form spherical particles 30-60 nm in diameter. Affinity-purified avidin is added, binding essentially irreversibly to the surface of the colloid. The solution is stabilized with human serum albumin and to avoid dissolution of the Co_2B particles passivated with potassium dichromate. The fluid is then concentrated by tangential filtration (Amicon, Lexington, MA) and purified by gel permeation chromatography on Sepharose CL-6B-200 (Sigma, St. Louis, MO). The MAC is adjusted to 20 OD_{400}/ml, filtered through a 0.45-μm Millex-HA filter unit (Millipore, Bedford, MA), and is ready for use. The entire procedure requires about 3 hours.

Bone marrow mononuclear cells are purified by discontinuous Percoll gradient centrifugation (5). The cells (5×10^7/ml) are incubated with cocktails of MAbs at concentrations previously determined to be saturating for 30 minutes at 4° in 10-mM phosphate-buffered saline (PBS) (pH 7.4) containing 2% fetal bovine serum (FBS). The cells are washed twice by centrifugation in PBS (2% FBS) and incubated at 5×10^7/ml with a combination of a biotinylated affinity-purified IgG fraction of goat antimouse immunoglobulins (B-GAM) at 100 μg/ml and biotinylated soybean agglutinin (B-SBA) at 100 μg/ml in PBS (2% FBS) for 30 minutes on ice. The cells are again washed twice and incubated at 5×10^7/ml with MAC (at 20 OD_{400}/ml) for 30 minutes on ice. The MAC procedure is shown in Figure 1. The cells are pelleted, resuspended at 5×10^7 cells/ml in PBS (2% FBS), and separated on a high-magnetic gradient column. The column (1.9 × 40 cm) is made by stacking 100 tinned steel screen disks (3/4-in diameter, .0065-in wire, 60 mesh; Tetko, Elmsford, NY) in a 20-ml syringe (Becton Dickinson, Rutherford, NJ) and fitting it with an external samarium cobalt permanent magnet (2.25 × 0.55 × 0.6 cm, grade 25, magnitude point parallel to a 0.6-cm dimension; Permag, Richardson, TX). The syringe is fitted with a rubber stopper at the top and a three-way stopcock at the bottom and is sterilized with ethylene oxide. The cells pass through the column in PBS (2% FBS) at 1 ml/minute, and the column is washed at 5 ml/minute with 100 ml of PBS (2% FBS). For clinical separations with larger numbers of cells, two such columns are arranged in tandem. The separation apparatus is depicted in Figure 2.

The cell suspensions are analyzed before and after MAC separation by flow cytometry and for clonogenic target cells and normal hematopoietic progenitors. The cells are stained with MAbs, B-GAM and B-SBA, and fluorescein isothiocyanate (FITC) avidin, and analyzed on either an Ortho 2150 or a Coulter Epics C flow cytometer. The control cells are treated similarly, except that the FITC avidin is preincubated with an excess of biotin. T cells are analyzed in a limiting dilution clonal assay we have previously described (6). Hematopoietic progenitors are analyzed by colony formation in methylcellulose for granulocyte-macrophage colony-forming units (CFUs-GM), erythroid burst-forming unit (BFU-E), and mixed colonies arising from granulocyte-erythrocyte-macrophage-megakaryocyte colony-forming units (CFUs-GEMM) and in agar

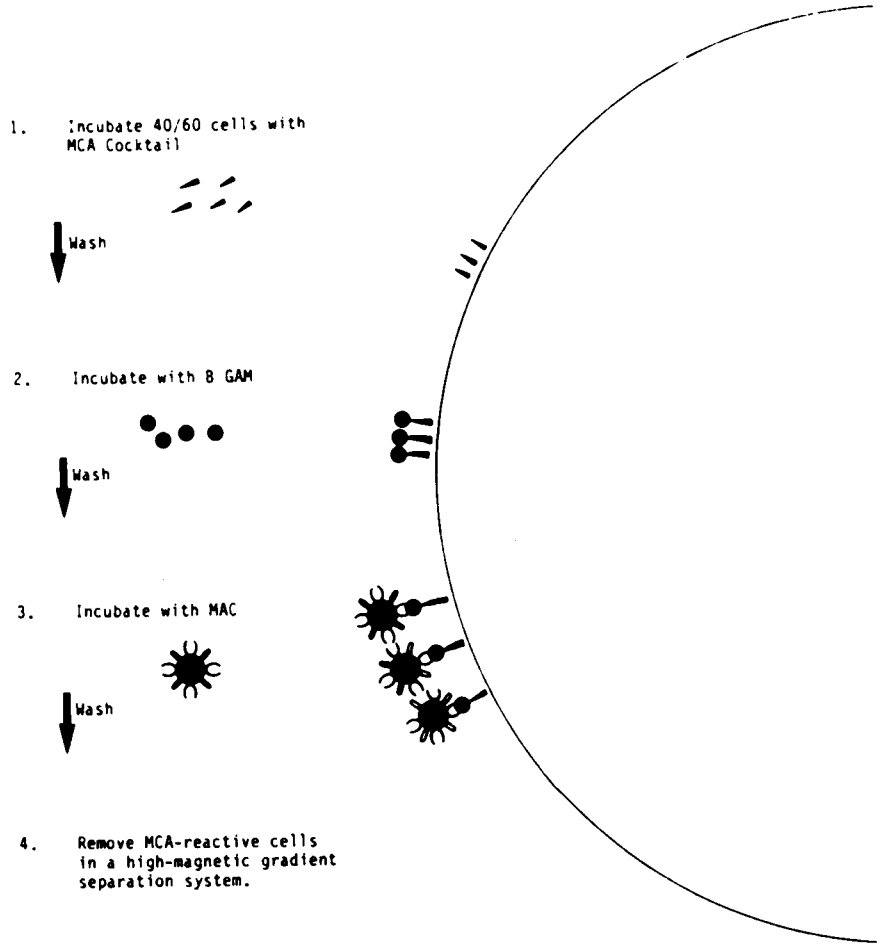

Figure 1. *As this schematic of the magnetic affinity colloid (MAC) procedure shows, bone marrow (BM) cells are incubated with a cocktail of monoclonal antibodies (MCA), washed, and incubated with biotinylated goat antimouse immunoglobulins (B GAM) and biotinylated soybean agglutinin. After washing, the cells are incubated with MAC, which binds to biotin on the cells.*

for CFU-GM assays as previously described (6). Limiting dilution analysis of granulocyte-macrophage precursors (LDA-GM), analysis of undifferentiated blast (UB) colonies, and colony assays of fresh acute leukemia cells are performed as described elsewhere (7-9).

The monoclonal antibodies OKT-3, OKT-4, OKT-8, OKT-11, and L-243 were purified from cultures of mycoplasma-free hybridomas (American Type Culture Collection, Rockville, MD). The CF-1 hybridoma line was a gift from Centocor (Malvern, PA). The CLB-gran mon/2, from cluster of differentiation

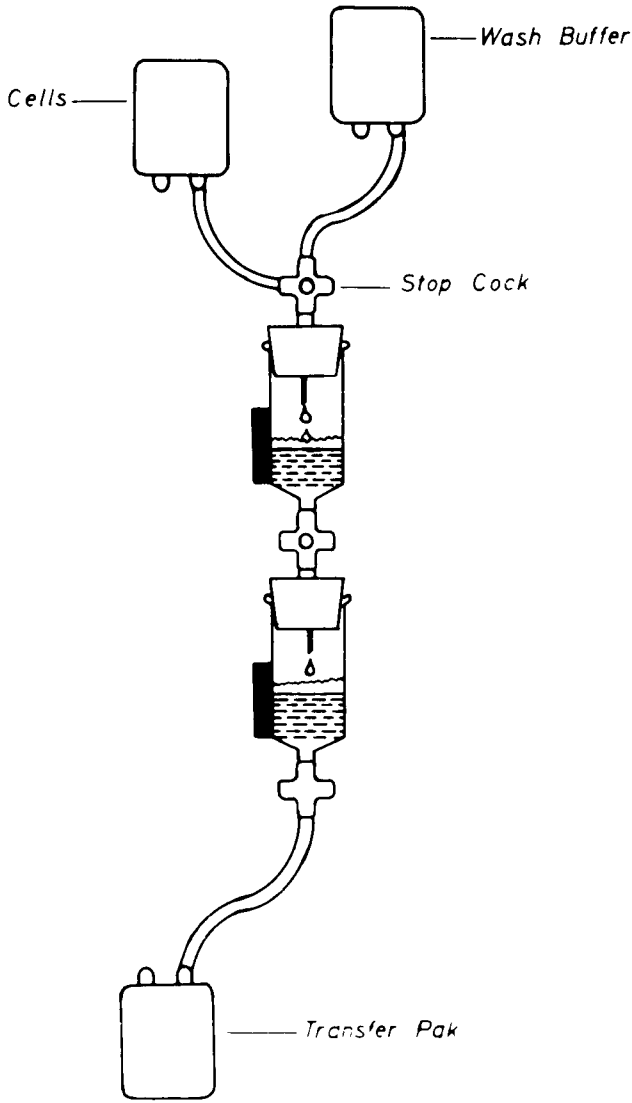

Figure 2. *After excess MAC is washed from cells, they are passed through a high-magnetic gradient separation column.*

(CD 13), and CLB-gran 2 cl.3B9 (CD 15) hybridoma lines were a gift from Dr. P. Tetteroo (CLB, Amsterdam, The Netherlands). Monoclonal antibodies J5, My4, My7, My9, B1, B2, B4, My8, Mo1, and Mo2 were purchased from Coulter (Hialeah, FL). OKB-7 and OKT-9 were purchased from Ortho (Raritan, NJ). BA-1 was a gift from Dr. T. LeBien (University of Minnesota Medical School, Minneapolis, MN), Du-ALL-1 was from Dr. R. Metzgar (Duke University Medical

School, Durham, NC), and PM-81 and AML-2-23 were prepared by one of us. SBA and avidin were purified as previously described (10,11).

Fresh samples of peripheral blood mononuclear cells were obtained by leukapheresis of patients with acute leukemia. The blast cells were isolated on Ficoll-Hypaque and incubated with saturating concentrations of MAbs diluted in PBS (2% FBS) for 30 minutes on ice. After two washes in PBS (2% FBS), the cells were incubated with 100 μg/ml of FITC and IgG fraction of goat antimouse immunoglobulin (FITC-GAM) (Cappel, Malvern, PA) for 30 minutes on ice. After two washes in PBS (2% FBS) the cells were analyzed for fluorescence intensity of cells gated on the blast region on either an Ortho 2150 or a Coulter Epics C. The negative region was set on cells stained with FITC-GAM alone. Cells were also stained with 100 μg/ml FITC-SBA.

RESULTS

The data obtained from phenotyping acute leukemia cells is shown in Tables 1 and 2. Thirty-one cases of acute myelogenous leukemia (AML) and eight cases of acute lymphoblastic leukemia (ALL) were examined with a panel of MAbs. The SBA was reactive with most of the AML (11/14) and ALL (4/5) cases tested, staining a median of 51% of the AML and 70% of the ALL blast cells. CF-1 reacted with all 31 cases of AML and all 7 cases of ALL, with medians of 70% and 88% of the blasts staining, respectively. My7 reacted with 23 of 25 AML samples (median, 65%) and PM-81 bound 20 of 22 AML samples tested (median, 68%). Du-ALL-1 reacted with 8 of 8 ALL cases tested (median, 48%), J5 bound 5 of 7 (median, 84%) and B4 reacted with 6 of 6 cases (median, 86%).

Reagents were combined in attempts to increase the reactivity of blast cells. In Tables 3 and 4 the percentage of reactive blasts increased with combinations of reagents. In one case of M_5 AML, the combination of CF-1, PM-81, and My7 reacted with 99.6% of the blasts. In one non-T ALL sample, the combination of CF-1 and J5 reacted with 99% of the blasts. From this analysis, we chose a cocktail for AML of SBA, CF-1, and MAbs from CD 13 and CD 15, and for ALL we chose a cocktail of SBA, CF-1, and MAbs from CD 9, CD 10, CD 19, and CD 24. We will use these antibodies for marrow purging.

We examined the efficiency of eliminating specific cell populations using clonogenic assays. When normal bone marrow mononuclear cells were treated with a cocktail (OKT-C) of OKT-3, OKT-4, OKT-8, and OKT-11, and MAC, we examined the elimination of clonogenic T cells using a limiting dilution assay (Table 5). We were able to remove an average of 2.6 logs of clonogenic T lymphocytes. With SBA and MAC, we could only remove 1.5 log, but with a combination of OKT-C, SBA, and MAC, we eliminated 3.9 logs of clonogenic T cells. Recoveries of hematopoietic progenitors were generally in excess of 50%.

We also developed a limiting dilution analysis for eliminating K562 cells from peripheral blood or marrow samples (Christopher L. Reading *et al.*, unpublished data). This analysis yields a clonogenic frequency for K562 cells of nearly 1.0. We examined the ability of a combination of MAbs CF-1 and PM-81

Table 1. Frequency and Percentage of Reactivity of Monoclonal Antibodies and Soybean Agglutinin With Acute Myelogenous Leukemia Blast Cells[a]

Diagnosis	SBA	CF-1	Ia	My4	My7	My8	My9	Mo1	PM-81
AML	1/1, 32	9/9, 70	7/8, 63	3/7, 37	7/7, 41	5/7, 55	7/7, 61	3/7, 70	4/6, 39
M_1	—	(54–96)	(29–96)	(23–38)	(29–96)	(24–83)	(29–95)	(37–72)	(35–86)
M_2	4/5, 44	7/7, 82	7/8, 60	0/4	4/4, 68	2/3, 65	4/4, 70	2/4, 42	4/4, 42
	(23–60)	(62–93)	(33–80)	—	(46–84)	(38–92)	(35–74)	(27–58)	(24–71)
M_3	—	1/1, 88	0/1, 0	—	—	—	—	—	—
M_4	2/4, 57	6/6, 57	4/5, 65	3/6, 28	5/6, 65	6/7, 33	5/6, 31	4/6, 38	6/6, 63
	(41–73)	(27–89)	(27–72)	(25–81)	(23–85)	(23–78)	(23–77)	(27–75)	(40–81)
M_5	4/4, 73	8/8, 76	9/9, 81	7/8, 86	7/8, 66	8/8, 79	8/8, 82	8/8, 62	6/6, 87
	(51–87)	(58–94)	(58–94)	(70–94)	(23–84)	(26–96)	(34–93)	(26–90)	(69–94)
Total	11/14, 51	31/31, 70	27/31, 69	13/25, 71	23/25, 65	21/25, 58	24/25, 66	17/25, 58	20/22, 68
	(23–87)	(27–96)	(27–96)	(23–94)	(23–96)	(23–96)	(23–95)	(26–90)	(24–94)

[a]*Values are the frequency of positive results (more than 20% reactive blasts), the median percentage of reactive blasts for the patients with positive results, and the range of reactivity for the patients with positive results (*in parentheses*).*

Abbreviations: SBA, soybean agglutinin; AML, acute myelogenous leukemia.

Table 2. Frequency and Percentage of Reactivity of Monoclonal Antibodies and Soybean Agglutinin With Non-T Acute Lymphocytic Leukemia[a]

Diagnosis	SBA	CF-1	Ia	Du-ALL-1	J5	B1	B2	B4	OKB-7	OKT-3	OKT-4	OKT-8	OKT-9	OKT-11
Non-T ALL	4/5, 70	7/7, 88	8/8, 86	8/8, 48	5/7, 84	0/6	0/6	6/6, 86	2/6, 44	0/8	2/8, 51	0/8	1/7, 52	3/8, 70
	(63–88)	(48–98)	(44–96)	(22–90)	(72–95)	—	—	(45–96)	(21–66)	—	(27–75)	—	(52)	(54–88)

[a]*Values are the frequency of positive reactions (more than 20% reactive blasts), the median percentage of reactive blasts for the patients with positive results, and the range of reactivity for the patients with positive results (*in parentheses*).*

Abbreviations: ALL, acute lymphocytic leukemia; SBA, soybean agglutinin.

Table 3. Percentage Reactivity of Soybean Agglutinin and Monoclonal Antibody Cocktails With Acute Myelogenous Leukemia Cells

Cell Type	Blasts (%)	CF-1	PM-81	My7	SBA	CF-1 PM-81	CF-1 My7	PM-81 My7	CF-1 SBA	CF-1 SBA My7	CF-1 PM-81 My7	CF-1 My7 My7
AML												
M_2	61	89.6	55.6	—	—	71.0	—	—	—	—	—	—
M_2	63.2	62.3	23.8	53.9	23.4	73.4	73.8	—	85.6	87.4	—	—
M_4	84.6	58.1	58.5	54.9	—	79.7	—	—	—	—	91.0	—
M_4	77.6	28.7	80.0	23.1	—	89.4	64.5	85.0	—	—	91.2	—
M_5	90.0	70.1	87.9	—	—	96.3	—	—	—	—	—	—
M_5	80.0	83.0	86.1	31.9	—	92.4	89.6	87.0	—	—	92.7	—
M_5	83.4	93.6	93.4	80.1	—	99.4	—	—	—	—	99.6	99.6
M_5	76.6	57.5	74.4	4.4	57.5	93.0	71.2	—	75.0	75.2	—	—

Table 4. Percentage Reactivity of Soybean Agglutinin and Monoclonal Antibody Cocktails With Non-T Acute Lymphocytic Leukemia Cells

Cell Type	Blasts (%)	CF-1	B4	J5	Du-ALL-1	SBA	CF-1 B4	CF-1 J5	CF-1 SBA
Non-T ALL	91.8	66.3	64.9	1.2	90.0	67.6	89.5	—	—
Non-T ALL	89.6	90.2	82.4	83.9	53.7	12.2	98.9	99.0	—

Table 5. Separations of Bone Marrow Cells With OKT-C, Biotinylated Soybean Agglutinin, or Both

Removal (%)				Recovery (%)						
Exp	% +	FMF	LDA-T	T Cells	CFU-GM	BFU-E	CFU-M	LDA-GM	UB	AGAR-GM
				OKT-C						
314	61.9	97.9	99.0	93.4	—	—	—	—	—	—
320	24.6	94.2	99.0	40.7	37	48	53	—	—	—
321	30.3	99	99.8	32.0	—	—	—	—	—	—
403	37.5	97.7	99.1	63.0	—	—	—	—	—	—
404	38.6	99	99.2	61.6	27	51	27	92	—	—
408	46.1	99	99.2	72.9	27	—	22	—	—	—
514	43.7	99	99.6	67.3	—	—	—	—	—	—
523	41.1	99	99.5	71.8	—	—	—	—	—	—
618	36.1	96.7	—	45.2	—	—	—	—	—	—
709	39.8	99	99.97	36.5	—	—	—	—	—	—
813	13.5	99	99.93	48.9	47	103	105	42	61.5	63.2
814	54.5	97.9	99.96	78.3	25	93	45	76	45.6	—
827	19.4	98.9	99.94	51.9	32	30	42	—	107	58.8
				B-SBA						
501	30.1	99	98	51.0	34	71	—	—	—	—
502	46.2	99	ND	62.0	72	100	—	91	—	—
506	10.7	89.3	96	73.3	95	55	64	—	—	—
508	11.1	97.5	95	63.0	66	81	75	—	—	—
				OKT-C + B-SBA						
710	52.7	99	99.98	21.4	—	—	—	42		
715	84.3	99	99.992	47.8	—	—	—	37		

Abbreviations: Exp, experiment; %+, percentage of positive cells; FMF, percentage of removal determined by flow microfluorimetry; LDA-T, T-cell limiting dilution assay; CFU-GM, granulocyte-macrophage colony-forming unit; BFU-E, erythroid burst-forming unit; CFU-M, mixed colonies; LDA-GM, granulocyte-macrophage limiting dilution assay; OKT-C, cocktail of the monoclonal antibodies OKT-3, OKT-4, OKT-8, OKT-11; B-SBA, biotinylated soybean agglutinin.

and MAC to eliminate K562 cells from mixtures (Fig 3). Starting with a mixture of 1 K562 cell/10 bone marrow mononuclear cells, the clonogenic analysis yielded a frequency of 1 in 14. After MAC separation, the frequency of K562 cells was 1 in 626,000. The analysis was still linear at this level of contamination. With the recovery of 40%, this represents a 5-log removal of K562 cells.

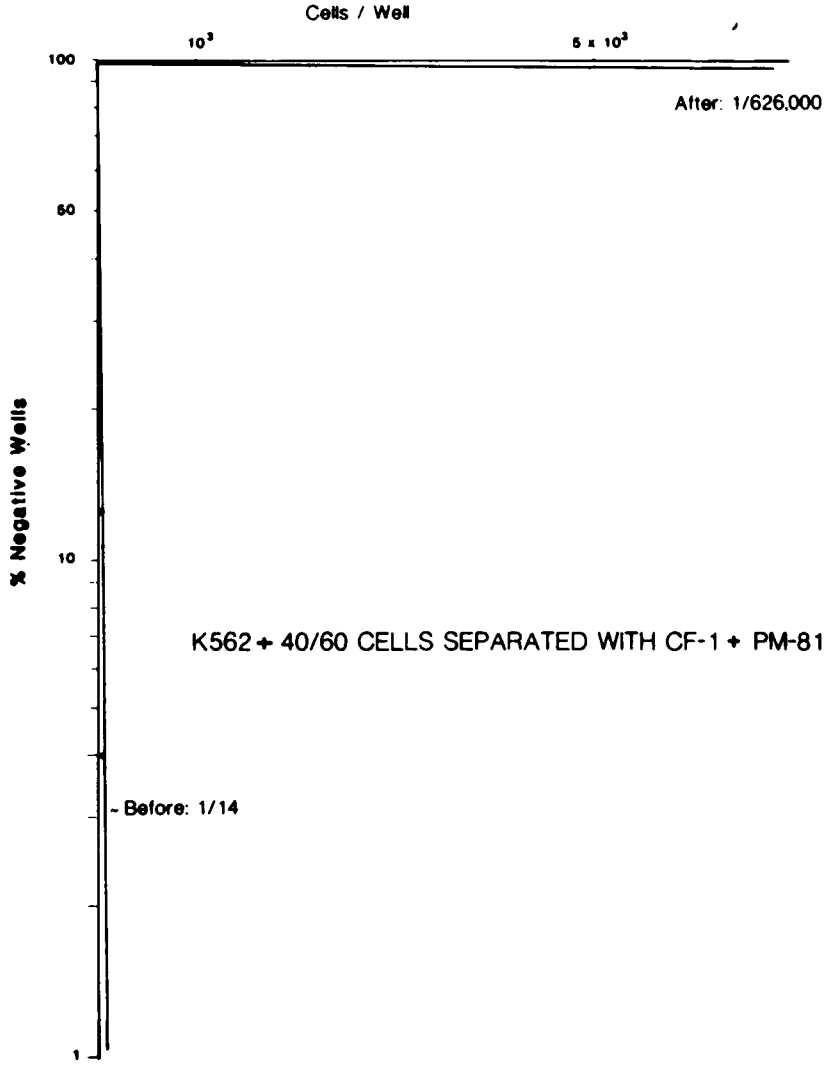

Figure 3. *Limiting dilution clonal analysis of K562 cells mixed with irradiated peripheral blood mononuclear cells before and after MAC separation with CF-1 and PM-81. The frequency of K562 clonogenic cells in the mixture before separation is 1/14 cells. After separation, the frequency is 1/626,000 cells. Considering the 40% cell recovery, this represents a 5-log removal of K562 cells: log [(.40)(1/626,000)]/(1/14) = −5.05.*

DISCUSSION

We have developed a simplified, reproducible separation procedure capable of eliminating 4-5 logs of MAb- and lectin-reactive cells. This method is practical for use in autologous transplantation. We are able to recover about 50% of the hematopoietic progenitors when we use reagents that do not react with these precursors. We have defined cocktails of reagents that spare hematopoietic progenitors and react with essentially all of the acute leukemic blast cells. Using CF-1 and PM-81 with MAC we have removed 99% of the leukemia colonies and clusters from three of three AML samples (data not shown). With these tools in hand we are ready for clinical trials. PM-81 is already in use for bone marrow treatment in vitro using complement-mediated cytotoxicity (12). We believe that a combination of MAC and chemopurging will be the most efficient method for removing residual acute leukemia cells from autologous marrow.

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4-Hydroperoxycyclophosphamide and Vincristine as Ex Vivo Bone Marrow Treatment for Acute Leukemia in Second Remission

Leonard J. Horwitz, Miklos L. Auber, Sangeeta Khorana, Sundar Jagannath, Karel A. Dicke, and Gary Spitzer

A major drawback of autologous bone marrow transplantation (ABMT) in acute leukemia is the likely presence of occult leukemic cells in the remission marrow (1). Physical, immunological, and pharmacological procedures or their combinations have been used in animal models to eliminate this leukemic cell contamination (2,3).

MATERIALS AND METHODS

The cyclophosphamide derivatives, Asta Z 7557 (mafosfamide) and 4-hydroperoxycyclophosphamide (4-HC), are among the drugs commonly used for ex vivo chemotherapy (chemopurging) of harvested marrow (4-7). Clinical studies of ex vivo chemotherapy with 4-HC (40-120 μg/ml) and ABMT suggest that a small percentage of acute myeloid leukemic (AML) patients, most of them in second complete remission (CR2), may achieve long-term disease-free survival. These patients have usually had a previous complete remission of relatively long duration (18 months or more). Ex vivo marrow treatment with 4-HC has been less successful for acute lymphoid

leukemic (ALL) patients than for AML patients (5,8). Ex vivo chemotherapy with 4-HC at higher doses has significant treatment-related mortality of approximately 20-30%, which are related to serious bacterial infection before recovery (8).

Because of the lack of effectiveness with ex vivo 4-HC in ALL, even at doses imparting severe myelotoxicity, we designed experiments to identify drugs or drug combinations that might have greater leukemic cell kill than 4-HC alone in acute leukemia while still having acceptable in vitro myelotoxicity.

RESULTS AND DISCUSSION

The drugs selected for investigation had demonstrated activity in acute leukemia with reversible or no myelotoxicity: vincristine (VCR), doxorubicin (also known as Adriamycin) (ADR), hydrocortisone (HY), etoposide (VP-16-213), L-asparaginase, bleomycin, spirogermanium, taxol, and dexamethasone. Preliminary results of these experiments have been reported by our group (9).

To compare the in vitro myelotoxicity of these drugs more equitably, we introduced the concept of equitoxic doses of drugs, expressed as percent inhibitory concentrations (IC) on colony-forming units granulocyte-macrophage (CFUs-GM). Spitzer and others (10) have shown that the number of CFUs-GM transfused during ABMT correlates significantly with the neutrophil recovery time; the minimum number of infused CFUs-GM necessary for adequate neutrophil recovery was found to be above 0.5×10^4/kg body weight. With the minimum CFU-GM dose, the median recovery to 1000 granulocytes/mm^3 is 22 days; below this CFU-GM dose, the median neutrophil recovery is 58 days. The rapidity of neutrophil recovery is, in turn, a major determinant of the morbidity and mortality following ABMT, especially after ex vivo treatment. Since, in our clinical experience, the number of CFUs-GM recovered from the average marrow harvest is approximately 4×10^4/kg body weight, the minimum number of CFUs-GM necessary for adequate neutrophil recovery (0.5×10^4/kg) would be approximately 12.5% of the average total number of CFUs-GM from a marrow harvest. Therefore, a reasonable upper limit for ex vivo chemotherapy, which should still result in acceptable neutrophil recovery, would be around the IC_{90} (i.e., 10% recovery) dose.

To determine the IC_{90} doses of the selected drugs, light density cells were first separated from nonleukemic bone marrow in gradients of Ficoll-Hypaque (specific gravity, 1.077 g/mm^3) or Percoll (40-60% interphase; specific gravity, 1.077 g/mm^3). Cells were then incubated with the chosen drugs at 5×10^6 cells/ml at 37°C for 1 hour. This incubation time, rather than the more common 30 minutes, was chosen to reduce any error caused by slight time variations, to minimize any variations in temperature equilibration, and to allow cell cycle-active drugs to express their effect better. Following

incubation, the cells were washed three times, resuspended, and cultured in a soft bilayer agar system at 1×10^5 cells/plate. The number of CFUs-GM was counted on each plate after 10 days of incubation at 37°C in a humidified 5% CO_2, 12% O_2 atmosphere. The dose-response curve was plotted and the IC_{90} dose was determined from its intercept of the 10% CFU-GM survival line.

After determining the IC_{90} dose for each drug, we investigated the kill of leukemic colony-forming cells. Limiting dilution analysis was used to establish the leukemic cell kill of each drug on four ALL and two AML cell lines. This assay has been described elsewhere (11). With an initial cell concentration of 10^5 cells/well, serial dilution factor of 5-6 wells used at each dilution and 10 serial dilutions per experiment, the maximum detectable log kill under our experimental conditions was 5.6 logs. In this system, a log kill difference of 0.9 is statistically significant ($P < .05$).

L-Asparaginase, bleomycin, spirogermanium, taxol, and dexamethasone were eliminated from further investigations either because of lack of leukemic cell kill or because of solubility problems. After comparing the leukemic cell kills of the other drugs tested at their IC_{90} dose, we concluded that none of these drugs was any more effective than 4-HC alone.

We thereupon chose to investigate drug combinations. Vincristine was selected to be part of each combination because of its known clinical activity in ALL and its ability to kill up to 2.5 logs on ALL cell lines in vitro while not exceeding even its IC_{70} dose. The following combinations were tested: VCR plus HY, VCR plus ADR, VCR plus VP-16-213, and VCR plus 4-HC. Table 1 shows the changes in calculated IC_{90} doses for these drugs as increasing doses of VCR are added. The interaction of VCR in the drug combinations was evaluated in greater detail by the isobologram analysis of Steel and Peckham (12). The toxicity of combinations of VCR plus HY, VCR plus ADR, and VCR plus VP-16-213 were either additive or superadditive on the normal bone marrow; even a small dose of VCR (0.1 μg/ml) could necessitate the other drug's being given at below its IC_{90} dose in order not to exceed the IC_{90}

Table 1. Percent of Granulocyte-Macrophage Colony-Forming Unit Survival Following Ex Vivo Chemotherapy on Normal Bone Marrow[a]

VCR (μg/ml) Added	4-HC	VP-16-213	ADR	HY
None	4.9	24.0	1.85	18.0
0.1	—	20.2	1.5	8.5
1	6.5	16.5	1.15	6.8
5	4.4	12.8	0.9	8.5

[a]*Based on five experiments, IC_{90} dose of drugs alone reflect only the patient population tested with combination drugs, and may differ somewhat from the overall population average.*

Abbreviations: VCR, vincristine; 4-HC, 4-hydroperoxycyclophosphamide; VP-16-213, etoposide; ADR, Adriamycin; HY, hydrocortisone.

isoeffect. On the other hand, the combination of VCR plus 4-HC was subadditive and even protective on normal marrow: doses of VCR below 1.0 μg/ml (and in some instances, 5 μg/ml) allowed 4-HC to be given in the combination at greater than its IC_{90} dose before achieving its IC_{90} isoeffect. The results of ex vivo chemotherapy on normal bone marrow of seven patients are reported in Table 2.

In contrast to bone marrow, the toxicity of VCR in combination with 4-HC was additive or superadditive on both ALL and AML cell lines. The isoeffect IC_{90} doses of VCR (1 or 5 μg/ml) plus 4-HC (5 μg/ml) was significantly superior to 4-HC alone at its IC_{90} dose of 5 μg/ml ($P < .05$) on ALL cell lines REH and KM3. The superiority was evident but did not become significant until VCR (5 μg/ml) was added on LAZ221, NALM-6, or AML cell line HL60, and no drug or combination was able to cause a 1-log reduction of K562 at its IC_{90} (paper submitted for publication).

Following the identification of the drug combination, VCR plus 4-HC, as protective of normal bone marrow yet significantly superior in leukemic cell kill to any single drug alone, clinical studies were initiated using VCR plus 4-HC for ex vivo marrow treatment in CR2 acute leukemia patients. A reserve marrow of at least 1×10^8 cells/kg body weight was stored. The patient subsequently completed a marrow harvest of $3\text{-}4 \times 10^8$ cells/kg body weight. Light density cells were separated on a Percoll gradient (40-60% interphase; specific gravity, 1.077 g/ml) and incubated in a slowly shaking water bath at 5×10^6 cells/ml at 37°C for 1 hour with VCR (1 μg/ml) plus 4-HC (5 μg/ml). Following incubation, the cells were washed with RPMI 1640 solution containing DNase (to avoid clumping), then filtered and frozen in dimethyl sulfoxide and autologous serum in 4.5-ml aliquots.

Table 2. Percent of Granulocyte-Macrophage Colony-Forming Unit Survival After Chemopurging Normal Bone Marrow[a]

4-HC (5 μg/ml) (x ± SEM)	VCR (1 μg/ml) + 4-HC (5μg/ml) (x ± SEM)	VCR (5 μg/ml) + 4-HC (5 μg/ml) (x ± SEM)
4.1 ± 3.4	4.1 ± 2.4	—
6.8 ± 3.2	37.8 ± 1.4	—
4.0 ± 1.5	9.9 ± 5.3	—
17.6 ± 2.3	31.4 ± 5.2	5.2 ± 1.3
1.9 ± 1.1	4.4 ± 4.4	1.3 ± 0.6
12.6 ± 9.8	20.3 ± 1.7	16.3 ± 2.6
18.6 ± 1.3	19.4 ± 1.5	7.4 ± 1.0
9.4 ± 2.6	18.2 ± 4.9	7.6 ± 3.2

[a] *Based on seven experiments.*

Abbreviations: 4-HC, 4-hydroperoxycyclophosphamide; x, mean; SEM, standard error of the mean; VCR, vincristine.

To date, four patients (one ALL and one mixed lineage in CR2, and two AML—one in first CR [CR1] and one in CR2) have had ex vivo marrow treatment. The final CFU-GM recovery was between 0.46 and 1.87 x 10^4/kg body weight in these patients (see Table 3). The CFU-GM recovery after ex vivo chemotherapy ranged from 5% to 25%, corresponding to an IC_{74-95}. Table 4 shows the sequential CFU-GM losses in our first clinical case—recovery was as anticipated from previous in vitro experiments. The three CR2 patients received CBV (cyclophosphamide, BCNU [carmustine], VP-16-213) preparation chemotherapy as described previously (13), followed by their purged marrow. Hematologic recovery was a neutrophil count of 100/mm^3 by posttransplantation days 13, 16, and 19; a neutrophil count of 500/mm^3 by days 15, 23, and 36; platelet count of 50,000/mm^3 was achieved by days 26, 31, and 36. The acceptable hematopoietic recovery in the transplanted patients suggests that the multipotent hematopoietic stem cell has not been seriously damaged, even after freezing.

Figures 1 and 2 show our overall ex vivo strategy for acute leukemic patients in CR2. To overcome chemotherapy-resistant cells, we plan to add magnetic separation, using monoclonal antibody (MAb) pools directed at specific leukemia types. The antibodies are bound to a cobalt magnetic affinity colloid (see Reading *et al.* "Magnetic Affinity Colloid Elimination of Specific Cell Populations From Bone Marrow," in this volume). Figure 1 shows the anticipated cell and CFU-GM losses when MAbs are combined with chemopurging. Figure 2 outlines the planned stepwise development of a safe and effective combined ex vivo regimen. Once we are confident that there is still no compromise of hematopoietic recovery on subsequent patients treated with ex vivo chemotherapy alone, the addition of immunomagnetic separation will be investigated.

Minimal progenitor cell losses are anticipated with MAbs directed against ALL cells. However, some antibodies reactive with myeloid leukemia cells would be expected to reduce CFU-GM numbers significantly. Both pools of MAbs would result in mechanical losses of up to 50% CFU-GM. Therefore, we

Table 3. Clinical Ex Vivo Purging[a]

	CFU-GM/kg x 10				
Patient	Aspirate	40–60% Percoll Interphase	Postpurging	% IC	% Total Recovery
1	28.96	9.47	1.87	80.3	6.5
2	37.69	12.44	0.56	95.5	1.5
3	5.05	2.33	0.62	73.6	12.2
4a	9.26	4.38	0.46	87.5	5.0
4b	1.21	1.03	0.08	92.2	6.6

[a]*Using vincristine (1 μg/ml) + 4-hydroperoxycyclophosphamide (5 μg/ml).*

Abbreviations: CFU-GM, granulocyte-macrophage colony-forming unit; IC, inhibitory concentration.

Table 4. Ex Vivo Purge Using VCR (1 μg/ml) Plus 4-HC (5 μg/ml) Combination

Patient (DB), Female, 50 kg		
Total number of cells collected		146.22×10^8 cells 2.92×10^8/kg
Buffy coat		127.02×10^8 cells 2.54×10^8/kg
		1448×10^4 CFU-GM 28.96×10^4/kg
Percoll 40/60 volume (57.8 ml) 29.65×10^6 cells/ml		17.13×10^8 cells 0.34×10^8/kg
		474×10^4 CFU-GM 9.47×10^4/kg
Purge volume (343 ml) 5×10^6 cells/ml		1273×10^8 cells 0.25×10^8/kg
Total CFU-GM		93.6×10^4 CFU-GM 1.87×10^4/kg
	=	IC 80.00% of mononuclear cell fraction, 6.5% total recovery of CFU-GM

Abbreviations: VCR, vincristine; 4-HC, 4-hydroperoxcyclophosphamide; CFU-GM, colony-forming units granulocyte-macrophage.

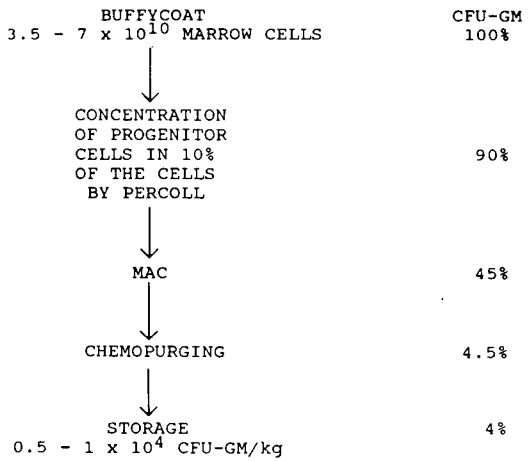

Figure 1. *Number of cells needed for ex vivo treatment of marrow from a patient (70-kg body weight) with 50 colony-forming units granulocyte-macrophage (CFU-GM)/10^5 unfractionated cells.*

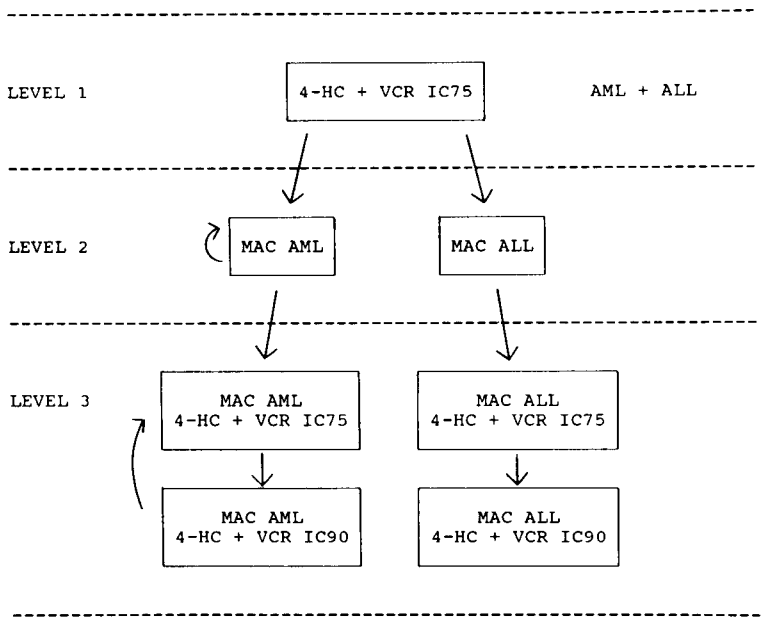

Figure 2. *Phase I studies of hematopoietic recovery (sequential) (4-HC, 4-hydroperoxycyclophosphamide; VCR, vincristine; IC, inhibitory concentration; AML, acute myeloid leukemia; MAC, magnetic affinity colloid).*

plan to introduce chemopurging with immunomagnetic separation initially at the IC_{75} dose level. If there are no significant toxicity or engraftment problems, the ex vivo chemotherapy dose will then be raised to IC_{90} levels. The arrows in Figure 2 show how modifications will be made at each step until a pool of MAbs with acceptable toxicity to normal precursor cells in combination with 4-HC and VCR is selected. In this way, an effective ex vivo marrow purging regimen for leukemia may be developed.

ACKNOWLEDGMENT

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Decontaminating Bone Marrow With Merocyanine 540, Mafosfamide, or Both

Maria Teresa Marchetti-Rossi, Filippo Centis, Nadia Talevi, Annunziata Manna, Giovanni Sparaventi, and Adolfo Porcellini

The real efficacy of purging procedures in autologous bone marrow transplantation (ABMT) has yet to be established. Although in acute nonlymphocytic leukemia (ANLL) the advantage of marrow purging is doubtful when autografting in the first complete remission (CR), there are some indications that cleansing the marrow is associated with improved survival in patients autografted in second CR (1). Several methods for eliminating clonogenic cells from autologous marrow have been attempted (2-6).

Merocyanine 540 (MC-540), a promising drug recently proposed as a bone marrow decontaminating agent, is a fluorescent dye that was originally developed as a sensitizing additive for photographic emulsions. It binds preferentially to fluidlike or cholesterol-free domains in the outer leaflet of the lipid bilayer of intact cell membranes (7). High-affinity binding sites for MC-540 are found on electrically excitable cells, leukemic cells, and at least some classes of immature blood cells (8,9). Excitation of membrane-bound dye with light of a suitable wavelength causes an increase in dye uptake, impairment of membrane functions, and eventually cell death (9).

Sieber and coworkers (10-12) have extensively studied the sensitivity of

hematopoietic precursors and pluripotent stem cells to MC-540-mediated photolysis and compared it to the much greater sensitivity of the lymphocytic leukemia cell line L1210, showing that this dye could be introduced as a cleansing agent in ABMT. In a study that used L1210 cells either in exponential growth phase or arrested in G_1 by thymidine-rich culture medium, it was demonstrated that MC-540 activity is cell-cycle independent (13).

The major problem in the marrow cleansing field, no matter what decontaminating agent is used, still remains determining its efficacy in terms of numbers of clonogenic cells eliminated from bone marrow. To evaluate the decontaminating capability of drugs effective in vitro, we developed a clonogenic assay that mixes established leukemic cell lines with normal bone marrow cells (14).

MATERIALS AND METHODS

Details of preparation of bone marrow cell suspensions, leukemic cell lines, purging agents (MC-540 and mafosfamide), decontamination procedures, and limiting dilution assay (LDA) have been previously described (14-16). In brief, to simulate remission bone marrow from leukemia patients, we mixed 2×10^5 (MC-540 studies) or 10^6 (mafosfamide studies) leukemia cells of the CCRF-SB or K562 lines (kindly supplied by J. Hansen, Fred Hutchinson Cancer Research Center, Seattle, WA) with a 19-fold excess (5%) of human bone marrow mononuclear cells (HBMMCs) and incubated them with MC-540 (15 μg/ml under fluorescent light exposure for 90 minutes) or mafosfamide (100 μg/ml/2×10^7 cells for 30 minutes). In another group of experiments, a combination of mafosfamide and MC-540 was investigated by contaminating a HBMMC suspension with 15% (or 3×10^6) clonogenic cells. After treatment, aliquots of the purged mixtures were dispensed in microculture wells to which graded numbers of the same leukemia lines were added using the LDA method.

These studies were undertaken to help define the effects of combination purging protocols on pluripotent stem cells. Day 11 spleen colony-forming units (CFUs-S) were assayed as previously described (16). In brief, after the incubation period without drugs, with either one of the drugs, or with both the drugs, cells were adjusted to 5×10^5 cells/ml, and 10^5 cells were injected into lethally irradiated mice. Each marrow suspension was assayed in a minimum of ten mice that had received 8.5 Gy from an x-ray apparatus as described previously (17). Spleen colonies were evaluated on day 11 as described by Magli *et al.* (18).

RESULTS

The strategy of LDA analysis in evaluating the number of leukemic cells surviving in a HBMMC mixture after treatment with mafosfamide or MC-540 has

Table 1. Cloning Efficiency of Two Cell Lines After Mafosfamide and Merocyanine 540 Treatment

Leukemia Cells (%)	Treatment	Residual Blasts/Well	CE (%)
CCRF-SB			
5	AZ	0	22
5	MC-540	0	13.4
15	AZ	0.6	9.8
15	AZ + MC-540	0.25	7.3
K562			
5	AZ	0.08	44
5	MC-540	0.11	40.3
15	AZ	1.75	1
15	AZ + MC-540	1.83	3.6

Note: Results were obtained from limiting dilution analysis of bone marrow mononuclear cells contaminated with 5% or 15% of CCRF-SB lymphoblasts or myelogenous K562 cells and subsequently treated with mafosfamide, merocyanine 540, or both. The wells containing 10E4 purged mixture cells and to which clonogenic cells of the same line were added using the limiting dilution technique were scored as positive (responders) or negative (nonresponders) according to whether the tritium-labeled thymidine uptake was greater or less than the threshold of the mean of the nonresponding microculture with no added clonogenic cells plus three times the standard deviation. Results were obtained by plotting the proportion of nonresponding wells against the number of clonogenic cells added to each well (see Fig 1).

Abbreviations: CE, cloning efficiency; AZ, mafosfamide; MC-540, merocyanine 540.

been described elsewhere (14,15). Table 1 and Figure 1 present the data obtained from LDAs of cleansed HBMMC and leukemic cell mixtures in which the equations of the line of best fit were derived using the maximum likelihood method. As shown in Table 1, the cloning efficiencies (CEs) of CCRF-SB and K562 cells, calculated as the slope of the regression line when the ordinate is the natural log of the percentage of nonresponders, were 22% and 44%, respectively, in the mafosfamide experiments, and 13.4% and 40.3%, respectively, in the MC-540 experiments. When the clonogenic cell contamination was increased by a factor of 3, the CE decreased in the mafosfamide studies and was not improved by incubating the cell mixtures with both agents.

The estimate of the number of leukemic cells still present in the cell mixtures after cleansing is represented by the x intercept of the maximum likelihood regression line. In the experiments shown in Table 1, complete decontamination of CCRF-SB cells in each well containing a mafosfamide- or MC-540-purged mixture of 10^4 cells was obtained, but leukemic cells survived after treatment. When the clonogenic cell frequency in the cell mixtures was increased to 15%, CCRF-SB and K562 cells survived the treatment.

Incubation of murine marrow cells with 100 μg/ml of mafosfamide caused a decrease in day 11 CFU-S colonies of about 50% of controls, and after

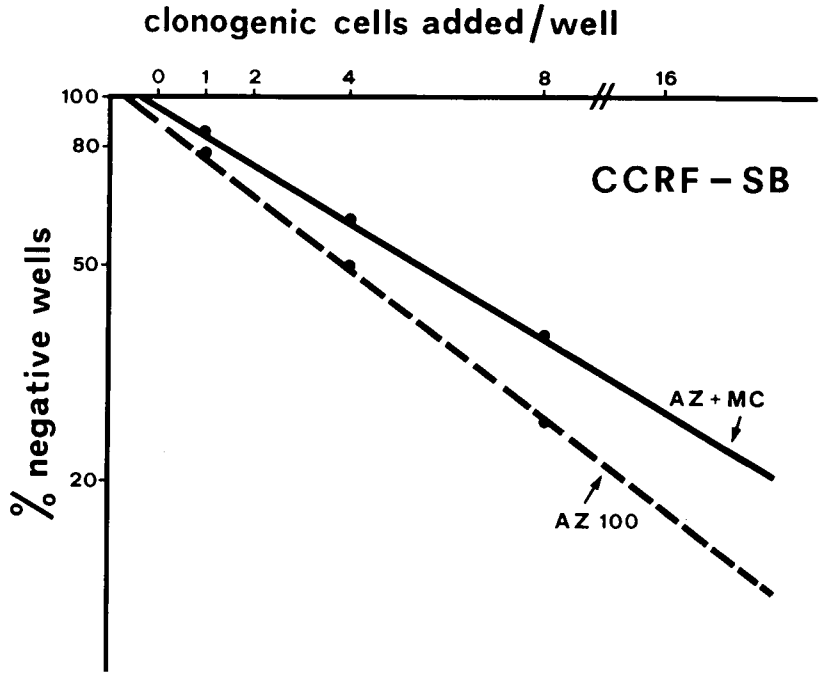

Figure 1. *Results obtained from limiting dilution analysis of bone marrow mononuclear cells contaminated with 15% of CCRF-SB lymphoblasts and treated with mafosfamide (AZ) alone or mafosfamide and merocyanine 540 (MC). The proportion of nonresponding wells is plotted against the number of clonogenic cells added to each well containing 10^4 purged mixture cells (see note to Table 1). The lines of best fit, as determined by the maximum likelihood regression analysis, x intercept values, and confidence limits (not shown) on the population regression line were originated by a MINUIT computer program in a VAX-750 computer.*

photosensitization with MC-540 75% of these cells were still present (Fig 2). Exposure of marrow cell suspensions to both agents, but in reverse sequence, again caused a 50% CFU-S inhibition.

DISCUSSION

It is assumed that the clonogenic cells are distributed randomly among the culture wells, according to the Poisson probability distribution; hence, the 0 term of the Poisson equation will indicate that the proportion of negative wells (i.e., ones in which no residual clonogenic cells remain after purging) is a negative logarithmic function of the number of leukemic cells added to each well. A regression line of best fit was established by the method of maximum likelihood to enumerate residual clonogenic cells remaining in the suspension after MC-540 photosensitization; these can be calculated as the x intercept of the maximum likelihood regression line.

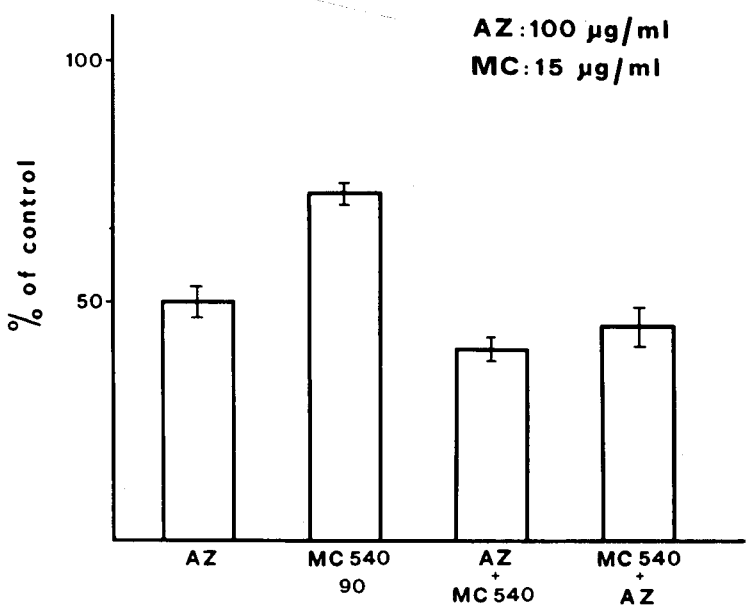

Figure 2. *Effect of mafosfamide (AZ), merocyanine 540 (MC; MC 540), or both on murine spleen colony-forming units on day 11. Values are means from three to six experiments plus or minus the standard error. Colony-forming units were derived from spleen colony assays in 22–31 surviving recipient mice. Results are expressed as a percentage of values from the control suspension.*

In our model, when the clonogenic cell frequency was 5% of the total cell number, both mafosfamide and MC-540 proved able to effectively eliminate all acute leukemia CCRF-SB lymphoblasts present in the cell mixtures. In the mixtures containing K562 cells, both agents yielded a result of 1 residual blast per 10^4 cells of cleansed mixture (i.e., 99.97% decontamination).

To define the decontaminating limits of these two agents, we tested both agents alone and in combination with cell mixtures in which the clonogenic cell frequency had been increased to 15%. In the case of CCRF-SB cells, mafosfamide was able to yield a satisfactory result, but with the K562 cells the results were dismal.

Combined treatment with mafosfamide followed by the photosensitizer MC-540 proved no longer toxic on pluripotent day 11 CFU-S (Fig 2). With K562 cells, the combined treatment was equally ineffective, but with the CCRF-SB line results improved when MC-540 was added to mafosfamide, residual blasts dropping from 0.6/well to 0.25/well. It should, however, be noted that the CE proved very low (Table 1; Fig 1), which may reflect a high number of residual leukemic cells. In this case, the increased proliferation rate of the residual leukemic cells, as measured by tritium-labeled thymidine uptake, can give rise to false-negative results and hence an unexpectedly high proportion of negative wells. In our microtray assay system, though cultures are refed periodically,

medium supply may become a limiting factor. In other words, our data, though apparently acceptable with the lymphoblasts, show that there might be a critical limit of leukemic cell frequency beyond which any cleansing procedure whatsoever is completely ineffective.

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CALLA-Negative Clonogenic Cultures in CALLA-Positive B-Cell Precursor Acute Lymphoblastic Leukemia

Fatih M. Uckun, John H. Kersey, Daniel A. Vallera, Norma K. C. Ramsay, Kevin G. Waddick, and Kazimiera J. Gajl-Peczalska

A variety of immunologic strategies is being evaluated for effective ex vivo purging of residual leukemic cells from autografts. The alternatives include treatment of marrow with monoclonal antibodies (MAbs) plus complement, with immunotoxins, and with MAbs linked to magnetic beads (1). For a MAb to be of therapeutic value in autologous bone marrow transplantation (ABMT) for acute lymphoblastic leukemia (ALL), it must be reactive with the majority of clonogenic ALL blasts in autografts. Choosing a MAb for ex vivo marrow purging has been exclusively based on the immunologic surface marker profiles of the bulk of marrow blasts at diagnosis or relapse. Given the high degree of heterogeneity in ALL, the immunophenotype of marrow blasts may not reliably predict the immunophenotype of clonogenic ALL cells. Currently, very little is known about the immunologic surface marker profiles of clonogenic blasts in ALL. The paucity of knowledge largely reflects the historic difficulties in cloning ALL blasts.

To learn more about these cells, we have developed colony assays that enable us to culture leukemic cells of B lineage (2,3) and T lineage from freshly

obtained bone marrow samples from ALL patients (4). These assays are devised to elucidate the immunologic and biologic features of clonogenic cells in ALL. Results of such studies will be extremely important in designing effective in vivo and ex vivo immunotherapy regimens for ALL patients. Here we report the results of a preclinical study examining common ALL antigen (CALLA) expression and immunophenotypic heterogeneity at the level of clonogenic B-lineage ALL blasts in B-cell precursor ALL.

MATERIALS AND METHODS

Freshly obtained bone marrow blasts from patients with B-cell precursor ALL were cultured in α-minimum essential medium supplemented with 0.9% methylcellulose, 15% fetal calf serum, 15% fresh human plasma, and 10% phytohemagglutinin-leukocyte-conditioned medium for 7 days at 37°C in a humidified atmosphere of 5% CO_2 in air as previously described (2). On day 7, blast colonies containing greater than 20 cells were counted on a grid using an inverted phase microscope. Subsequently, all colonies were pooled for immunologic analysis of colony blasts as described below.

Bone marrow mononuclear cells were immunophenotyped by indirect immunofluorescence and flow cytometry as previously described (2) using a broad panel of MAbs, including BA-3 (anti-CD 10), B43, and BA-1 (anti-CD 24). Samples were scored as positive when 25% or more of cells bound the antibody used. Results of all cases studied were negative for surface immunoglobulin and the T-lineage markers CD 2,5,7 (not shown) but reacted with at least two of the three MAbs listed above. The surface marker profiles of colony blasts were analyzed on acetone-fixed cytospin slides by indirect and direct immunofluorescence as previously described (2-4). The percentage of cells expressing each marker was determined using a Zeiss fluorescent microscope. Quantitative data were obtained from examination of 100-200 cells for each case.

RESULTS AND DISCUSSION

Fresh marrow blasts from 50 patients with ALL of B lineage were evaluated for blast colony formation: cultures were positive in 33 patients (3). The immunologic marker profiles of colony blasts were consistent with those for B-cell precursor ALL, but they frequently differed from the immunophenotype of uncultured fresh bone marrow blasts. We summarize in Table 1 the differences in expression of three distinct B-lineage surface determinants—namely, CD 10/CALLA, B43, and CD 24—on colony blasts and fresh marrow blasts from successfully cultured cases.

Notably, two major immunologic groups could be identified based on the expression of CALLA on colony blasts. In the first group (22 patients, 67%), cultures of pooled leukemic B-cell precursors were positive on day 7 for CALLA.

Table 1. Differences in Expression of B-lineage Surface Determinants on Fresh Marrow Blasts and Colony Blasts From Patients With B-cell Precursor Acute Lymphoblastic Leukemia

	Fresh Marrow Samples		Pooled Blast Colonies on Day 7	
Surface Determinant	Positive Cases (%)	Mean of Positive Blasts (%)[a]	Positive Cases (%)	Mean of Positive Blasts (%)[a]
CALLA/ CD 10, BA-3	100	83	67	40
B43	100	86	94	69
CD 24, BA-1	100	84	94	66

[a] *All 33 cases were included in calculating the mean percentage of positive blasts.*

Table 2. Immunophenotypic Comparison of Cultured B-cell Precursors From Primary and Secondary Blast Colonies

Surface Determinant	Fresh Marrow Blasts	Primary Colony Blasts	Secondary Colony Blasts
Patient 2			
CD 10/CALLA	+++	+++	+++
B43	+++	++	+++
CD 24/BA-1	+	++	+++
Patient 4			
CD 10/CALLA	+++	+++	+++
B43	+++	+++	+++
CD 24/BA-1	+++	+++	+++
Patient 6			
CD 10/CALLA	++	–	–
B43	+++	+++	+++
CD 24/BA-1	+++	+	+
Patient 7			
CD 10/CALLA	+++	–	–
B43	ND	++	++
CD 24/BA-1	+++	+	+

Note: –, 0–24% of cells positive; +, 25–49% of cells positive; ++, 50–74% of cells positive; +++, 75–100% of cells positive; ND, not determined.

In the second group (11 patients, 33%), colony blasts did not express CALLA. In both groups, cultures of colony blasts were always positive for at least two distinct B-lineage markers but did not react with a T-lineage MAb. In replating experiments, we found that the immunophenotype of secondary colony blasts was identical to that of primary colony blasts.

We compare in Table 2 B-lineage surface marker expression of blasts from primary and secondary colonies with expression of fresh marrow blasts in four representative cases (nine were analyzed). Most important, colony blasts positive for CALLA yielded only secondary leukemic B-cell precursor colonies

positive for CALLA, and colony blasts negative for CALLA yielded only secondary colonies negative for CALLA. The secondary plating efficiencies of CALLA-positive and CALLA-negative colony blasts were comparable; therefore, the described culture conditions did not provide a selective growth advantage for CALLA-negative over CALLA-positive leukemic B-cell precursors, and CALLA expression was not essential for the proliferative activity of leukemic B-lineage lymphoid progenitor cells in this colony assay system. Taken together, these findings suggest the existence of at least two immunologically distinct (i.e., CALLA-positive and CALLA-negative) clonogenic cell populations in CALLA-positive B-cell precursor ALL.

In more recent experiments, fresh marrow blasts from patients with B-cell precursor ALL were stained with two colors for B43 and CALLA antigens, and fluorescence-activated cell sorter sorted select blast populations were cultured in the B-cell precursor colony assay system that was positive for B43 and CALLA as well as positive for B43 and negative for CALLA. Blast colony formation was observed in both CALLA-positive and CALLA-negative cultures, providing the initial direct evidence for the existence of CALLA-negative clonogenic leukemic cells in CALLA-positive B-cell precursor ALL (Fatih M. Uckun *et al.*, unpublished observations). These experimental findings are also supported by the phenotypic shifts relative to CALLA expression (i.e., loss or acquisition of CALLA at relapse) observed by a number of investigators during the clinical course of some patients with B-cell precursor ALL (5,6).

In order to evaluate further the immunophenotypic heterogeneity of cultured ALL blasts, we used a broad panel of MAbs reactive with B-lineage markers. In Table 3 we show our findings from seven representative cases. Cultures were positive for TdT, HLA-DR, B43, BA-1; were positive and negative for CALLA; but lacked surface immunoglobulin, consistent with B-cell precursor ALL. Notably, colony blasts from each case had a slightly different immunophenotype. This marked intrapatient and interpatient variation in the expression

Table 3. Immunophenotype of B-cell Precursor Acute Lymphoblastic Leukemia Colony Blasts

Patient	TdT	HLA-DR	B43	B4 CD 19	Leu 14 CD 22	BA-1 CD 24	BA-3 CALLA/ CD 10	Clg	slg
1	+++	+++	+++	++	–	+++	+	–	–
2	+++	+++	++	++	–	++	+++	–	–
3	+++	+++	++	+++	++	+++	–	++	–
4	+++	+++	+++	+++	+++	+++	+++	–	–
5	+++	+++	++	+++	–	+++	++	–	–
6	ND	ND	+++	ND	ND	+	–	–	–
7	ND	ND	++	ND	ND	+	–	–	–

Note: –, 0-24% of cells positive; +, 25-49% of cells positive; ++, 50-74% of cells positive; +++, 75-100% of cells positive; ND, not determined.

of B-lineage surface determinants on colony blasts indicate that, most likely, a pronounced immunologic heterogeneity exists also at the level of clonogenic blasts in B-cell precursor ALL. Our data strongly argue for the use of MAb cocktails directed against multiple distinct surface determinants, over the use of a single MAb, for effective ex vivo marrow purging in B-cell precursor ALL. Our current studies focus on determining the composite immunophenotype of leukemic progenitor cells in order to define the most promising MAb cocktail and the most effective ex vivo marrow purging strategy.

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Acetaldophosphamide—A New Stable Aldophosphamide Analogue for Ex Vivo Chemotherapy of Leukemia

Miloslav Beran, Yuqiang Wang, and David Farquhar

Latent contamination of remission bone marrow with malignant cells is a potential hazard when such marrow is used for autologous rescue after high-dose chemotherapy. Although not proved necessary beyond doubt, in vitro treatment of such marrow is logical. A drug used for such purging should ideally kill leukemic cells yet spare bone marrow stem cells. Here we report the in vitro hematopoietic effects of the new stable aldophosphamide analogue, acetaldophosphamide (A-ALD) and compare its activity with that of 4-hydroperoxycyclophosphamide (4-HC).

MATERIALS AND METHODS

Drugs

We have synthesized A-ALD at our institution. It has a molecular weight of 379, and when diluted in phosphate-buffered saline (PBS) (pH 7.2), it has a half-life of 52 hours at 37°C. Before use, it was diluted in PBS and used either fresh or stored at −20°C and used after thawing. The 4-HC was a gift from Asta-Werke (Bielefeld, Federal Republic of Germany). It was used immediately after dilution in PBS.

Bone Marrow

Human bone marrow was obtained from consenting volunteers. The light density bone marrow (LDBM), containing all progenitor cells and devoid of RBCs was obtained using Ficoll-Hypaque gradient separation (density, 1.080 g/cm^3). Cells were washed twice in PBS containing 5% fetal calf serum (FCS) before being used in experiments.

Assay for Granulocyte-Macrophage Colony-forming Units

Cells were resuspended in Iscove's modification of Dulbecco's medium (IMDM) and supplemented with 20% FCS and 0.3% agar (Difco Laboratories, Detroit, MI). One-milliliter portions were plated in each of three 35-mm Petri dishes on top of a 1-ml feeder layer consisting of IMDM supplemented with 20% FCS, 0.5% agar, and 10% human placenta-conditioned medium as a source of colony-stimulating factor. Cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air. At the end of a 7-day incubation, clones of 50 or more cells were scored. Plates were then incubated for another 8 days and clones rescored on day 15 using identical criteria.

Long-term Bone Marrow Suspension Cultures

A two-step technique was employed as described previously (1,2). In the first step, feeder layer cells were prepared by incubating 1×10^7 LDBM cells/25-cm^2 flask (Corning Glass Works, Corning, NY) in IMDM supplemented with 12.5% FCS, 12.5% horse serum, and 5×10^{-6} M hydrocortisone sodium succinate. After 4 days *all* nonadherent cells were carefully removed, and feeder layers were used for support of either nontreated or A-ALD-treated autologous bone marrow. Cultures with feeder layers only were used to monitor possible "background" production of granulocyte-macrophage colony-forming units (CFUs-GM) by cells originally entrapped within such feeder layers.

After reseeding the feeder layers with autologous LDBM cells treated with A-ALD, cultures were continued at 37°C in 5% CO_2 in air. Every 3 or 4 days 90% of media was exchanged, and the cultures were assayed for the presence of $CFU\text{-}GM_{7d}$ by plating cells from aliquots of media (usually 0.2 ml) in 1-ml semisolid media in triplicate cultures as described above.

Leukemic Cell Lines

The myelogenous leukemia cell line KBM-3 was established in our laboratory from blood of a patient with relapsed monocytic leukemia. It was maintained in IMDM supplemented with 15% FCS by passages every 3 or 4 days and seeded at initial density of 3×10^5 cells/ml. The doxorubicin-resistant subline (KBM-3/DOX) was developed by repeated pulse exposures (1 hour) of the parent cell line to gradually increasing concentrations of doxorubicin. When used for drug studies, both cell lines were in the exponential growth phase.

Assay on Hematopoietic Toxicity of A-ALD Ex Vivo

Washed LDBM cells were resuspended at desired density in 37°C PBS containing Ca^{++} and 5% FCS along with various concentrations of A-ALD. Cells were then incubated in a 1-ml 37°C water bath at ambient atmosphere for 1 hour. Thereafter the cells were diluted with 10 volumes of ice-cold PBS and washed twice to remove the drug excess. They were assayed either immediately in a semisolid agar system for survival of CFU-GM_{7d} and CFU-GM_{15d} or were grown in suspension cultures on autologous feeder layers of marrow stromal cells to evaluate the kinetics of CFU-GM regrowth.

Leukemic cells from various leukemic cell lines were treated in an identical manner. To study the influence of RBC contamination on the antileukemic activity of A-ALD, we added washed human RBCs (to a hematocrit of 0.8%) along with various concentrations of the drug. The IC_{50} and IC_{90} (doses required to kill 50 and 90% of cells, respectively) of the drug were calculated based on marrow exposure to at least four different concentrations of A-ALD or 4-HC for 1 hour at 37°C.

RESULTS

Sensitivity of CFU-GM_{7d} and CFU-GM_{15d} to A-ALD and 4-HC

The main aims of these studies were 1) to compare the sensitivity of CFU-GM_{7d} and CFU-GM_{15d} for each drug, 2) to compare the toxicity of A-ALD with that of 4-HC under identical conditions, and 3) to evaluate whether variations in cell concentrations during drug exposure influence the survival of both classes of CFU-GM to a comparable degree.

The results are presented as dose-response curves on Figures 1 and 2; the IC_{50} and IC_{90} values are summarized in Table 1. It is apparent that A-ALD, like 4-HC, kills CFUs-GM in a dose-dependent manner with a mode of action compatible with that of an agent acting independent of cell cycle. The slopes of survival curves for both classes of CFUs-GM and both drugs could almost be superimposed after drug exposure of bone marrow at a low cell concentration (2×10^6/ml) (Fig 1). After exposure of bone marrow at a higher cell concentration (10×10^6/ml), the slopes plotted for marrow treated with 4-HC are less steep, indicating a decreased toxicity. These observations are reflected in IC_{50} and IC_{90} values (Table 1). On a weight basis, A-ALD is two to four times more toxic than 4-HC; on a molar basis, the A-ALD efficacy is still three to five times higher.

Effect of A-ALD on Clonogeneic Leukemic Cells

When exposed to A-ALD under identical conditions—cell density, pH (7.2), and temperature (37°C)—clonogeneic cells of KBM-3 and KBM-3/DOX lines were two to two and one-half times more sensitive than marrow CFU-GM_{7d} (Table 2). Parental, "sensitive," and doxorubicin-resistant leukemic cells were

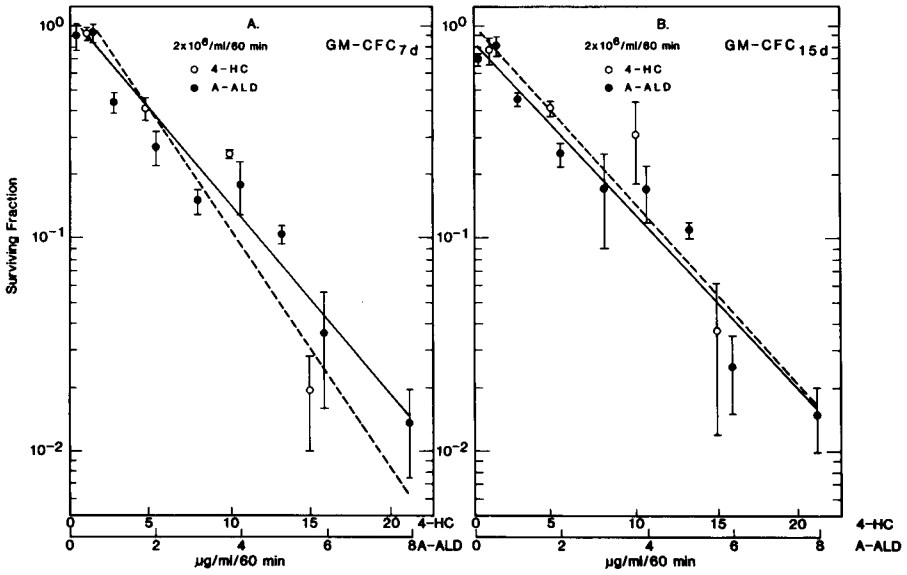

Figure 1. *Survival of human marrow-derived granulocyte-macrophage colony-forming cells (GM-CFC) at 7 days or 15 days as a function of increased doses of acetaldophosphamide (A-ALD) or 4-hydroperoxycyclophosphamide (4-HC). Bone marrow was exposed to each drug at 2 × 10⁶ cells/ml. Each value represents the mean plus or minus the standard deviation of triplicate cultures.*

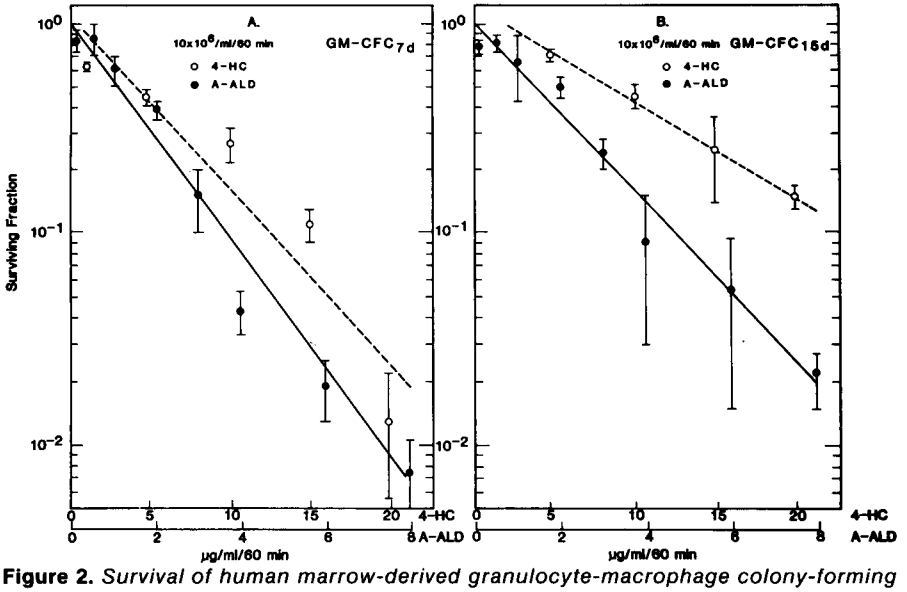

Figure 2. *Survival of human marrow-derived granulocyte-macrophage colony-forming cells (GM-CFC) at 7 days or 15 days as a function of increased doses of acetaldophosphamide (A-ALD) or 4-hydroperoxycyclophosphamide (4-HC). Bone marrow was exposed to each drug at 10 × 10⁶ cells/ml. Each value represents the mean plus or minus the standard deviation of triplicate cultures.*

Table 1. In Vitro Activity of Acetaldophosphamide and 4-Hydroperoxycyclophosphamide on Human Bone Marrow

	Low Concentration (2×10^6 cells/ml)		High Concentration (10×10^6 cells/ml)	
Drug	$CFU\text{-}GM_{7d}$	$CFU\text{-}GM_{15d}$	$CFU\text{-}GM_{7d}$	$CFU\text{-}GM_{15d}$
IC_{50}				
A-ALD	1.50	1.00	1.10	1.40
4-HC	4.43	4.25	4.29	4.70
	2.95	3.71	3.90	6.43
IC_{90}				
A-ALD	5.75	3.71	3.65	4.70
4-HC	14.14	12.71	13.43	23.50
	2.46	2.99	3.68	5.00

Source: Data calculated from Figures 1 and 2.

Note: CFU-GMs represent colonies of 50 or more cells.

Abbreviations: CFU-GM, granulocyte-macrophage colony-forming unit; A-ALD, acetaldophosphamide; 4-HC, 4-hydroperoxycyclophosphamide.

Table 2. Effect of Acetaldophosphamide on Three Types of Cells

Tissue	IC_{50} (10×10^6 cells/ml)	Therapeutic Ratio[a]	IC_{90} (10×10^6 cells/ml)	Therapeutic Ratio[a]
Human marrow				
$CFU\text{-}GM_{7d}$	2.12	—	5.30	—
AML cell line				
(KBM-3)	0.82	2.50	2.25	2.36
AML cell line				
(KBM-3/DOX)	1.12	1.89	2.50	2.12

Abbreviations: CFU-GM, granulocyte-macrophage colony-forming unit; AML, acute myelogenous leukemia; DOX, doxorubicin.

[a] *IC of $CFU\text{-}GM_{7d}$/IC of AML cells.*

equally sensitive to A-ALD, confirming the lack of cross-resistance between doxorubicin and A-ALD in this cell system.

Sparing Effect of A-ALD on CFU-GM Progenitor Cells

Bone marrow was treated with A-ALD, which caused gradual depletion of $CFU\text{-}GM_{7d}$. Immediately after exposure to a concentration of 12 μg/ml, no $CFU\text{-}GM_{7d}$ could be detected using agar culture assay, plating 5×10^5 cells/dish in triplicate dishes. Thus, theoretically, no more than 5 $CFU\text{-}GM_{7d}$ could be seeded into cultures of 9×10^6 cells present in each flask containing the autologous marrow-derived adherent cell layer as a supportive microenvironment. Flasks containing untreated cells and flasks containing an adherent cell layer only served as positive and negative controls, respectively. Results (summarized in Table 3) show the reappearance of $CFU\text{-}GM_{7d}$ after 1 week of

Table 3. Regrowth of CFUs-GM From Acetaldophosphamide-depleted Bone Marrow

Treatment With A-ALD (μg/22 × 10^6/ml/60 min)	CFUs-GM/Culture (× 10^3)			
	Day 0	Day 6	Day 21	Day 28
A-ALD-treated cultures				
0	29.5	30.7	2.1	0.7
6	3.6	33.7	12.0	3.7
10	0.5	17.4	10.3	3.3
12	0	7.1	6.9	4.3
Feeder layer				
0	0	ND	0.10	0.12

Abbreviation: CFUs-GM, granulocyte-macrophage colony-forming units.

culture. The initial increase in A-ALD-treated cultures is largely proportional to the number of surviving CFU-GMs. The CFU-GM_{7d} compartment in cultures of untreated control marrows is maintained at initial size. After 3 and 4 weeks, however, the size of the CFU-GM compartments in A-ALD-pretreated CFU-GM-depleted cultures actually overshoots the size in controls. In control cultures containing feeder layers, the number of CFU-GM released and detected was less than 1% of CFUs-GM in flasks containing depleted marrow treated with 12 μg/ml of A-ALD.

DISCUSSION

Our results demonstrate that the newly synthesized aldophosphamide analogue acetaldophosphamide acts on both classes of human marrow-derived CFUs-GM as 4-HC does but is more toxic by weight and on a molar basis. To determine whether its ex vivo effect depends less on cell concentration during drug exposure than does that of 4-HC (3) will require further comparative studies. Data showing survival of CFU-GM_{15d} in marrow exposed at a higher cell density seem to indicate such a possibility. Our work with A-ALD-treated marrow grown in long-term suspension cultures clearly indicates A-ALD's selective effect on various cell compartments. Regrowth kinetics of CFU-GM_{7d} in cultures depleted of these cells by A-ALD strongly favors its sparing effect on CFU-GM precursor cells, the putative stem cells. Our most recent studies of early regrowth kinetics of CFUs-GM after their depletion by A-ALD further support the theory of CFU-GM repopulation from their progenitor cell pool; absence of such regrowth in marrow depleted of CFU-GM to a comparable degree by x-irradiation and maintained under identical conditions (M. Beran, unpublished results) lends further support to selective action of A-ALD. When compared with regrowth of CFUs-GM in long-term cultures of marrows depleted by 4-HC (2), the CFU-GM recovery after A-ALD appears both more rapid and more extensive. Whether this sparing effect is the result of an

aldehyde dehydrogenase-mediated detoxification described for 4-HC (4) remains to be determined.

Our studies revealed A-ALD's profound antileukemic effect on leukemic clonogeneic cells, with an ex vivo therapeutic ratio of two to two and one-half times that of normal marrow CFUs-GM. Moreover, we have shown absence of cross-resistance to doxorubicin. This is an important property, considering that many leukemic cells have been exposed to anthracyclines during chemotherapy in vivo preceding marrow collection for autologous rescue. In contrast to 4-HC, A-ALD can be easily prepared in bulk quantities, and it is highly stable in neutral buffer solutions with a half-life of 52 hours at pH 7.2 and 37°C, a property of considerable importance for ex vivo chemotherapy procedures. A-ALD is converted into its active form by esterases; whether the presence of such an activation step might further add to the selectivity of its action remains to be determined. After activation into aldophosphamide it exists in equilibrium with 4-HC, and its major metabolites are phosphorodiamidic mustard and acrolein. Like 4-HC, its toxicity might be modulated by aldehyde dehydrogenase-mediated detoxification, a possibility which is currently being investigated.

In conclusion, A-ALD is a novel, stable aldophosphamide analogue that is active in vitro with significant antileukemic potential and sparing effect on primitive CFU-GM progenitors (stem cells?). It holds significant potential for ex vivo chemotherapy of marrow used for autologous bone marrow transplantation.

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Detecting Residual Disease in Bone Marrow

P. Herve and M. Favrot

Residual disease in the bone marrow is defined as tumor cells that persist in the marrow after rounds of induction and consolidation chemotherapy. Undetectable with traditional cytomorphology, the cells fall below the threshold of that perceptible phase when tumor cells are still identifiable by cytology.

During complete remission, tumor or leukemic cells are drowned in the normal marrow cells. To count them would be a matter of pure speculation; for example, when diagnosing acute leukemia, the number of leukemic cells is estimated to be 10^{12}. If one considers that the treatment intended initially reduces this population 3 logs, then 10^9 leukemic cells will be distributed in the total bone marrow. An autologous bone marrow harvest then, which corresponds to 2% of the total marrow volume, will be contaminated by $1\text{-}2 \times 10^7$ leukemic cells.

In autologous bone marrow transplantation, defining the quality of a complete remission and evaluating the possibility of relapse is fundamental to choosing the optimal period for harvesting and to improving the quality of the graft as well as the possible efficiency of in vitro treatments—whether pharmacologic or immunologic.

The prerequisite to detecting residual disease in acute leukemia in non-Hodgkin's malignant lymphoma or in a solid tumor (containing medullary micrometastasis) is identifying the characteristics of the malignant cell at the time of diagnosis. This identity card identifies all the specific markers of the

tumor cell. Qualitative and quantitative assessment of residual disease involves several fields such as cytogenetics, molecular biology, cell culture, and immunology combined with standard optical microscopy or flow cytometry and cytomorphometric analysis in automated systems.

The aims of recent local and national French meetings have been to analyze previously described techniques in order to define their methodological constraints, to compare the reproducibility and reliability of their results, and to search for a process to increase their sensitivity through possibly combining several methods to detect residual disease. Further, all techniques are not equal in detecting residual disease. They differ in complexity, reliability, sensitivity, and type of results (Table 1). Appraising these methods requires a multiparametric analysis including cost and daily clinical application (1).

Table 1. Panel of Bone Marrow Residual Disease Detection Methods

Bone Marrow Detection Markers	Leukemia	At Diagnosis	Before Bone Marrow Harvesting	Clonal Marker
Immunology				
CD2-CD8	T-ALL	++	–	–
CD11-CD15	ANLL	+	–	–
CD9, CD10, CD19, CD20, CD24	Non-T-ALL	++	–	–
Antigen density (CALLA)	C-ALL	(+)	(+)	–
Chromosome analysis[a]				
t(4;11)	ALL (5%)	++	++	++
t(9;22)	ALL (6–19%)			
t(1;19)	pre-B-ALL (30%)			
t(15;17)	AML (M_3)			
Aneuploidy (flow cytometry)	ALL/ANLL	+	+	+
Mutation at the DNA level	ANLL	+	?	+
Clonal DNA rearrangement				
T_B genes	T-ALL	+	?	+
Heavy chain Ig gene	Non-T-ALL			
Light chain Ig gene	Non-T-ALL			

Note: ++, high value; +, intermediate value; (+), low value; –, no information.

[a]*These are examples.*

Abbreviations: CD, cluster of differentiation; T-ALL, T-cell acute lymphoblastic leukemia; ANLL, acute nonlymphocytic leukemia; CALLA, common acute lymphoblastic leukemic antigen; C-ALL, common acute lymphoblastic leukemia; ALL, acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia; Ig, immunoglobulin.

AVAILABLE TECHNIQUES

Cytomorphology and Immunohistochemistry

Although the techniques of cytomorphology and immunohistochemistry are not conclusive, they do, nevertheless, provide a lot of information on the structure of tumor cells. A differential diagnosis between tumor cells and reactive cells after chemotherapy is sometimes difficult to obtain. Cytohistology benefits from the contribution made by immunology when the same cells in immunophenotype or in cytology (smear or cut sections) are examined. Immunohistochemistry can identify isolated tumor cells that traditional medullary histology cannot detect.

Immunology

Although a panel of more than 800 monoclonal antibodies (MAbs) grouped together in 19 clusters of differentiation exists, none of these MAbs is actually specific to either the leukemic or tumor cell (2). MAbs supply an immunologic phenotype of the target cell, prove its origin from a given cellular lineage, and locate the cells that fail to acquire the properties of mature cells. The choice of MAbs with a therapeutic aim (ex vivo treatment of the marrow) depends on the initial phenotype. Cell cryopreservation of the initial tumor plays a vital part that should not be omitted. These serve as references in atypical relapses and in the choice of the ex vivo treatment.

Cytogenetics

Cytogenetics (3-5) identifies chromosomal abnormalities (translocations in particular) of great interest in acute lymphoblastic leukemia (ALL) when a prognosis is made. We have a very useful pure clonal marker that tracks down residual disease and confirms the malignant origins of suspect cells. The technical conditions in setting up the culture (dominated by the delay separating harvest from culture—less than 60 minutes in ALL) determine the success of the karyotype. More than 50 mitoses have to be analyzed in order to increase the chances of finding an abnormal mitosis.

Flow Cytometry

Flow cytometry (6,7) discriminates between cells according to size; cytoplasmic density; and fluorescent surface membrane, cytoplasmic, or nuclear markers. With it, the threshold of sensitivity in detecting an aneuploidy (of variable frequency according to the malignant proliferation concerned) depends on the DNA index (which corresponds to the relationship between the quantity of DNA in the tumor cell and the quantity of DNA in a control cell). The threshold lies between 1% and 5%. The specific and nonspecific fixation of some MAbs can be distinguished when using double labeling with different fluorochromes and phycoerythrin. The variations in antigenic density sometimes allow a normal cell to be discriminated from a malignant cell. In instances in

which this is possible, the simultaneous search for a membrane marker with one aneuploidy allows the threshold of detection to drop below 0.1%. The flow karyotype technique enables an analysis of many cells and, as well as being objective, it sometimes allows the selection of abnormal chromosomes.

Cell Culture

In cell culture, a residual malignant clone emerges (3,8,9), but culture systems do not allow absolute selectiveness in cloning tumor colony-forming units (CFUs) and normal CFUs. We use cell culturing for three reasons: 1) to study factors that control the development of leukemic or tumor CFUs; 2) to test the efficiency of ex vivo treatments; and 3) to detect and quantify residual leukemic or tumor cells in a bone marrow in remission. The clonal nature of the colonies observed by using cytogenetics (when the marker is identifiable), immunology, and molecular biology have to be proved.

The ideal cell culture system adapted to the study of residual disease in acute leukemia should allow the residual leukemic cells to be detected and a selected growth phase of those cells to appear immediately recognizable. The choice of cell culture aims at assessing the frequency of a rare occurrence but one that is detectable through the knowledge of growth conditions. To appraise the marrow purging methods, we believe the study of tumor CFUs in patient samples is a more reliable approach than the study of CFUs in cell lines, which, being artificial, are distinguished in terms of kinetics, phenotype, and clonality. The tumor CFUs, which are drowned in normal marrow cells, cannot have a high clonal rate. In addition, with specific markers, a combination of MAbs can be offered to separate tumor CFUs from normal CFUs (the elimination of a part of normal cells is accompanied by an elimination of a concentration of leukemic CFUs).

Molecular Biology

The contribution of molecular biology (3, 10-12) has changed traditional techniques drastically. Rearrangements of immunoglobin genes (heavy and light chains) in B-type clonal proliferations can be studied with the Southern blot method. In T-cell clonal proliferations, the rearrangements of α and β genes (more generally the β gene) of the T receptor of the antigen are studied. The threshold of detection is low (1-5%). With the Southern blot test, 50 μg of DNA is the minimum required (corresponding to $1\text{-}5 \times 10^7$ nucleated cells). This method is therefore unsuited to the study of small cell fractions, such as one of an isolated colony.

The oncogenes have a physiological function and serve as codes for proteins that enter into cellular proliferation and differentiation. When their manifestation is abnormal or disorganized, an abnormal proliferation can be observed that might then lead to a malignant growth. Mutated oncogenes can transcribe an abnormal protein that, when manifested at the cellular membrane level, could lead to the production of specific MAbs of this protein. In this case, a

truly specific immunologic tool of the tumor cell would be obtained. The study of a mutation at the DNA level of an oncogene shows high sensitivity when detecting residual disease, but its threshold of detection still remains to be determined. The identification of RNA transcripts by in situ hybridization would allow a considerable increase in sensitivity and specificity of detection methods.

Systems of Image Analyzers

More or less automated systems of image analyzers linked to data processing allow detection and cytological analysis of isolated cells that an ordinary microscope does not. One abnormal cell among 10^6 normal ones can be detected with a multiparametric microscope adapted to the search for rare occurrences (using a wide-angle lens, for example) and relayed to a computer. The principle is that by computerizing specific characteristics of the tumor cell (size, density, fluorescence, enzymatic activity, chromatin structure, for example) one may compare all the sample cells with it.

APPLICATION OF TECHNIQUES

The application of these techniques has been assessed in different malignant diseases in which marrow involvement is either frequent or constant (medullary micrometastasis of some solid tumors). The following malignant diseases were topics of discussion in relation to these techniques at recent local and national French meetings: neuroblastoma, small cell lung cancer, non-Hodgkin's malignant lymphoma, and acute lymphoid and nonlymphoid leukemia (1).

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Pharmacological Manipulation to Reverse Drug Resistance and Protect Hematopoietic Stem Cells During Purging

Anna Janowska-Wieczorek and Carol E. Cass

We are pursuing pharmacological approaches to increase selective killing of neoplastic cells during in vitro purging for autologous bone marrow transplantation (ABMT). Antitumor drugs alone may not be sufficiently selective purging agents, particularly in marrow from patients who have undergone extensive chemotherapy and whose grafts may contain drug-resistant neoplastic cells. Recent studies of drug-drug interactions in cultured leukemia and lymphoma cell lines and in a number of other neoplastic cell lines have provided a rationale for in vitro manipulation of drug uptake by cells within marrow grafts to enhance the selective toxicity of certain antineoplastic drugs.

A large number of neoplastic cell lines have now been identified, which, though selected for resistance to a single heterocyclic drug, have developed cross-resistance to a variety of other unrelated heterocyclic drugs by virtue of decreased net uptake of drug (for reviews, see 1,2). Although the clinical importance of multiple-drug resistance has yet to be determined, molecular markers associated with resistance have been detected in the malignant cells of cancer patients (3). Membrane-active agents (e.g., verapamil) have been found to sensitize drug-resistant cultured neoplastic cells, including many leukemia and lymphoma cell lines, by increasing net uptake of drug (1,4), raising the

possibility that certain types of clinical resistance can be circumvented by coadministration of such agents. Before these pharmacological manipulations can be examined clinically in ABMT, selectivity must be established by demonstrating that verapamil does not also sensitize normal human hematopoietic stem cells to the anticancer drugs involved in multidrug resistance. If not, coadministration with drugs frequently associated with multidrug resistance (e.g., the vinca alkaloids) may lead to selective killing of drug-resistant cells during the purging procedure.

A second approach involves the use of potent inhibitors of nucleoside transport, such as nitrobenzylthioinosine (NBMPR), to selectively protect hematopoietic stem cells from tubercidin (7-deazaadenosine), a nucleoside analogue (5,6) with considerable activity against neoplastic cell lines (7,8). We (9,10) and others (11,12) have observed that a number of cultured cell lines differ in their sensitivity to NBMPR inhibition of nucleoside transport. Thus, depending on the relative sensitivities of neoplastic and hematopoietic stem cells to NBMPR or other potent inhibitors of nucleoside transport (dilazep, dipyridamole), it should be possible to use such inhibitors, which by themselves are nontoxic, to protect normal hematopoietic stem cells but not neoplastic stem cells from the cytotoxic effects of tubercidin. Nucleoside transport systems and their sensitivity to specific inhibitors have recently been reviewed (13,14).

In this work we present evidence that pharmacological strategies to both circumvent resistance of neoplastic cells and protect hematopoietic stem cells may indeed find broad application in ABMT.

MATERIALS AND METHODS

Heparinized bone marrow aspirates were obtained from the sternum of patients undergoing cardiac surgery. The light-density fraction was obtained by centrifugation of mononuclear cells on 60% Percoll. For studies with vincristine and verapamil, 6×10^5 cells were incubated for 4 hours (37°C) in Iscove's modification of Dulbecco's medium (IMDM) containing graded concentrations of vincristine (0.1-100 μM), verapamil (2.5-80 μM), or both. Cells were then washed with drug-free media and plated in mixed-colony assay (15,16) in 1 ml of IMDM supplemented with 0.8% methylcellulose, 30% human plasma, 1 unit erythropoietin, and 5×10^{-5} M mercaptoethanol. Human pluripotent hematopoietic and granulocyte-macrophage colony-forming units (CFUs-GEMM and CFUs-GM) and erythroid burst-forming units (BFUs-E) were counted after incubation for 14 days at 37°C in 5% CO_2 in air. For studies with nucleoside transport inhibitors and tubercidin, 6×10^5 light-density fraction marrow cells were preincubated for 30 minutes (37°C) with inhibitor (3 μM NBMPR, dilazep, or dipyridamole) in IMDM and then, still in the presence of transport inhibitor, incubated for 1 hour (37°C) in graded concentrations of tubercidin (0.001-100 μM). Cells were then washed and plated in mixed-colony assay as above.

Cultured mouse leukemia L1210/C2 cells, human promyelocytic leukemia HL-60/C1 cells, and HeLa cells were maintained as described previously (7,17,18). Cell lines were demonstrated to be free of mycoplasma by conventional techniques. Detailed procedures have been presented elsewhere for assessing drug effects on proliferation and colony-forming ability of L1210/C2 cells (7) and HeLa cells (17), for synchronizing HeLa cells by mitotic detachment (19), and for measuring uptake of tritium-labeled vincristine.

The tritium-labeled vincristine was purchased from Moravek Biochemicals, Brea, CA, and was repurified within 4 days before use by high-performance liquid chromatography as described elsewhere (18). Nitrobenzylthioinosine was prepared (20) from 6-thioinosine provided to Dr. A. R. P. Paterson by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Tubercidin was purchased from Sigma Chemical Co., St. Louis, MO. Dilazep and dipyridamole were gifts to Dr. A. R. P. Paterson from Hoffmann-La Roche, Basel, Switzerland, and Boehringer, Ingelheim, Burlington, Ontario, respectively.

RESULTS AND DISCUSSION

Circumvention of Drug Resistance

Verapamil, along with a number of other calcium antagonists, is one of a heterogeneous group of agents that have been shown to overcome multiple-drug resistance in experimental tumor systems (1). Tsuruo *et al.* (4) demonstrated that these agents, which lack significant toxicity by themselves, potentiate the activity of vincristine and doxorubicin (Adriamycin) against both drug-resistant and drug-sensitive human hematopoietic cell lines during long-term exposures, apparently by enhancing net uptake of drug. While the sensitizing effects of verapamil on drug-resistant cells are well established, relatively little information is available concerning its effects on drug-sensitive cells in short-term exposures. Yalowich *et al.* (21) reported that verapamil enhanced the activity of etoposide and vincristine but not doxorubicin against cultured K562 cells during 1-hour exposures to both drugs. We have examined the effects of coadministration of verapamil and vincristine on HL-60 cells, which are highly sensitive to vincristine toxicity (18), and have found that verapamil significantly increased drug uptake, with the greatest effects seen between 40 and 80 μM of verapamil (Table 1; see also 22). That finding raised the possibility that verapamil might also potentiate the effects of vincristine against other nonresistant cell types, including normal human hematopoietic stem cells.

Exposure of human bone marrow cells to graded concentrations of verapamil alone (2.5-80 μM) did not affect the growth of CFU-GM, BFU-E, and CFU-GEMM colonies (Fig 1). When verapamil (5, 10, and 20 μM) was coadministered with graded concentrations of vincristine (0.1-100 μM), the inhibition of colony formation was not significantly different from the inhibition

Table 1. Effect of Verapamil on Uptake of 0.1 μM Tritium-labeled Vincristine by HL-60 Cells

Verapamil (μM)	Cell-associated Vincristine (pmol/10^6 cells)		
	15 min	2 hr	4 hr
0	3.4	5.6	6.9
2	3.9	6.1	6.9
5	3.9	7.1	8.0
10	6.1	7.8	9.6
20	6.9	10.6	10.3
40	9.1	11.4	12.0
50	11.8	13.5	13.6

Note: Actively proliferating HL-60/C1 cells (3 × 10^5 cells/ml) were combined with equal amounts of growth medium containing tritium-labeled vincristine and verapamil at twice the test concentrations. Cultures were incubated at 37° C, and at the times indicated triplicate 1-ml samples were withdrawn and assayed for radioactivity as described previously (21).

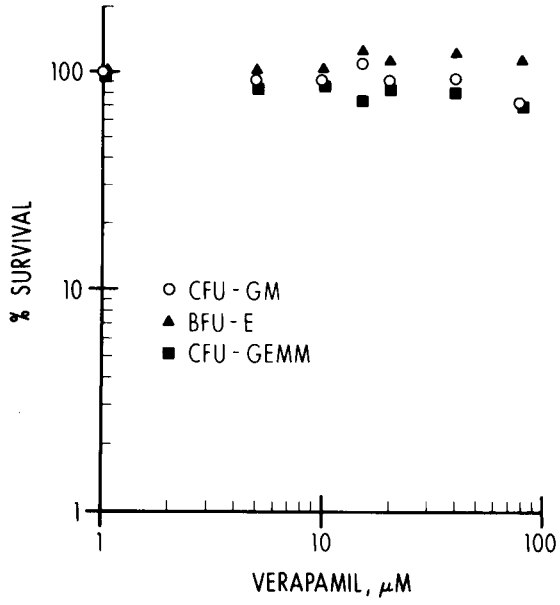

Figure 1. *Effects of verapamil alone (2.5–80 μM, 4 hr, 37° C) on the formation of CFU-GM, BFU-E, and CFU-GEMM colonies. Each symbol represents the mean value of eight experiments.*

seen with vincristine alone (Fig 2) (22). These results suggest that the strategy of employing verapamil to circumvent resistance to vincristine, and possibly other drugs associated with the multiple-resistance phenotype, during in vitro purging can be attempted without significant risk to normal hematopoietic stem cells. Yalowich *et al.* (21), who also found that short exposures to verapamil did not enhance vincristine's toxicity against CFU-GM cells, reported enhanced toxicity of etoposide against CFU-GM cells.

Selective Protection of Normal Stem Cells

We have previously shown that the cytotoxicity of nucleoside analogues depends on the presence of a functional nucleoside transporter in the

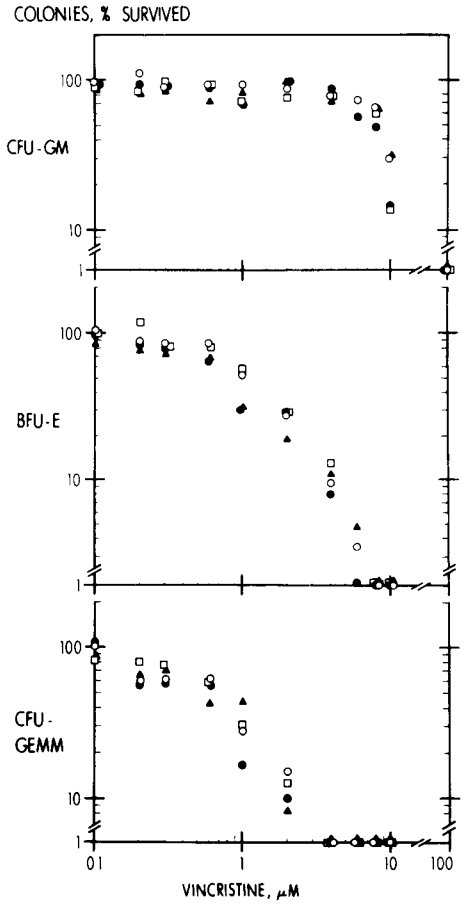

Figure 2. *Effects of vincristine alone (*solid circles*) and with 5 μM (*open circles*), 10 μM (*solid triangles*), and 20 μM (*open squares*) verapamil on CFU-GM, BFU-E, and CFU-GEMM colony formation (mean values of five to ten experiments). Differences in values for vincristine with and without verapamil are not statistically significant.*

membrane of the target cell (23,24). Coadministration of NBMPR protected cultured human lymphoblastoid RPMI 1640 cells from the toxicity of a heterogeneous array of nucleoside analogues (23), and transport-defective murine lymphoma AE1 cells were insensitive to several cytotoxic nucleosides (24). It has become apparent that cell types differ significantly in their relative sensitivities to NBMPR (9-12), suggesting that inhibitors of nucleoside transport may exhibit sufficient selectivity to protect dose-limiting normal tissues from particular cytotoxic nucleosides.

Tubercidin is a nucleoside antibiotic that exhibits a variety of biochemical effects, including inhibition of energy metabolism, de novo synthesis of purines, methylation reactions, and synthesis of nucleic acids and proteins (5,6). Tubercidin is accepted as a permeant by the NBMPR-sensitive, equilibrative transport systems of cultured cells, and results of detailed kinetic studies indicate that it is as good a substrate as adenosine and deoxyadenosine, the preferred natural substrates (25). Tubercidin is highly cytotoxic, exhibiting significant activity against cultured L1210 leukemia cells during short in vitro exposures (Table 2) (7). In addition, tubercidin is not phase specific, because HeLa cells, synchronized by mitotic detachment, were equally sensitive during G_1 and S phase (Table 3). The toxicity of tubercidin was greatly reduced by prior treatment of mice with a prodrug of NBMPR, thereby significantly increasing its therapeutic index in the treatment of the L1210 leukemia (26,27).

Tubercidin is also highly toxic to normal human hematopoietic multipotential (CFU-GEMM) and committed (CFU-GM) progenitor cells (Fig 3). No CFU-GM and CFU-GEMM colonies were seen after exposure of bone marrow cells to tubercidin alone at concentrations ≥ 1 μM. In contrast, pretreatment of bone marrow cells with inhibitors of nucleoside transport significantly diminished tubercidin cytotoxicity. More than 60% of CFU-GM and CFU-GEMM colonies survived exposure to 10-μM tubercidin after pretreatment with 3 μM NBMPR (Fig 3A), indicating a substantial reduction in uptake of tubercidin by

Table 2. Effect of Tubercidin on Viability of Cultured L1210 Leukemia Cells

Tubercidin (μM)	Colony-forming Ability (% control ± SD)[a]
0	100.0 ± 15.0
0.1	43.1 ± 10.5
0.2	17.6 ± 2.1
0.3	4.3 ± 1.0
0.4	0.14 ± 0.01

Note: Actively proliferating L1210/C2 cultures (10^5 cells/ml) were exposed to tubercidin at 37° C for 2 hrs and then assayed for colony-forming ability as described previously (9). The absolute cloning efficiency of untreated cultures (100 cells/tube) was 57.3 ± 8.6.

[a] *Percentage of control values plus or minus standard deviation (mean ± SD; n = 20).*

Table 3. Effect of Tubercidin on Viability of HeLa Cells in G_1 and S Phase

Condition	Colony-forming Ability (% control ± SD)[a]	
	G_1 phase	S phase
Without drug	100.0 ± 9.0	100.0 ± 11.1
Tubercidin (1 μM)	55.7 ± 5.1	49.7 ± 4.8
Vincristine (0.05 μM)[b]	64.8 ± 7.7	21.5 ± 6.3

Note: HeLa/S3 cells were synchronized by mitotic detachment (19), plated in 60-mm culture flasks, and subjected to 1-hr drug exposures during G_1 (4 hr after detachment) and S (15 hr after detachment) phases. Colonies were scored after 14 days of incubation at 37° C. The absolute plating efficiencies of untreated G_1 and S-phase cultures (100 cells/plate) were 50.3 ± 4.5 and 50.3 ± 5.6, respectively.

[a] *Percentage of control values plus or minus standard deviation (mean ± SD; n = 20).*

[b] *Data, from Cass* et al. *(8), illustrate the effects of an agent whose activity is specific to cell cycle.*

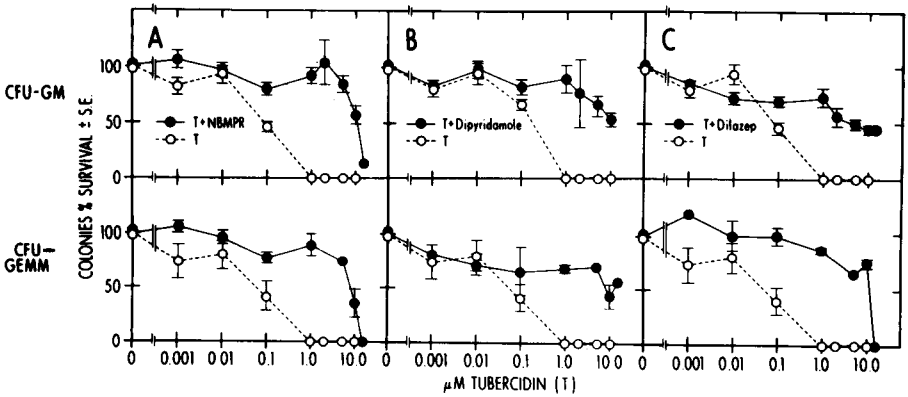

Figure 3. *Effects of various concentrations of tubercidin alone (*open circles*) and with a fixed concentration (3 μM) of an inhibitor of nucleoside transport (*solid circles*) on the survival of CFU-GM, BFU-E, and CFU-GEMM colonies (mean ± SE; n = 5) (*A, *NBMPR;* B, *dipyridamole;* C, *dilazep).*

stem cells. Pretreatment of bone marrow cells with dipyridamole (Fig 3B) spared about 50% of CFU-GM and CFU-GEMM colonies from toxicity during 1-hour exposures to 10-μM tubercidin, and pretreatment with dilazep (Fig 3C) spared about 40% of CFU-GM and 70% of mixed colonies.

To be successful, pharmacological purging techniques should simultaneously preserve hematopoietic stem cells and achieve a 5- to 6-log kill of neoplastic cells. We have demonstrated that human hematopoietic stem cells are sensitive to specific, tight-binding inhibitors of nucleoside transport and can thus be protected against tubercidin and probably against other nucleosides

with antineoplastic activity. Of the large variety of compounds that inhibit nucleoside transport, the S^6 derivatives of 6-thioinosine, including NBMPR, and the nonnucleoside inhibitors dilazep and dipyridamole have been the most studied as probes of nucleoside transport systems (9,13,14,28). Although a few cultured cell lines can be completely protected against cytotoxic nucleosides by transport inhibitors, the existence of cell types that exhibit partial sensitivity to the transport inhibitors suggests that conditions that are completely protective for normal hematopoietic stem cells but not for neoplastic cell types can be determined. Unfortunately, there is little information concerning the relative sensitivities of the nucleoside transport systems of normal human cells, though we do know that nucleoside transport in human erythrocytes is highly sensitive to inhibition by NBMPR, dilazep, and dipyridamole (14). Wiley *et al.* (29,30) have quantitated NBMPR binding sites on leukemic myeloblasts and lymphoblasts and on normal granulocytes and lymphocytes and have suggested that transport across the cell membrane is a limiting factor in the effectiveness of therapy with cytarabine.

In conclusion, we demonstrated that NBMPR, dilazep, and dipyridamole had significant protective activity for hematopoietic stem cells during in vivo short-term exposures to tubercidin. Further investigations are in progress to define the relative sensitivities of human leukemic and lymphoma cells to these transport inhibitors and to establish selective conditions for bone marrow purging.

ACKNOWLEDGMENTS

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ID. Chronic Myelogenous Leukemia

Transient Restoration of Partial Philadelphia Chromosome Negativity After Autografting in Chronic Myeloid Leukemia

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Evidence suggests that considerable numbers of Philadelphia chromosome (Ph^1)-negative hematopoietic stem cells may be present, albeit in quiescent state, in the marrow of newly diagnosed patients with chronic myeloid leukemia (CML) (1). Some of these Ph^1-negative stem cells may also be present in the circulation. Presumably, the clonal proliferation of Ph^1-positive cells depends on their ability to suppress the growth of these Ph^1-negative (putatively normal) stem cells. Because in certain clinical circumstances a Ph^1 positive-negative mosaicism has been recognized (2) and because in other circumstances patients have been restored to partial or complete Ph^1 negativity by administration of cytotoxic drugs (3,4), we thought that high-dose chemotherapy (or chemoradiotherapy) followed by autografting with blood-derived stem cells collected at diagnosis might give similar results.

An additional rationale for developing this protocol was that we thought the probability of a patient entering the blastic phase of the disease might be directly proportional to the number of leukemic stem cells in the body, a number that might be little affected by administration of cytotoxic drugs or

radiotherapy at a standard dosage but much more affected, even eradicated, at a high dosage. Adequate hematopoiesis could then be reestablished by transfusion of a relatively small number of autologous stem cells. Such treatment might delay the onset of transformation and thereby prolong life.

We now update our preliminary experience (5) with the use of high-dose chemotherapy and autografting in 13 patients with CML treated before the onset of transformation.

PATIENTS AND METHODS

Since April 1984 13 patients with Ph^1-positive CML in chronic phase were treated with cytoreductive therapy followed by autografts of peripheral blood buffy coat cells that had been collected at the time of diagnosis and stored for 1-42 months (Table 1). The technique for collection, cryopreservation, and reconstitution of cells has been described elsewhere (6).

There were five women and eight men. Their median age was 34 years (range, 29-49 years). One patient (number 4) had not received any previous treatment; another patient (number 3) had received only standard conditioning treatment preceding transplantation. The other 11 had received hydroxyurea, busulphan with or without thioguanine, or thioguanine alone. The median duration of the chronic phase before the autograft was 18 months (range, 1-42). Three patients (numbers 1-3) who received daunorubicin, cyclophosphamide, total body irradiation, and an unsuccessful allogeneic transplant then received autologous buffy coat cells. (See details in Table 1.)

Ten patients were treated with busulphan (4 mg/kg) orally daily for 4 consecutive days followed after 24 hours by melphalan (60 mg/m^2) given by bolus intravenous injection. Forty-eight hours after the last dose of chemotherapy the frozen peripheral blood buffy coat cells were taken to the bedside, rapidly thawed, and resuspended in balanced Hartman's solution. They were then given by slow intravenous injection. A total nucleated cell dose of 10×10^8/kg body weight was given to all patients except one (number 2) who received 20×10^8/kg buffy coat cells. The experimental nature of the procedure was explained to all the patients. The median duration of follow-up was 17 months (range, 2-29 months).

Full blood counts, bone marrow aspirates, and cytogenetic analyses were performed by standard methods at regular intervals. In some cases granulocyte-macrophage progenitor cells (CFUs-GM) were established in agar culture (7). For cytogenetic analysis individual colonies were transferred into 50 μl of 0.075 M KCl and plated on polylysine-coated slides. After hypotonic treatment of the colonies, the cells were fixed in a three-step procedure and examined with and without Giemsa banding.

Table 1. Thirteen Patients With CML Autografted in Chronic Phase: Results of Cytogenetic Analysis

Patient Number	Sex/ Age (yr)	CP Duration Before Autograft (mo)	Primary Rx	Preautograft Rx	Survival (mo)	Postautograft Rx	Ph¹-negative Metaphases (%)[a]					
							Before Autograft	After Autograft (wk)				
								4–8	8–16	16–32	32–54	54+
1	F/36	42	BU	D/C/TBI	28+	NFT	0	33	33	25	0	
2	M/49	10	HU	D/C/TBI	15+	Interferon at 1 month	0	88	66	0	0	
3	M/34	10	None	D/C/TBI	9+	NFT	0	0	0	0		
4	F/29	1	None	BU, MEL	17+	NFT	0	16	—	90	100	93[b]
5	M/32	30	HU	BU, MEL	23+	2A/G at 13 months; NFT	0	20	13	10	0	
6	F/39	25	TG	BU, MEL	24+	NFT	0	—	23	3	—	
7	M/33	12	BU/TG	BU, MEL	10	LBT at 6 months	0	10	6	—		
8	M/32	30	BU/TG	BU, MEL	24+	2A/G at 10 months; interferon at 1 month	0	15	—	7	3	100[c]
9	F/29	15	HU	BU, MEL	14	LBT at 8 months	0	5	—			
10	M/33	20	HU	BU, MEL	16+	NFT	0	0	0	0		
11	M/31	6	HU	BU, MEL	16+	HU at 10 months	0	0	0	10		
12	F/49	10	HU	BU, MEL	4+	NFT	0	0	0			
13	M/45	8	HU	BU, MEL	3+	NFT	0	25				

Abbreviations: CML, chronic myeloid leukemia; CP, chronic phase; BU, busulphan; D/C/TBI, daunorubicin, cyclophosphamide, and total body irradiation; NFT, no further treatment; HU, hydroxyurea; MEL, melphalan; 2A/G, second autograft; TG, thioguanine; LBT, lymphoid blast transformation.

[a] *Number of metaphases examined ranged from 5 to 30.*

[b] *Twenty-eight of 30 examined.*

[c] *Five of five examined.*

RESULTS

There was early evidence of trilineage engraftment in all 13 patients. However, one patient (number 12) required an additional autograft without preconditioning at day 84. Two other patients required blood transfusions up to 4 months after the autograft. The mean duration of neutropenia (neutrophils $< 500 \times 10^8$/l) in the 12 patients (excluding number 12) was 30 days (range, 20-48 days) and of thrombocytopenia (platelets $< 50 \times 10^9$/l) was 39 days (range, 33-60 days). In one of two patients who had prolonged thrombocytopenia platelet antibodies were discovered; the platelet count increased after treatment with corticosteroids. Apparent clinical normality (improved well-being, impalpable spleen) with normal values for peripheral blood count and normal results on bone marrow aspirate studies (normal fat spaces, normal cellularity of trails, absence of myelocyte peak) was restored in the peripheral blood and bone marrow for a median of 8 months (range, 2-30 months).

Cytogenetic analysis showed less than 30% Ph^1-negative metaphases in the marrow after hematopoietic recovery in ten of the thirteen patients treated. The percentage of Ph^1-negative cells remained at zero or diminished and disappeared over 5-8 months in nine of the patients. Patients 5 and 8, whose cytogenetic studies showed conversion from Ph^1 negativity to persistent Ph^1 positivity associated with a transition of their disease from morphological normality to early chronic phase disease, were autografted on a second occasion (one 8 months and the other 13 months) after the first autograft. One of the two had a recurrence of Ph^1-negative metaphases in the marrow 4 months after the second autograft. This Ph^1 negativity seen in five of five metaphases analyzed was associated with a rising WBC count (96×10^9/l). He is currently being treated with α-interferon. The other patient who received a second autograft remains Ph^1 positive.

In the case of the patient (number 4) who had received no previous treatment, Ph^1-negative metaphases appeared 4-6 weeks after the autograft but were not observed in 20 metaphases, all of which were Ph^1-positive, analyzed 5 months after the autograft. However, they reappeared at 7 months and were still present 12-17 months after the autograft. Cells from CFU-GM cultures from this patient at 11 and 12 months after the autograft showed only Ph^1-negative metaphases. Rearrangement of the *bcr* gene, which was present at diagnosis, was not present in the Ph^1-negative metaphases obtained after 8 months. Cytogenetic analysis 17 months after the autograft showed the presence of a minority population (2/30) of Ph^1-positive cells.

Lymphoid blast transformation occurred in two patients 6 and 8 months after the autograft. Both subsequently died of resistant leukemia after autograft despite intensive chemotherapy appropriate to adult lymphoblastic leukemia. Eleven patients are alive, seven requiring no chemotherapy, up to 28 months after their autograft. Their median survival after the autografting is 15 months (range, 3-28 months).

There were few complications of the chemotherapy. All patients had temporary chemotherapy-induced mucositis. Grand mal convulsions occurred in two cases on day 3, and thereafter patients were treated with prophylactic phenytoin from day 1 (8). In three patients amphotericin B-responsive fungal infections developed, and in eight bacterial infections responsive to antibiotics developed. There were no treatment-related deaths.

DISCUSSION

We do not yet know whether the induction of Ph^1 negativity will prolong the duration of the chronic phase of CML, but we have found it to be a regular, though usually a partial and transient, phenomenon. Deliberate attempts to induce the regeneration of Ph^1-negative hematopoietic cells in chronic phase CML by intensive treatment intended to ablate the dominant Ph^1-positive population have not been successful in prolonging the duration of the chronic phase (9). Our observation that cryopreserved buffy coat leukocytes administered to rescue a patient whose human leukocyte antigen-matched allograft failed to grow led to the appearance of Ph^1-negative metaphases during the early months after autografting suggested that autografting in the chronic phase of CML offered a possible way of prolonging the duration of the chronic phase and of investigating the circumstances in which Ph^1-negative cells had a growth advantage. In the work reported here our intention was to see whether autografting affects the duration of the chronic phase and to determine the frequency of appearance and the duration of persistence of Ph^1-negative metaphases in the postautograft period.

Engraftment was rapid in all patients except one who received a supplementary autograft on day 80 because of continuous pancytopenia (persistent WBC count of only $1 \times 10^9/l$, platelets $< 10 \times 10^9/l$, and a continuing blood transfusion requirement). No therapy-related deaths were seen. The convulsions related to high-dose busulphan therapy appear to have been prevented in subsequently treated patients by anticonvulsant therapy. Eleven patients survive in good health, seven requiring no treatment, which may or may not be of clinical advantage.

The occurrence of lymphoid blast transformation in two patients 6 and 8 months after autograft could mean the autograft expedited the growth of a previously transformed cell population or that it induced the transformation.

Ph^1-negative hematopoiesis has been seen after autografting with blood-derived buffy coat cells or autologous bone marrow. The origin of the Ph^1-negative cells seen in the patients in this series is uncertain, but it is likely that they arise from the autograft and indicate a temporary growth advantage over the Ph^1-positive cells in hypoplastic bone marrow. In this circumstance it is possible that Ph^1-negative stem cells react better to homeostatic influences than Ph^1-positive cells. In patient 4, however, the delayed and prolonged Ph^1-negative hematopoiesis could have been endogenous.

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Mitoxantrone, Cytarabine, and Melphalan as Cytoreductive Therapy Before Autografting in Blast Crisis of Chronic Myeloid Leukemia

F. Brito-Babapulle, D. Catovsky, D. A. Galton, C. Arthur, and J. M. Goldman

Currently available treatments for the myeloid blast crisis of chronic myeloid leukemia (CML) are largely ineffective. Once blast crisis supervenes, survival is usually short (8-12 weeks) (1). Since 1977 we have performed autografts using cryopreserved peripheral blood buffy coat cells to expedite hematologic recovery after marrow-ablative therapy in the blast crisis of CML (2). Previous programs failed because of the toxicity of the preautograft cytoreductive treatment and the recurrence of blast proliferation (3). In an attempt to improve the cytotoxic drug combination, we decided to assess the efficacy of mitoxantrone, cytarabine, and melphalan as cytoreductive treatment prior to autograft. The mitoxantrone and cytarabine were chosen because of encouraging results with their use in refractory and relapsed acute myeloid leukemia (4).

PATIENTS AND METHODS

Ten patients (three women, seven men) with blast crisis of CML were treated. Typing of the blast cells using cytochemical and immunological techniques identified two cases as in lymphoid blast transformation (LBT) and

eight as myeloid (two basophilic, two monocytic, one myelomonocytic, one megakaryocytic, two undifferentiated). Two of the ten patients had received bone marrow transplants for chronic phase (CP) CML 2 and 4 years previously and had relapsed with myeloid blast crisis. Two other patients had Philadelphia chromosome-negative CML. The median age of the group was 51 years (range, 32-59 years). Two patients had hypertensive heart disease and another had had a splenectomy 2 years before undergoing autografting. The median duration of the chronic phase before the onset of blast crisis was 3.3 years (range, 0.3-6.5 years). The median white cell count at transformation was $100 \times 10^9/l$ (range, $40-250 \times 10^9/l$) with over 50% of blasts in the blood. Mitoxantrone ($12\ mg/m^2$) was given by half-hour infusion daily on days 1-5 and cytarabine ($1\ g/m^2$) was given twice daily by 2-hour infusion on days 1-3. Melphalan ($120\ mg/m^2$) was given by bolus injection on day 7, and an autograft of cryopreserved peripheral blood buffy coat cells ($10 \times 10^8/kg$) was infused on day 9 in six patients and on day 11 in four. Once the chronic phase was restored, maintenance chemotherapy with vincristine (weekly), prednisolone (for 5 days every 4 weeks), oral methotrexate, and mercaptopurine (daily) was given to the patients with LBT.

The technique for collection, cryopreservation, reconstitution, and infusion of buffy coat cells has been described (5). Two patients whose infusions failed to engraft by day 23 received another dose of $10 \times 10^8/kg$ of buffy coat cells on day 25. The second chronic phase was defined as clearance of all blast cells from the peripheral blood and less than 5% blast cells in the bone marrow.

RESULTS

All patients were restored to a second CP. Engraftment occurred at a median of 18 days (range, 12-40 days). This includes the two patients who had a supplementary autograft at day 25. The median duration of the second CP was 6+ months (range, 5-14+ months). Localized bone pain, which responded to radiotherapy (30 Gy in ten fractions), occurred in three patients whose blood and bone marrow remained in CP. Blasts recurred in the blood and bone marrow in three other patients 5, 6, and 8 months after autograft. A second attempt at cytoreduction and autograft with the same chemotherapeutic regimen was carried out in two of the three. Both patients died with pancytopenia and disseminated fungal infections. The third patient received mitoxantrone and cytarabine alone because the supply of cryopreserved cells was exhausted. His leukemia returned to a CP that lasted for 2 months. Two other patients died in accelerated phase, one of a cerebrovascular accident 6 months after autograft and the other of septicemia and renal failure 13 months after autograft. Five patients are alive with second CP of blood and bone marrow (range, 5-14 months).

All patients had nausea, vomiting, and mucositis. Seven patients had fungal infections (2 had disseminated candidiasis, 3 had gastrointestinal

candidiasis, and 2 had aspergillus pneumonia). Five of these infections responded to therapy with amphotericin B. Asteatosis (severe pruritus and skin desquamation) developed in two patients. No drug-induced cardiovascular or cerebellar toxicity was seen.

DISCUSSION

Current regimens of treatment effective in ablating the myeloid blasts of CML are usually toxic and cause much morbidity and mortality (6). Less toxic regimens result in a short duration of the second CP with early recurrence of blasts. The regimen we used had acceptable toxicity and was effective in substantially reducing the populations of both lymphoid and myeloid blasts. However, blasts recurred in the blood after 6 months in 30% (three of ten treated). It is of interest that localized bone pain appeared in three patients despite the autograft procedure. Although most of the patients in the study had disease of poor prognosis (elderly patients, basophilic and megakaryoblastic cell types, and blast crisis following bone marrow transplantation), the blasts appeared to be sensitive to the chemotherapeutic regimen used and toxicity was not excessive. The unusually high incidence of fungal infections prompted us to incorporate the use of prophylactic intravenous amphotericin B (20 mg i.v. on alternate days). The regimen described appears to be less toxic than previous preautograft cytoreductive schedules (3) and may prolong survival in some patients with blast crisis of CML.

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Autologous Transplantation of Philadelphia Chromosome-Positive Cell-Depleted Marrow in Chronic Granulocytic Leukemia

Josy Reiffers, Dominique Maraninchi, Gerald Marit, Marie-Francoise Bertheas, Philippe Bernard, Didier Blaise, Bernard David, Gerard Lepeu, Francoise Feuillatre, Gisele Novakovitch, Wen Zong-Qi, Jean-Antoine Gastaut, Antoine Broustet, and Yves Carcassonne

Philadelphia chromosome (Ph^1)-positive chronic granulocytic leukemia (CGL) is a clonal myeloproliferative disorder that progresses uniformly from a stable chronic phase lasting 3 to 4 years to a blastic transformation, which marks the terminal stage of the disease. Allogeneic bone marrow transplantation is the only way to eradicate the malignant clone and cure CGL patients, but this method is applicable only to patients under 50 years of age who have a human leukocyte antigen (HLA)-identical sibling donor (1).

Autologous transplantation of blood-derived or marrow cells may be used as treatment of blast crisis. In most patients, a second chronic phase may be restored. However, a recurrent transformation is usually observed within 6 months after transplantation, so that only a few patients have benefited from this technique (2,3). More recently, Goldman and coworkers (4) proposed

autologous bone marrow transplantation (ABMT) during the chronic phase of the disease. The rationale for this strategy was based on several considerations. First, among patients with CGL in transformation, those who received autografts during the accelerated phase survived longer than those who did so during overt blast crisis (5,6). Second, Ph^1-negative hematopoiesis could be restored in some patients (6,7), confirming the results obtained by Coulombel *et al.* (8) who demonstrated the presence of Ph^1-negative precursors in long-term culture systems; Ph^1-negative restoration could prolong the duration of the chronic phase (4). Third, high-dose chemoradiotherapy during the chronic phase could reduce the size of the stem cell compartment and thereby reduce the chance of transformation (4).

Although in vitro purging methods failed to eradicate Ph^1-positive cells in CGL bone marrow harvests or buffy coats, a transient disappearance of Ph^1-positive metaphases (partial or complete) was commonly reported in patients given high-dose chemotherapy during the chronic phase (9). This suggested that a marrow harvest could be performed during this Ph^1-negative period and cryopreserved marrow could be used for autologous transplantation.

We have up to now performed transplantations in four patients during the chronic phase of CGL with marrow purged in vivo by high-dose melphalan for which antileukemic efficacy has been demonstrated in CGL (10,11).

PATIENTS AND METHODS

Four patients (median age, 25 years; range, 12-38 years) with Ph^1-positive CGL received high-dose melphalan (HDM, 140 mg/m^2) after a 9- to 84-month period in stable chronic phase. This was followed by prolonged cytopenia with fewer than 0.5×10^9 granulocytes/l lasting 16-50 days (median, 31.5 days). Marrow collection was performed 28-62 days (median, 57 days) after HDM, as soon as the WBC and platelet counts reached 2×10^9/l and 100×10^9/l, respectively. Harvested marrow cells were cryopreserved using dimethyl sulfoxide, then stored in liquid nitrogen. A few weeks later, patients underwent ABMT after a standard conditioning regimen with cyclophosphamide (120 mg/kg) and total body irradiation (10-12 Gy). A median of 1.55×10^8 (range, 0.8-3.8 cells) nucleated cells/kg was transfused containing a median of 2.1×10^4 (range, 0.75-3.8 cells) colony-forming units of granulocyte-macrophage (CFU-GM)/kg.

RESULTS

In two patients the cells failed to engraft. Both had received transplanted marrow containing 100% Ph^1-positive cells. Of these two patients, one returned to a stable chronic phase after a second transplantation of peripheral blood stem cells collected during the original evaluation. He is still alive 8 months after initial ABMT. The other patient had a second autologous transplantation of

marrow cells collected 4 months after HDM treatment, but these also failed to engraft. However, she is still alive despite pancytopenia 9 months after the initial ABMT. No marrow metaphases were obtained after transplantation.

Two patients who underwent autografts with marrow containing a mixture of Ph^1-positive and Ph^1-negative cells experienced prompt engraftment. One patient (patient 1, Table 1) had a minority of Ph^1-negative metaphases (20/53) in transplanted marrow. She had a normal WBC count 4 months after ABMT. Cytogenetic studies revealed 50% and 40% Ph^1-negative marrow metaphases 2 and 3 months after transplantation. The karyotype showed 96% Ph^1-positive marrow cells when a typical hyperleukocytosis was detected 4 months after transplantation. This patient is still in stable chronic phase 7 months after ABMT and 46 months after diagnosis. Ph^1-negative marrow metaphases have disappeared.

For the other patient (patient 2, Table 1), marrow with a majority of Ph^1-negative cells (33/44) was transplanted. The WBC count is still normal 18 months after ABMT. More than 500 marrow metaphases were examined during the first 6 months following transplantation, and very few Ph^1-positive cells were found. But afterwards, the percentage of Ph^1-positive marrow cells increased slowly, attaining 64% 1 year after transplantation.

DISCUSSION

High-dose melphalan was used to treat some patients with Ph^1-positive CGL during the chronic phase to reduce the Ph^1-positive contingent. No effect was observed in two patients in whom 100% Ph^1-positive marrow cells persisted and in whom ABMT subsequently failed to engraft. In one patient, the number of thawed CFU-GM cells (0.75×10^4 CFU-GM cells/kg) infused, was low. This number was not abnormal for the other patient, who received 3.8×10^4 CFU-GM/kg. Such graft failures have been reported in CGL after ABMT but not after transplantation of blood-derived stem cells (7), and the failure might be explained by the particular fragility of CGL marrow progenitors in the freezing process (12).

After HDM treatment, the two other patients achieved partial remission defined by the disappearance of a substantial proportion of Ph^1-positive marrow

Table 1. Number and Proportion of Ph^1-Negative to Ph^1-Positive Marrow Metaphases After Autologous Bone Marrow Transplantation

Months After ABMT	1–2	3	4	5–6	7–9	9–12
Patient 1	5/10 (50%)	12/30 (40%)	1/24 (4%)	—	0/31	—
Patient 2	—	190/191 (99.5%)	253/257 (98.5%)	105/106 (99%)	40/50 (80%)	36/100 (36%)

Abbreviation: ABMT, autologous bone marrow transplantation.

metaphases. After they received transplants of in vivo purged marrow containing, respectively, 1 and 2.9 x 10^4 thawed CFU-GM cells/kg, engraftment was very prompt in both cases. After transplantation, one of the patients had a mixture of Ph^1-positive and Ph^1-negative cells for 4 months until the typical features of the chronic phase returned. After ABMT, the percentage of Ph^1-negative marrow cells found in this patient never exceeded substantially the percentage of Ph^1-negative cells transplanted. The other patient achieved "clinical" remission after transplantation and is still in continuous clinical remission 18 months after transplantation.

Such a Ph^1-negative status has previously been reported in patients with CGL in transformation undergoing autologous transplantation of marrow or blood cells (see review, 13). The latter case confirms the proliferative advantage of Ph^1-negative precursors transplanted to the patient, an advantage that may be the result of selective cryoinjury of Ph^1-positive progenitors (14). The results observed in our patient differ from those of other reports in two remarkable aspects: the number of Ph^1-positive cells present after transplantation was very low and the duration of clinical remission was very long, suggesting that the preclinical phase of CGL was restored. Moreover, autologous transplantation may have prolonged the chronic phase. This encouraging result was obtained in the one patient who received a transplant only 16 months after diagnosis of CGL (versus 18, 45, and 91 months for the other patients), so that ABMT, if used, should be considered early in the course of CGL (15).

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Autologous Bone Marrow Transplantation and Chronic Myelogenous Leukemia

P. Chervenick and H. Kantarjian, Chairmen

DR. A. ZANDER: What is the rationale of your conditioning regimen?

DR. F. BABAPULLE: I think, as you know, most chemotherapeutic regimens are not based on very good reason. We use large doses of busulfan and we decided that we should also incorporate another alkylating agent—some people use cyclophosphamide, we chose to use melphalan.

DR. ZANDER: You observed 4 out of 13 patients with delayed platelet engraftment. How does this compare with your previous study in blast crisis using buffy coat cells? Have you had similar problems with platelet recovery?

DR. BABAPULLE: We have had similar problems with platelet engraftment. And, it may actually be that our human pluripotent stem cell numbers in the cryopreserved peripheral blood buffy coat cells are not adequate.

DR. W. PETERS: When were the marrows harvested?

DR. BABAPULLE: They were not marrows, they were peripheral blood, cryopreserved buffy coat cells.

DR. PETERS: Would you comment on the degree of mucositis that you saw using busulfan and melphalan in combination?

DR. BABAPULLE: I think that the degree of mucositis was compounded by the fact that we used melphalan, which is very mucotoxic.

DR. K. DICKE: What was the conditioning regimen in the double transplantation?

DR. BABAPULLE: The first conditioning regimen was 10 out of the 11 patients, Cytoxan and total body irradiation, and for the second autografting, high-dose melphalan with 140 mg/m^2.

DR. C. GORIN: Just a short comment. I am not surprised that you had marrow engraftment failures. We had two failures. I just wanted to say that in our experience with 15 patients, we had about 40% engraftment failures, and I believe that these experiences have been shared by the Seattle group some years ago. So, for some reason, there is a major difference in marrow grafting and peripheral blood stem cell grafting in chronic myelogenous leukemia. In contrast, we have no graft failure in the 32 patients who have received transplants with peripheral blood stem cells.

DR. ZANDER: If there are no more questions, then we can move to the next session.

IIA. Hodgkin's Disease

Natural History of Relapsed Hodgkin's Disease and Results of the Bloomsbury Autograft Studies

J. Gribben, A. H. Goldstone, D. C. Linch, B. Vaughan-Hudson, and G. Vaughan-Hudson

The prognosis of patients with advanced Hodgkin's disease has improved dramatically with the development of combination chemotherapy. DeVita and colleagues (1) have shown that more than 50% of patients with advanced Hodgkin's disease are cured with chemotherapy alone. However, the fate of those patients who fail first-line therapy or who relapse has been more difficult to establish. Bonadonna and coworkers (2) developed ABVD (Adriamycin [doxorubicin], bleomycin, vinblastine, dacarbazine) to meet the needs of patients who were resistant to MOPP (mechlorethamine, Oncovin [vincristine], procarbazine, prednisone) therapy, and his group reported a 59% complete remission (CR) rate with an estimated 5-year survival of 38% and advocated the use of MOPP/ABVD alternating therapy in stage IV disease (3). However, attempts to confirm these results in other heavily pretreated patients have had limited success (4). It has previously been shown also that high-dose therapy with autologous bone marrow transplantation (ABMT) can produce a high response rate in advanced relapsed Hodgkin's disease, but most studies have been carried out in patients with very advanced disease (5,6).

Analysis of data from the European Bone Marrow Transplantation Group (EBMTG) has clearly shown in non-Hodgkin's lymphoma that patients treated early in the evolution of their disease do better than those who have become multiply resistant to chemotherapy and that those patients who undergo transplantation in CR as consolidated therapy have a clear survival advantage over those treated in relapse. This conclusion is in accord with that drawn in the early experience of allogeneic transplantation in acute leukemia (7). Clearly, such an approach is not suitable in Hodgkin's disease, and we have attempted to identify groups of patients considered to be at risk who might be considered eligible for ABMT.

PATIENTS AND METHODS

British National Lymphoma Investigation

Three hundred forty patients with advanced (stages III and IV) Hodgkin's disease were entered into the British National Lymphoma Investigation (BNLI) MOPP/LOPP (lomustine [CCNU], vincristine, procarbazine, prednisone) study, 1979-1984 (8). We have studied the fate of patients whose treatment failed, examining their survival from starting second-line therapy (Fig 1). There was no significant difference between patients whose primary treatment failed but who achieved partial response and those who relapsed. These patients had a 2-year survival rate of 75% and a 5-year survival rate of

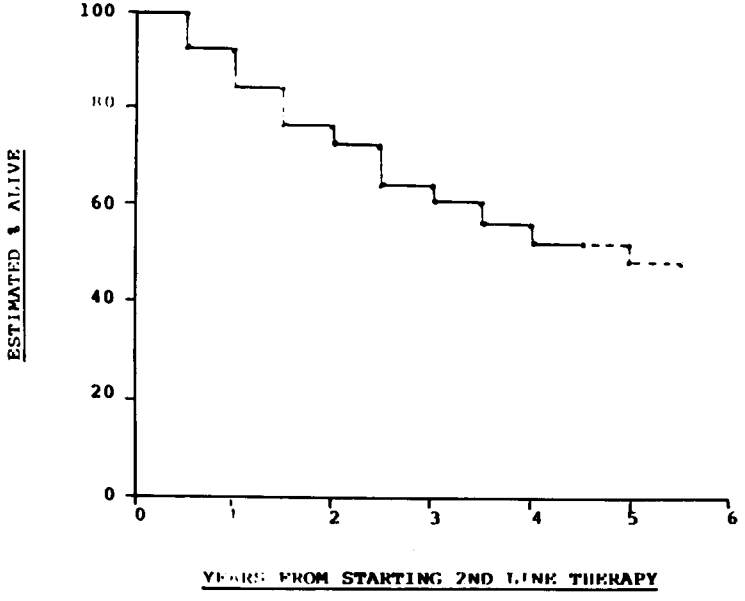

Figure 1. *Survival of patients less than 50 years of age whose first-line chemotherapy failed.*

50%. A small group of patients (8%) had no response to first-line therapy and had a 2-year survival of less than 25%. Most of these patients died within 6 months, however, and therefore would not have been eligible for ABMT. If one excludes from analysis those who died early in this group, then the 2-year survival rate was 50% and the 5-year rate less than 25%.

Various factors were coanalyzed to identify a poor-risk group of patients whose primary treatment failed. Those factors found to be significant were stage III and IV disease, the presence of B symptoms, an erythrocyte sedimentation rate greater than 39 mm in the first hour, and grade II histological findings. Patients having these characteristics represented about 30% of all whose treatment failed. These patients had a 2-year survival rate of 60% and a 5-year rate of 25% (Fig 2).

If one took all those whose first-line therapy failed and gave second-line therapy—either radiotherapy or a different chemotherapy—then 89/144 (62%) entered second CR. Of those who achieved a second CR, the survival rate from commencing second-line chemotherapy was 90% at 2 years and 70% at 5 years, with no difference between those receiving radiotherapy or a second chemotherapy (Fig 3). Those who failed to achieve a second CR had a 2-year survival rate of 60% and a 5-year rate of 25%.

The fate of patients whose alternating frontline chemotherapy failed is much less well known, though Santoro and coworkers reported on a small

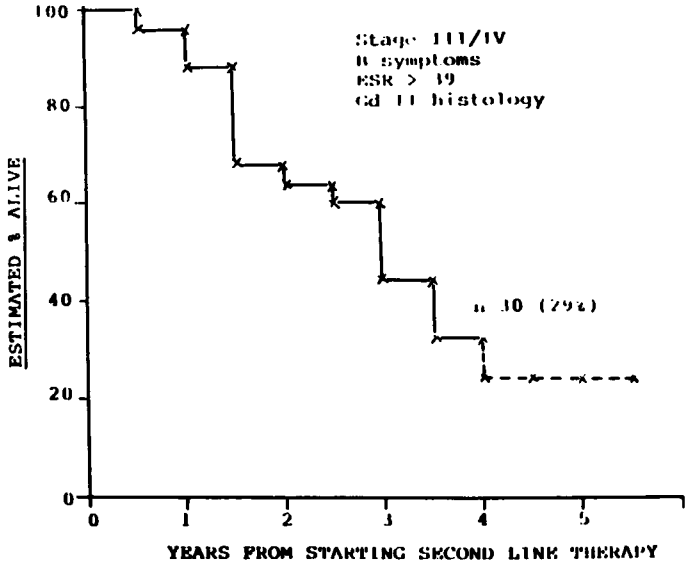

Figure 2. *Survival of patients with poor prognosis who were less than 50 years of age whose first-line therapy failed. Factors indicating a poor prognosis include stage III or IV disease, the presence of B symptoms, an erythrocyte sedimentation rate (ESR) greater than 39 mm in the first hour, and grade II histological findings.*

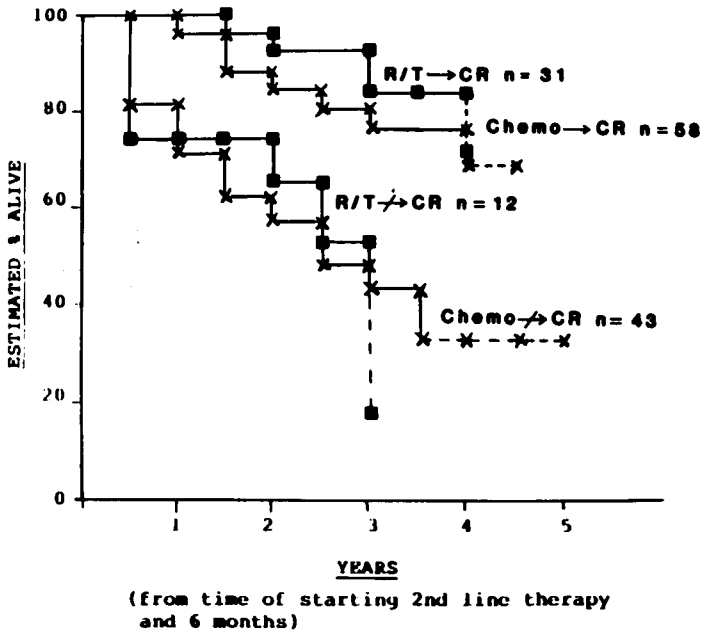

Figure 3. *Survival of patients less than 50 years of age who have failed first-line chemotherapy according to response to second-line therapy (R/T, radiotherapy; CR, complete remission; Chemo, chemotherapy).*

group of patients who relapsed on MOPP/ABVD (8). The BNLI LOPP/EVAP study has not yet sufficiently progressed to examine those patients whose treatment with this regimen failed, but these patients may be suitable also for salvage therapy by ABMT.

Middlesex Hospital Studies

Thirty-two patients with Hodgkin's disease have been treated with intensive myeloablative therapy (IMT) and ABMT at our center between August 1982 and December 1986. Twenty-nine (22 males and 7 females) were evaluable post-ABMT. The median age was 29 years, and 76% were between the ages of 15 and 30 years. Histological findings included nodular sclerosing (21), mixed cells (7), and lymphocyte predominance (1). No patients had bone marrow involvement as assessed by study of bilateral iliac crest trephine biopsy specimens. All patients were in relapse. Two patients (7%) had primary resistant disease showing relapse through first-line alternating chemotherapy (LOPP/EVAP [etoposide, vinblastine, Adriamycin, prednisone]). The remaining 27 patients had undergone at least two regimens of salvage therapy, including localized radiotherapy in 16 patients (55%). Nine patients (31%) were in responding relapse (i.e., were showing partial response to salvage chemotherapy at the time of ABMT). The remaining 18 patients

(62%) were showing no response to salvage chemotherapy at the time of ABMT and were classified as having resistant relapse. Autologous marrow was harvested and cryopreserved as previously described (9). No in vitro manipulation of bone marrow was carried out in an attempt to eliminate minimal residual disease.

The ablative regimen used is shown in Table 1. Eighteen patients received BCNU (carmustine), etoposide, ara-C (cytarabine), and melphalan (BEAM), six received University College Hospital (London) (UCH I), two received UCH I and II, one patient received cyclophosphamide and total body irradiation (TBI), and two patients received only TBI. All patients were assessed clinically and by repeated computed axial tomography (CAT) scans beginning at 3 months after ABMT. Four patients (13.8%) died during the aplastic phase, and sepsis was the cause of death in all four. Median time to recovery of a leukocyte count of 1×10^9/l was 18 days (range, 10-32 days), to a neutrophil count of 0.5×10^9/l was 22 days (range, 12-34 days), and to a platelet count of 50×10^9/l was 26 days (range, 13-43 days).

Eleven patients had CR after ABMT, five from the responding relapse group and six from the resistant relapse group. Four patients have subsequently relapsed from CR at 7, 8, 10, and 25 months after ABMT. Two patients died at 9 and 16 months. Two others are still in relapse at 16 and 46 months post-ABMT. One patient died at 8 months of acute cardiac failure.

Table 1. Ablative Regimen

Regimen	Day 1	2	3	4	5	6	7
UCH I							
Cyclophosphamide (1.5 g/m²)	x	x	x				
Carmustine (300 mg/m²)	x						
Cytarabine (100 mg/m²)	xx	xx	xx	xx			
ABMT						x	
UCH II							
Methotrexate (1.0 g/m²)	x						
Carmustine (300 mg/m²)	x						
Cytarabine (100 mg/m²)	xx	xx	xx	xx			
ABMT						x	
BEAM							
BCNU (carmustine) (300 mg/m²)	x						
Etoposide (50 mg/m²)	xx	xx	xx	xx			
ara-C (cytarabine) (100 mg/m²)	xx	xx	xx	xx			
Melphalan (140 mg/m²)						x	
ABMT							x

Abbreviations: UCH, University College Hospital; ABMT, autologous bone marrow transplantation.

Postmortem examination showed no evidence of disease recurrence at the time of death. Six patients are alive and disease-free at 7, 16, 17, 18, 28, and 39 months post-ABMT. The median follow-up of these 11 patients is 15 months. We show in Figure 4 the disease-free survival of this group.

Eleven patients had disease that only partially responded to IMT, four from the responding relapse group and seven from the resistant relapse group. Three patients have died of progressive disease at 5, 6, and 7 months post-ABMT. The remaining eight patients are alive at 3, 6 (3 patients), 7 (2 patients), 8, and 12 months post-ABMT. Four patients have shown further resolution of their disease on CAT scanning after initial follow-up at 3 months. One patient whose response we defined as partial at 3 months has no evidence of disease at 6 months posttransplantation with no further therapy given. One patient has a mediastinal mass that has remained unchanged for 9 months and may not have active disease. Two patients received localized radiotherapy to sites of residual disease and subsequently entered CR. The median follow-up of this group is 6 months.

The overall survival rate was significantly improved in those who achieved CR after ABMT compared with that of those who did not ($P < .001$) (Fig 5). Two patients were not evaluable after ABMT, and one patient has shown no response to IMT.

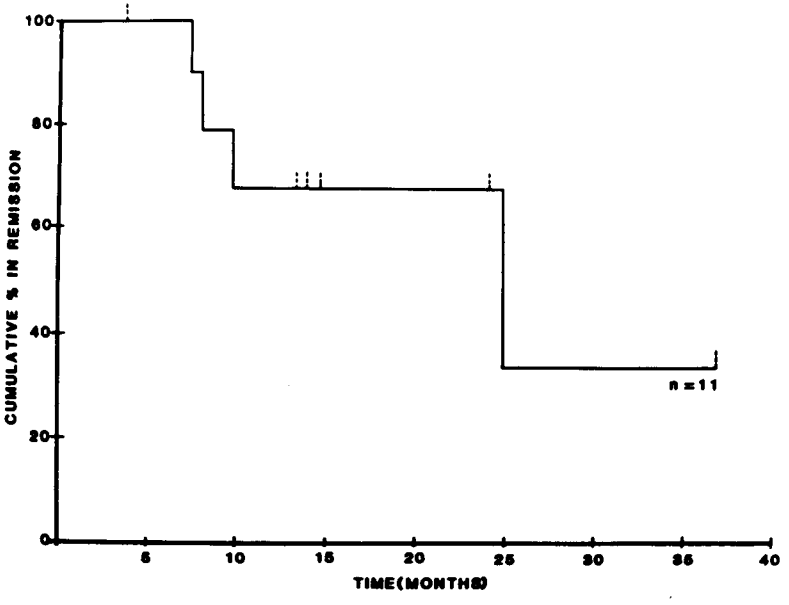

Figure 4. *Disease-free survival of 11 patients with Hodgkin's disease who underwent autologous bone marrow transplantation and achieved complete remission.*

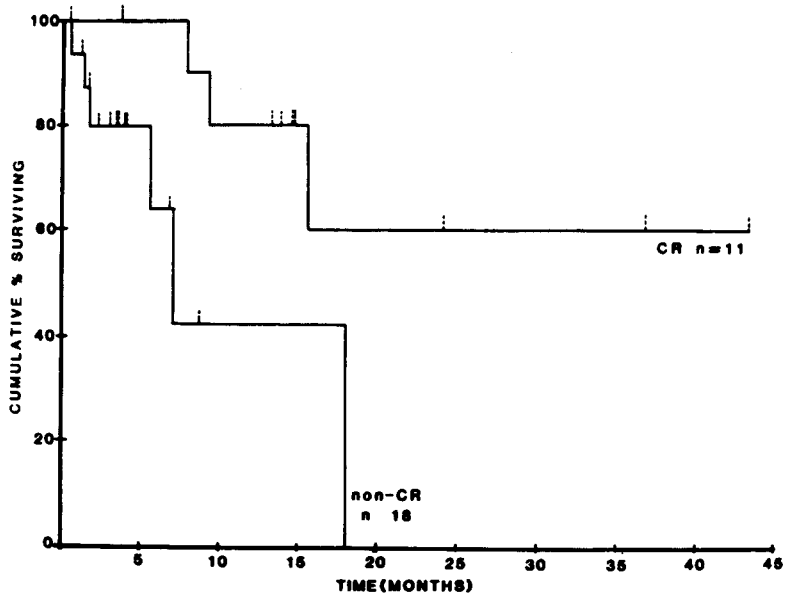

Figure 5. *Overall survival of those with Hodgkin's disease who achieved complete remission (CR) after autologous bone marrow transplantation compared with those who did not. Overall survival of the first group is significantly better (P <.001).*

CONCLUSION

Our own group of patients with very advanced Hodgkin's disease illustrates the potential difficulties of studies of high-dose therapy in these patients. Many remain alive for a long time with active Hodgkin's disease. In a study of 69 Hodgkin's patients reported to the EBMTG we attempted to indicate factors we found by multivariant analysis that influenced survival after ABMT. A patient's sex, age, histological findings, stage at presentation, site of disease at ABMT, or time from presentation to ABMT were found not to be significant (10). The only prognostic factor emerging as significant so far is whether the patient achieves CR or not after ABMT.

We have used a chemotherapy protocol rather than TBI in our recent studies for various reasons. First, TBI can be problematic with subsequent pneumonitis if the patient has received previous mantle radiotherapy (11). Second, there is no evidence from EBMTG data of any advantage of TBI over combination chemotherapy in non-Hodgkin's lymphoma (NHL) (9). Third, using chemotherapy as conditioning opens up the possibility of using double autografts in some patients.

Most of our patients were grafted when their disease status was too advanced to achieve a respectable result from ABMT. The BNLI data indicate that once failure of a first salvage regimen is established, the patient's

prognosis is already known to be poor. In the future it seems sensible to investigate ABMT, comparing it with conventional therapy, in Hodgkin's disease in the following groups of patients: 1) patients with poor prognosis (see above) whose first-line therapy fails; 2) patients whose alternating first-line chemotherapy fails; and 3) patients whose disease fails to respond to any two sequential modalities of therapy.

There may be an additional role for local boost radiotherapy, either before or after the graft (11), though analysis in NHL suggests that the bulk of the mass may be critical (T. Philip *et al.*, unpublished data). Finally, another parallel with NHL may be that patients who never enter remission (i.e., those who have primary refractory disease) may never do well with any therapy, including ABMT (12).

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Updated Results of CBV and Autologous Bone Marrow Transplantation for Hodgkin's Disease

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We developed a high-dose chemotherapy combination of cyclophosphamide, BCNU (carmustine), and VP-16-213 (etoposide) (CBV) with autologous bone marrow rescue and reported our experience in the treatment of leukemia, lymphoma, and solid tumors (1-3). The preliminary experience with this combination in patients with Hodgkin's disease failing both mechlorethamine, Oncovin (vincristine), procarbazine, and prednisone (MOPP)-like and Adriamycin (doxorubicin), bleomycin, vinblastine, and dacarbazine (ABVD)-like regimens has been very encouraging (4,5). We report here the updated results on 62 patients undergoing treatment at both the University of Nebraska Medical School in Omaha and The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston.

PATIENTS AND METHODS

Sixty-two patients with relapsed Hodgkin's disease were treated with high-dose chemotherapy and autologous bone marrow transplantation (ABMT) at two referral institutions. Informed consent for participating in the study was obtained from each patient.

The median age was 28 years (range, 15-56 years). There were 36 males. The median time from diagnosis to transplantation was 31 months (range, 6-111 months). The performance status in Zubrod's scale was 0 (36 patients), 1 (18 patients), and 2 or 3 (8 patients). No patient had major organ failure at the time of entry in the study. The median number of previous chemotherapy regimens patients had failed was two (range, 1-5 regimens). All but three patients had received both a MOPP-like regimen and a doxorubicin-containing program. Twenty-nine patients had received an etoposide-containing combination chemotherapy.

Twenty-four patients had not achieved a complete remission (CR) from the initial chemotherapy with or without additional radiation therapy. Thirty-three patients were progressing on the last salvage therapy. Forty-one patients had previous radiation therapy.

The preparative regimen was CBV (4). The total dose of etoposide was 450 mg/m^2 in the first patient; the dose was escalated to 600 mg/m^2 in 21 patients, 750 mg/m^2 in 19 patients and, finally, 900 mg/m^2 in 21 patients. ABMT was performed on day 7. One patient with bone marrow disease had received marrow from a human leukocyte antigen matched and mixed lymphocyte culture nonreactive sibling. Further details on chemotherapy administration and treatment of Hodgkin's disease have been previously reported (4,5).

RESULTS

The overall survival for all 62 patients with relapsed Hodgkin's disease treated with CBV and ABMT is shown in Figure 1. The median survival is 26 months, with 33 patients alive at the time of this analysis.

Sixty patients had measurable disease at the time of high-dose chemotherapy, while two were intensified in third complete remission (CR3). Twenty-seven (45%) of the 60 patients went into CR, while 15 (25%) achieved a partial remission (PR). Six patients in PR received additional radiation therapy and have achieved CR; five of these remissions were durable at 12+, 12+, 13+, 26+, and 31+ months. Eight of the 27 patients in CR and one of the two patients intensified in CR3 have relapsed. One patient died of cytomegalovirus and *Pneumocystis carinii* pneumonia 5 months after bone marrow transplantation while still in remission, as shown by the autopsy. Figure 2 shows the disease-free survival for all 62 patients. Twenty-four patients are alive and free of disease with a median follow-up time of 19 months.

Patients who achieve a complete response have an 85% projected 2-year

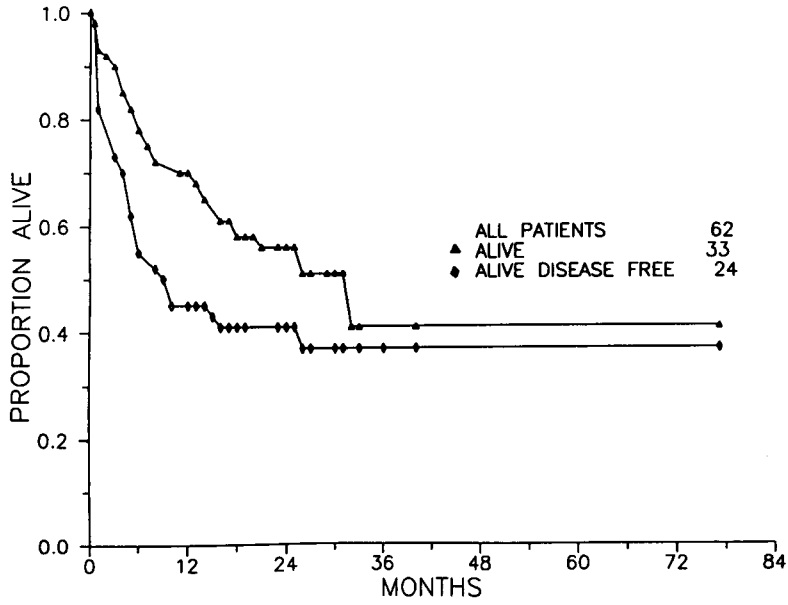

Figure 1. *Survival and disease-free survival for all patients receiving CBV (cyclophosphamide, BCNU [carmustine], VP-16-213 [etoposide]) plus autologous bone marrow transplantation in relapsed Hodgkin's disease.*

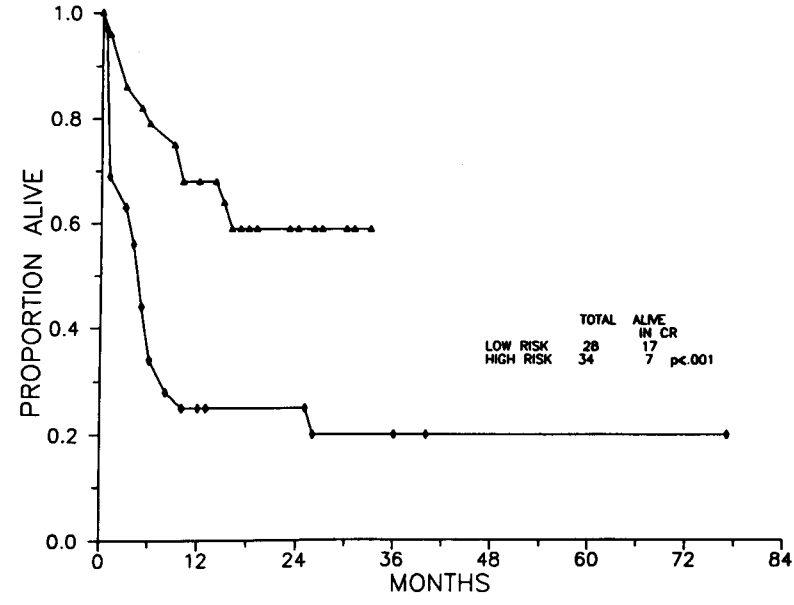

Figure 2. *Freedom from progression by risk groups receiving CBV (cyclophosphamide, BCNU [carmustine], VP-16-213 [etoposide]) plus autologous bone marrow transplantation for Hodgkin's disease.*

survival rate, while patients achieving a partial response have a median survival of 16 months, and unresponsive patients, a median of 3 months.

Toxicity

Four patients who died of toxic reactions received a dose of etoposide (900 mg/m^2). Other toxic reactions encountered were as follows: Nausea and vomiting were seen in 83% of the patients following administration of carmustine on the first day but were usually well controlled with antiemetics during the subsequent days of chemotherapy administration. Hematuria requiring blood product support occurred in less than 20% of the patients. Almost all patients had febrile episodes during neutropenia that required intravenous antibiotic therapy. Ten patients had radiographic evidence of pneumonia and 16 patients had bacteremia during the neutropenic period. Three patients had diffuse gallium uptake by the lungs noted at 1 month posttransplantation, which resolved within a couple of months. Clinical evidence of pulmonary fibrosis was not seen in any patient. One patient had congestive heart failure requiring diuretics, digoxin, and after-load reducing agents.

High-dose CBV chemotherapy produced significant neutropenia and thrombocytopenia. One engraftment failed. This patient had autologous marrow collected before her marrow relapse at the time of transplantation.

DISCUSSION

High-dose CBV and ABMT induced CR in 45% of the patients who had persistent or relapsed Hodgkin's disease after receiving a MOPP-like regimen and a doxorubicin-based program; 75% of these CRs are durable from 1 to 5+ years. These results confirm the preliminary data of the 30 patients reported previously (4,5).

Hodgkin's disease is a highly responsive tumor to both chemotherapy and radiation therapy. Patients who relapse after both MOPP- and ABVD-like regimens still respond to third-line salvage chemotherapy, like CAD (CCNU [lomustine], melphalan, desacetylvinblastine amide [vindesine]) (6), CEP (lomustine, etoposide, prednimustine) (7), or MIME (mitoguazone, ifosfamide, methotrexate, etoposide) (8); however, the remissions are not durable.

Several investigators have tried high-dose chemotherapy and ABMT (9-18). With the dose escalation, an increase in the CR rate and durability of remissions has been noted. Most patients with relapsed Hodgkin's disease have had radiation therapy and drugs with known pulmonary toxicity, such as bleomycin, before high-dose therapy. Therefore, programs based on total body irradiation have had higher incidences of treatment-related deaths (12,14,18).

In conclusion, high-dose CBV and ABMT should be considered an

important treatment modality for patients with MOPP-resistant or relapsed Hodgkin's disease, failing both MOPP- and ABVD-like regimens.

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High-Dose Chemotherapy and Autologous Bone Marrow Transplantation in Resistant Advanced Hodgkin's Disease

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A high proportion of patients with untreated advanced Hodgkin's disease may be cured with MOPP (mechlorethamine, Oncovin [vincristine], procarbazine, prednisone) therapy alone (1) or MOPP combined with other intermittent, non-cross-resistant oncolytic regimens, such as ABVD (Adriamycin [doxorubicin], bleomycin, vinblastine, dacarbazine) (2-5) or ABV (ABVD without dacarbazine), or CAD (CCNU [lomustine], melphalan, vindesine) (6). Despite these results, there are still patients whose disease is refractory to first-line chemotherapy or who relapse soon after responding. For such cases, the optimal management remains to be established, even if new second- and third-line salvage therapy result in a 20-25% 3-year disease-free survival (7,8). For patients whose disease is refractory to or who relapse soon after second- or third-line protocols, the prognosis is very poor (9). However, some encouraging results have been obtained with high-dose chemotherapy and autologous bone marrow transplantation (ABMT) (10), including ours with 28 patients. We report those results here.

MATERIALS

Patients

Between July 1981 and November 1986, 28 patients with advanced stage Hodgkin's disease received high-dose chemotherapy and ABMT. Criteria for entry to the protocol were refractory or relapsed Hodgkin's disease and adequate bone marrow function in the absence of marrow involvement.

The clinical characteristics of the patients are shown in Table 1. Median age was 27 years (range, 15-44 years), and 16 patients were males. At diagnosis, the most frequent histological subtype showed nodular sclerosis (18). Other patients had mixed cells (7), diffuse large cells (2), and predominant lymphocytes (1). All patients had advanced disease (1, stage III; 27, stage IV), and 22 patients had constitutional symptoms. Eight patients had a Karnofsky performance score of 60% or more, and 20 had scores of 50% or less. At the time of ABMT, 20 patients still on their salvage therapy program had progressive disease, and only eight patients had had a relapse off therapy. The most frequent sites of relapse were lungs or the mediastinum and lungs. First-line

Table 1. Patient Characteristics

Characteristics	No. of Patients
Hodgkin's disease sites	
Mediastinum and lung	20
Bone and lung	3
Liver and lung	3
Liver, lung, and bone	1
Previous treatment	
MOPP, CcVPP, + RT	1
MOPP, ABVD, + RT	15
MOPP, ABVD, RT, CEP, + other drugs	12
Best response to previous therapy	
CR duration ≥12 months	8
CR duration <12 months	6
Never CR	14
Status at high-dose chemotherapy and ABMT	
Refractory to standard therapy	14
Resistant relapse[a] after salvage therapy	10
Responding relapse[b] after salvage therapy	4

Abbreviations: MOPP, mechlorethamine, vincristine, procarbazine, prednisone; CcVPP, CCNU (lomustine), vinblastine, procarbazine, prednisone; RT, radiotherapy; ABVD, Adriamycin (doxorubicin), bleomycin, vinblastine, dacarbazine; CEP, CCNU (lomustine), etoposide, prednimustine; CR, complete remission; ABMT, autologous bone marrow transplantation.

[a] *Transplantation carried out in drug-resistant or progressive disease.*

[b] *Transplantation carried out following complete response to first salvage therapy.*

chemotherapy for 27 patients was alternating or sequential therapy with MOPP and ABVD and radiation (5); for the other patient it was MOPP and CcVPP (CCNU [lomustine], vinblastine, procarbazine, prednisone) and radiotherapy. Fourteen patients who had MOPP and ABVD therapy received ABMT upon relapse; 11 patients received second-line chemotherapy based on CCNU (lomustine), etoposide, and prednimustine (CEP) (11,12) or CCNU (lomustine), melphalan, and vindesine (CAD) (13) (one case). Fourteen patients never achieved a complete remission (CR) with first-line therapy, and six additional patients had a short-lived response (<12 months).

High-Dose Therapy

In all patients a central venous catheter was inserted 12 hours before high-dose chemotherapy. The patients had single rooms, received oral nonadsorbable antibiotics and antimycotics, and were hydrated until day 5 after bone marrow reinfusion. Urine output was maintained at 150 ml/hour. The high-dose chemotherapy consisted of cyclophosphamide (6 g/m^2 on 2 days), etoposide (600 mg/m^2 on 2 days), and BCNU (carmustine) (600 mg/m^2 on 2 days), all given intravenously. In the last six cases, the total doses of etoposide and carmustine were escalated to 1,000 mg/m^2 and 800 mg/m^2, respectively, in 4 days, and ABMT was done on day 7, after a 2-day rest (Table 2).

The median time from bone marrow collection to transplantation was 3 days (range, 2-6 days). The median number of nucleated cells reinfused was 1.6 x 10^8 cells/kg body weight (range, 0.5-3.4 x 10^8/kg). No patient's bone marrow was purged.

Table 2. The High-Dose Regimen With Autologous Bone Marrow Transplantation

Drug and Dose	Administration	Day						
		1	2	3	4	5	6	7
Mesna		x	x	x	x			
Cyclophosphamide (1.5 g/m^2)	Five-minute push i.v.	x	x	x	x			
Etoposide (250 mg/m^2)	Ninety-minute i.v. diluted in 500-ml dextrose	x	x	x	x			
Carmustine (200 mg/m^2)	Five-minute push i.v. (half dose); half dose i.v. diluted in 500-ml dextrose	x	x	x	x			
Autologous bone marrow transplantation	Forty-eight hours after the last chemotherapy administered							x

Post-ABMT Evaluation

Patients were evaluated at day 30 and monthly thereafter. A CR was defined as the disappearance of clinical and radiological evidence of Hodgkin's disease. Partial remission was defined as a reduction in measurable disease of 50% or more for at least 1 month. Patients who died less than 30 days after ABMT were not considered evaluable for tumor response unless there was evidence of progressive disease or evidence at autopsy of remission. No patient in CR underwent maintenance treatment, but while in partial remission, patients always underwent radiotherapy. Duration of CR was measured from the maximal response time until the relapse. Survival was calculated from the beginning of high-dose chemotherapy.

RESULTS

Three patients died within 15 days of ABMT. In two cases the autopsy showed persistence of Hodgkin's disease; in the last case, the bone marrow failed to reconstitute. Disease in seven patients failed to respond, and they died with progressive disease within a few months.

Twenty patients (71.4%) achieved a response and 15 (53.6%) a CR. One patient died of toxicity, but CR was confirmed at autopsy. Six out of 15 patients who achieved CR subsequently relapsed at 3, 4 (two patients), 5, 7, and 28 months. Nevertheless, eight patients who achieved CR are alive and off therapy, and five of them are long-term disease-free survivors (at 14, 26, 28, 41, 55, and 64 months). The overall results are shown in Table 3.

High-dose therapy produced significant neutropenia and thrombocytopenia in these heavily pretreated patients. All patients had a WBC count less than 0.5×10^9/l for a median of 16 days (range, 9-33 days) and a platelet count less than 10×10^9/l for a median of 18 days (range, 13-57 days). All patients experienced fever during neutropenia that warranted intravenous antibiotic and antimycotic therapy. Three patients had severe mucositis, and two other patients had generalized herpes simplex virus, which was well controlled with intravenous acyclovir.

Nausea, vomiting, and liver enzymes and alkaline phosphatase level elevations were observed in all patients. Two patients had a carmustine lung toxicity that resolved within 10 months. Cardiotoxicity, as assessed by clinical evaluation, echocardiography, and (in four cases) calculation of preejection period/left ventricular ejection time, was not observed.

DISCUSSION

Such remarkable progress has been made in the last few years in treating Hodgkin's disease that the increasing frequency of "cured" patients has stimulated a significant number of studies on long-term iatrogenic morbidity. Despite these changes, the optimal management of Hodgkin's disease proving

Table 3. Results by Clinical Characteristics

Characteristic	Patients (n = 28)	Response to High-dose Chemotherapy CR (%)	PR (%)	NR (%)
Overall	28	15 (53.9)	5 (17.8)	8 (28.5)
High-dose chemotherapy				
Carmustine	2	2 (100)	—	—
CVB	26	13 (50)	5 (19.2)	8 (30.8)
Histological findings				
LP	1	1 (100)	—	—
NS	18	9 (50)	5 (27.7)	4 (22.3)
MC	7	4 (57)	—	3 (42.9)
LD	2	1 (50)	—	1 (50)
Karnofsky performance status				
≥60%	8	7 (87.5)	1 (12.2)	—
≤50%	20	8 (40)	4 (20)	8 (40)
Sites				
Mediastinum and lung	20	12 (60)	3 (15)	5 (25)
Liver and lung	3	—	1 (33.3)	2 (66.6)
Lung and bone	3	1 (33.3)	1 (33.3)	1 (33.3)
Lung, bone, and liver	1	1 (100)	—	—
Best response to previous therapy				
First CR duration >12 months	8	6 (75)	1 (12.5)	1 (12.5)
First CR duration ≤12 months	6	2 (33.3)	3 (50)	1 (16.6)
Never CR	14	7 (50)	1 (7.9)	6 (42.1)
Response to salvage therapy				
Resistant relapse	10	3 (33.3)	1 (6.7)	6 (60)
Responding relapse	4	3 (75)	1 (25)	—

Abbreviations: CR, complete remission; PR, partial remission; NR, no remission; CVB, cyclophosphamide, VP-16-213 (etoposide), BCNU (carmustine); LP, lymphocyte predominance; NS nodular sclerosing; MC, mixed cellularity; LD, diffuse large cell.

resistant or causing the patient to relapse after primary intensive therapy remains to be established. At present, several salvage protocols have been tested, but the results are controversial and require caution in interpretation (14). The ABVD protocol has been used by the Milan group on 70 consecutive patients immediately after evidence of MOPP resistance. In the Milan studies CR was achieved in 54% and partial remission in 14%, for a global response of 68% (3,11). Similar findings were recently reported by Papa *et al.* (15) and Piga *et al.*

(16). Inferior results have been achieved by a recent multi-institutional German study that randomized MOPP-resistant Hodgkin's disease patients to receive either ABVD or a new four-drug regimen called DBVCy (daunorubicin, bleomycin, vincristine, cyclophosphamide). The CR rate for patients treated with ABVD was 11% compared with 24% for DBVCy therapy. The median duration of response to both combinations was less than 5 months (17).

Another second-line salvage protocol is the B-CAVe (bleomycin, CCNU [lomustine], Adriamycin [doxorubicin], vinblastine) tested at Stanford University in 52 MOPP-resistant patients (18). Twenty-one out of 52 patients achieved a CR, and 25% had 5 years' freedom from progression. For both ABVD and B-CAVe protocols, the incidences of CR and disease-free survival were related to absence of systemic symptoms and disease extent. Recently, Einhorn *et al.* (19) tested the new second-line protocol VABCD (vinblastine, Adriamycin [doxorubicin], bleomycin, CCNU [lomustine], dacarbazine) in 18 MOPP-refractory patients. Eight of 18 patients entered CR, and 5 of 8 responders maintained a CR from 5 to 36 months (19).

An unresolved matter is the role of third-line combination chemotherapy in the salvage of patients whose disease is refractory or who relapse soon after treatment with MOPP and ABVD protocols. They are CEP (12), CAD (13), and MIME (mitoguazone, ifosfamide, methotrexate, etoposide) (9). With these protocols, the CR rate varied from 13% to 40%, and absence of extranodal involvement was the only statistically significant factor predicting CR; in addition, the use of third-line regimens may result in a 10% disease-free survival 1 year later (9,11). There is, however, a large group of patients with malignant disease who do not achieve a remission or they relapse after a few months and then respond poorly even to third-line chemotherapy protocols such as CEP, CAD, or MIME.

The aim of our study, started more than 5 years ago, was to improve salvage therapy results in patients with highly treated advanced Hodgkin's disease by high-dose chemotherapy and ABMT rescue. From this pilot study, we have shown that it was possible to achieve a high response rate (71.4%) after high-dose chemotherapy with a very low proportion of early deaths in 28 patients who had resistant Hodgkin's disease with very poor prognosis. Similar results have been obtained by others on similar patients. A recent review reported that a total of 41 patients had been evaluated and that 21 (51%) had achieved CR (14). Ten patients relapsed early and three died in remission. Unfortunately, an assessment of disease-free survival in the nine patients still alive and well is limited because of the short observation period (<12 months). However, there are four patients that have survived in CR 24-48 months.

Spitzer *et al.* (20) reported the results of high-dose chemotherapy in 33 adult patients with Hodgkin's disease whose disease failed to respond after extensive prior chemotherapy and irradiation. Twenty-two patients (67%) had progressive disease at their last salvage chemotherapy at transplantation, and 48% had extranodal disease. Among the 22 patients who had previously

achieved CR, 13 had a maximum interval of disease-free survival of less than 12 months.

Jagannath and colleagues (21) have used high-dose carmustine in combination with cyclophosphamide and etoposide to treat 30 patients with refractory advanced Hodgkin's disease or Hodgkin's disease in relapse. More than half of the patients relapsed with disease at extranodal sites and had constitutional symptoms; most had been heavily pretreated with multiple salvage chemotherapy regimens and radiotherapy. High-dose chemotherapy with cyclophosphamide, etoposide, and carmustine induced CR in 15 patients and partial responses in 10 patients. Eleven patients are still in CR, one of whom has had an unmaintained remission for more than 44 months.

Recently, Goldstone (22) described the European Bone Marrow Transplantation Group's experience with ABMT in Hodgkin's disease: 63% of 60 evaluable patients achieved CR and 29% a partial remission for a total response of 92%. The conclusions were that, despite the advanced nature of the disease of most patients, the results are excellent in terms of CR rate; however, the relapses remain a major problem.

All these results, as well as those concerning small groups of patients (24-33), are similar to ours and prove the validity of such procedures in inducing CR, but the high rate of relapses suggests that it may be useful to treat these patients earlier in first relapse or in second CR after standard therapy or in first CR in very high-risk patients with Hodgkin's disease. We are working on optimizing the protocol of ABMT in first relapse after the MOPP and ABVD protocol. Of course, the question of whether high-dose chemotherapy and ABMT will eventually supersede conventional new salvage chemotherapy may be answered only after controlled clinical studies.

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Treating Advanced Hodgkin's Disease With Intensive Chemoradiotherapy and Autologous Bone Marrow Transplantation

S. N. Wolff, G. L. Phillips, J. W. Fay, C. F. LeMaistre, R. H. Herzig, and G. P. Herzig

Although substantial progress has been made in treating patients with advanced Hodgkin's disease with combination chemotherapy, the patient with recurrent disease remains a challenge. In the patient whose frontline chemotherapy has failed, therapeutic options include retreatment with the initial regimen (if the disease-free interval was greater than 1 year) or non-cross-resistant chemotherapy (if the disease-free interval was less than 1 year) (1,2). Although complete remissions can be achieved, few patients achieve durable remission, especially if their initial disease-free interval was short. For those patients whose second-line therapy fails, the long-term outlook is bleak.

One of the proven methods of improving the effectiveness of antineoplastic therapy is dose intensification (3,4). We have now treated 27 patients with advanced Hodgkin's disease whose former extensive chemotherapy failed. We report here that durable complete remission can be achieved in a moderate proportion of patients with Hodgkin's disease with the use of intensive chemoradiotherapy and autologous bone marrow transplantation.

MATERIALS AND METHODS

Twenty-seven patients were treated (median age, 31 years; range, 15-57 years). All had undergone previous chemotherapy, and seven (26%) had undergone prior standard radiation therapy. Prior chemotherapy was extensive, the median number of different chemotherapy agents used by each patient prior to intense therapy being eight; only two patients (7%) had received fewer than seven drugs. Only two patients could be classified as not being resistant to initial therapy (they had disease-free intervals greater than 1 year) or as not having salvage chemotherapy with a regimen non-cross-resistant to the initial combination chemotherapy that failed. The median number of sites of disease was two (range, one to seven), disease generally being limited to the mediastinum or peripheral lymph nodes.

Patients were treated on a succession of protocols. All patients received cyclophosphamide (60 mg/kg/day) intravenously for 2 days (days −5 and −4) followed by total body irradiation (TBI). Total body irradiation was initially given as 10 Gy for one fraction (day 1) or fractionated as 2 Gy twice a day for 3 days (days −3, −2, and −1) for a total dose of 12 Gy (FTBI). Involved-field radiotherapy (IFR), as 20 Gy given over 10-14 days before cyclophosphamide and TBI was administered, provided greater than 90% of the tumor could be safely encompassed in standard radiation ports. Patients who had received prior standard radiotherapy were generally not eligible for the IFR boost because excessive cumulative doses to normal tissues could be anticipated with the subsequent TBI. Nineteen patients (70%) received IFR, cyclophosphamide, and FTBI.

Marrow was collected, cryopreserved, and infused as previously described (5). Transplantation took place the day after the patient completed TBI (day 0).

Complete remission was defined as the disappearance of tumor according to all indexes and survival to day 30. Disease-free survival and total survival rates, calculated from day 0 and analyzed as of September 1, 1986, were estimated by the product limit method of Kaplan and Meier (6).

RESULTS

Nineteen patients (70%) had a complete remission. The other eight patients had a lower or no response to therapy. Six patients (22%) died as a result of therapy-induced toxicity (interstitial pneumonia, infection, or hemorrhage), four of whom were in complete remission. Four of the complete responders have relapsed, leaving 11 patients still in continued complete remission 3-47 months after transplantation. Of the four patients who relapsed, one is alive at 48 months in a second remission induced by local radiation therapy, two are alive at 8 and 28 months with progressive disease, and one has died at 42 months. All patients with less than a complete response to therapy have died of progressive disease or toxicity. The estimated probability of disease-free survival, with all deaths in remission counted as owed to tumor, is

23% at 38 months after bone marrow transplantation (Fig 1). The estimated probability of overall survival is 36% at 42 months. The subgroups of patients receiving or not receiving IFR have estimated probabilities of remaining disease-free of 29% and 0%, respectively (Fig 2).

Extramedullary toxicities in this patient population were not generally different from those experienced by other patients undergoing intense chemo-radiotherapy. The median (and range) of the duration of a neutrophil level below 500/μl and platelet level below 20,000/μl were 26 days (13-140) and 37 days (10-180), respectively.

DISCUSSION

Bone marrow transplantation allows the administration of cytotoxic chemotherapy beyond standard doses up to levels of severe extramedullary toxicity, resulting in survival rate improvement in patients with some hematopoietic neoplasms. The most commonly used transplantation regimen has been cyclophosphamide and TBI, though regimens not containing TBI have been reported (7,8). Hodgkin's disease is an attractive tumor model for such treatment because two very active standard treatments for Hodgkin's patients are the alkylating drugs (such as cyclophosphamide) and radiation therapy,

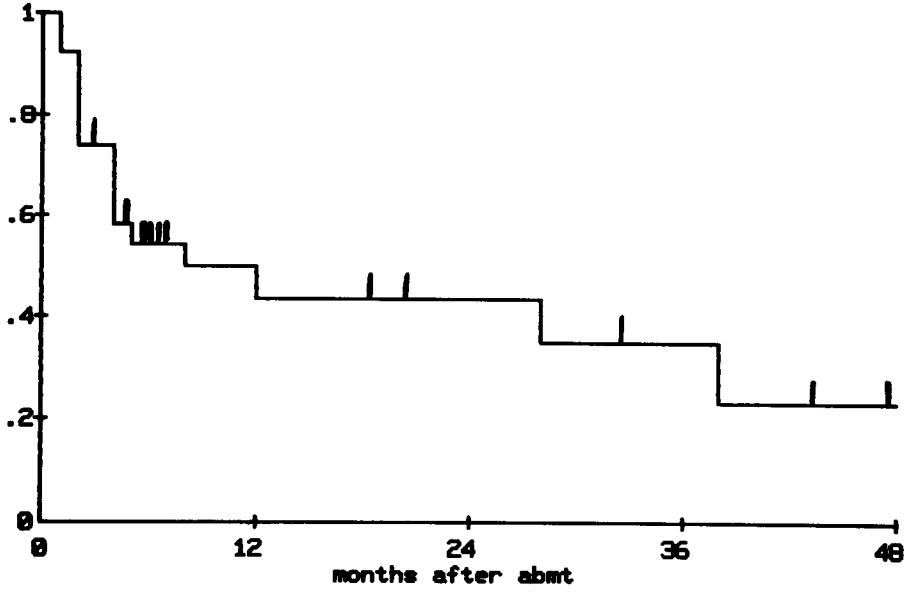

Figure 1. *The actuarial probability of disease-free survival for all patients treated with cyclophosphamide, total body irradiation, and autologous bone marrow transplantation with or without involved-field radiation therapy. Marks on line indicate patients still in remission. The probability of being disease free at 38 months after treatment is 23%.*

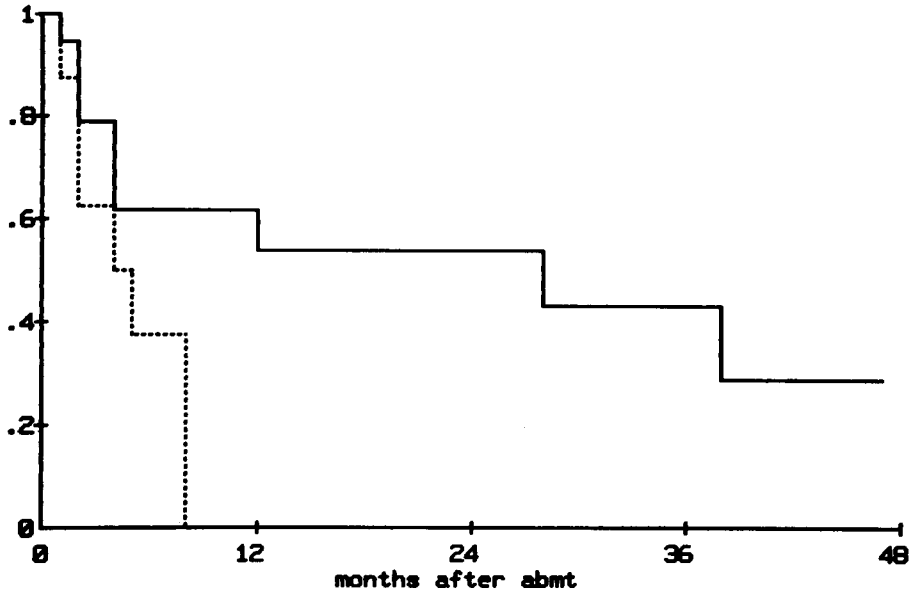

Figure 2. *The actuarial probability of disease-free survival for patient subgroups receiving or not receiving involved-field radiation before cyclophosphamide, total body irradiation, and autologous bone marrow transplantation. The solid line represents those patients who received involved-field radiation, and the dotted line represents those patients who did not receive involved-field radiation. The estimated actuarial probability of disease-free survival is 29% and 0%, respectively.*

both of which are part of a transplantation regimen that has proven successful. The 27 patients we treated with cyclophosphamide, TBI, and cryopreserved bone marrow transplantation were heavily pretreated with chemotherapy, and a few patients had had standard radiation therapy. All had had a limited prognostic outlook from standard salvage chemotherapy. Most patients had in fact undergone two regimens prior to consideration for transplantation. Although treated on a succession of protocols, involving single-fraction TBI, multiple-fraction TBI, and IFR, most patients (i.e., 70%) received a single-treatment regimen consisting of IFR followed by cyclophosphamide and FTBI.

At the latest analysis, the overall probability of survival and disease-free survival was 36% at 42 months and 23% at 38 months, respectively. Only analyzing patients who received IFR, the disease-free survival is estimated to be 29%. Although there are no long-term disease-free survivors in the patient group that did not receive IFR, the study design does not allow for meaningful comparisons.

One of the limiting factors concerning intense antineoplastic therapy is the prolonged duration of cytopenia and the occasional occurrence of fatal extramedullary toxicity. In our group of patients, the only toxicity that appeared to be increased compared with toxicity in other experiences was myelotoxicity

(9,10). Our patients endured 4-5 weeks of severe cytopenia. One of the factors possibly involved in this delayed hematopoietic recovery is the substantial chemotherapy patients received prior to bone marrow harvest. Inasmuch as TBI is considered to be the prominent marrow-ablative component of this transplantation regimen, less severe marrow suppression could be achieved with less myelotoxic therapy. A recent report describes a non-TBI-containing regimen that has promising results (11). Another possible benefit of a non-TBI-containing regimen is that it allows patients with prior extensive mantle or midline irradiation to undergo intense therapy (such prior therapy eliminates the possibility of future use of TBI). The advantage of TBI is, however, that radiation therapy is probably the most active antineoplastic agent for Hodgkin's disease.

In summary, Hodgkin's disease, considering this report and others, may be another neoplastic disease that can be successfully treated with intense chemoradiotherapy. Although the proportion of patients in our study having prolonged disease-free survival was only 23%, this outcome still represents improvement over results with other more conventional salvage treatments, especially considering that the patients were extensively pretreated and drug resistant. We encourage use of intensive therapy earlier in the course of Hodgkin's disease before the development of extensive drug resistance.

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Hodgkin's Disease

J. O. Armitage and S. Jagannath, Chairmen

DR. S. JAGANNATH: Are there any specific questions for Dr. Goldstone?

DR. K. DICKE: Tony (Dr. Goldstone), you mentioned in one of your slides the possibility of complete remission after failing frontline chemotherapy. And then in your last slide you mentioned that the indications for bone marrow transplantation are immediately after failure of the alternating ABVD-MOPP program. In my opinion, that is contradictory.

DR. A. "TONY" GOLDSTONE: The first slide you are alluding to showed the subgroup that goes easily into complete remission (CR) after failure from MOPP alone and those should be left alone because their ultimate prognosis is good. This is the difference between Hodgkin's disease and non-Hodgkin's lymphoma (NHL) in this regard because the Hodgkin's disease will still have a good prognosis at that stage and the NHL will not. I am not certain of the ultimate prognosis because I don't have the data of a large group of ABVD-MOPP induction patients; however, I suspect that the long-term survival of failures to alternating frontline chemotherapy on any sort of salvage regimen would be approximately 15-20% maximum. This first slide has to do with MOPP failures only.

DR. JAGANNATH: Any other questions for Dr. Goldstone?

DR. H. VRIESENDORP: Dr. Goldstone, have you looked at the influence of the involvement of bone marrow at initial presentation or at relapse on survival of these patients?

DR. GOLDSTONE: The answer for Hodgkin's disease is no. We have looked, in terms of autografting, at those patients with NHL who had bone marrow involvement at diagnosis versus those who did not. We were not able to do a very complex multivariant analysis. As far as NHL is concerned, if you have marrow involvement at diagnosis and then you don't at autologous bone marrow transplantation (ABMT), it really doesn't further affect your prognosis after autologous transplantation. But I don't have any data for Hodgkin's disease.

DR. VRIESENDORP: Just as a general question, is there any experience with ABMT in patients whose marrows are contaminated with Hodgkin's disease?

DR. GOLDSTONE: I don't know.

DR. J. ARMITAGE: We've treated several people with marrow involvement using peripheral blood stem cells and the peripheral blood stem cells successfully reconstituted the marrow. The patients we've treated have for the most part been with fairly advanced disease and the follow-up is not very long, therefore, it is not possible to make any comments about outcome.

DR. S. WOLFF: Our criteria for harvesting marrows was that the marrow at the time of harvest should be histologically normal. We have two long-term disease-free survivors who had a history of marrow involvement before some type of chemotherapy and had histologically normal marrow at the time of marrow harvest.

DR. A. ZANDER: Just one comment on bone marrow involvement and CBV with circulating stem cell rescue, which was done in one patient as the fifth salvage treatment. The tumor response was a partial remission but the important observation, I think, was a very fast hematopoietic recovery with a platelet count of more than 50,000 by day 14 posttransplantation and granulocyte count of more than 500 by day 10. The hematopoietic recovery is shortened by 10 days compared with Sundar's (Dr. Jagannath) data.

DR. F. HAGEMEISTER: I would like to go back to a comment made by Dr. Goldstone regarding the good prognosis of the patients who do not respond to MOPP. I would like to know what kind of regimen is used to salvage those patients because our experience at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, using an ABVD-like regimen that we call *ABDC* has CCNU instead of vinblastine, and has yielded very poor results with only about 25% of the patients being salvaged.

DR. GOLDSTONE: Yes, in that protocol, MOPP versus LOPP, when patients did not respond, it was open for investigators to use whatever chemotherapy protocol they wished. So there was a variety of protocols being used including ABVD and IMVP-16. The subgroup that went rapidly into second remission is only a very small percentage of the total group that failed to respond. The group that goes into second CR in two courses must be identified because they only represent 20% or less of those who failed to respond.

DR. VRIESENDORP: Dr. Jagannath, I was wondering, when you looked at your factors, which predicted the outcome of transplantation in Hodgkin's disease, whether you looked at the effect of bulky disease? The reason I ask is because we've looked at our patients with Hodgkin's disease and, like you, we haven't found that their initial response to chemotherapy predicted for response if you took into account bulky disease. We've done 10 patients now without bulky disease and of the 10, 7 are alive and disease free. Median follow-up was around 1 year. Our results of non-Hodgkin's disease are similar.

DR. JAGANNATH: Yes, tumor burden at the time of transplantation is one of the factors looked into. These patients who are treated with CBV and tumor burden are of importance. The tumor burden definition is a bit complicated. Any patient who had more than a 5-cm tumor bulk on any one side or a patient who had extensive abdominal disease (i.e., simultaneous involvement of para-aortic and pelvic disease or any involvement of mediastinum, even if not huge) was prone to have a poor prognosis by this treatment, which was very significant. Patients with mediastinal involvement, whatever size at the time of transplant, had prior radiation therapy to the mediastinum, therefore, patients who had mediastinum disease have a somewhat poorer prognosis.

DR. J. CAHN: Did you find any difference in terms of response rate in patients who had relapsed in previously irradiated fields?

DR. JAGANNATH: Involved-field relapses are not significant.

DR. WOLFF: Hodgkin's disease is a notoriously indolent disease in which patients can remain alive for prolonged periods of time with active illness, and I think that to critically evaluate the effect of a regimen, you have to look at event-free survival and not just the overall survival.

DR. HAGEMEISTER: Steve (Dr. Wolff), you can estimate that about 70% of the complete responders, which were about half the patients, stay well. So you can anticipate it's going to be about 30%.

DR. D. HEARD: I may have missed these comments earlier, but I just wanted to say something about peripheral blood stem cells in Hodgkin's disease. We have now done a couple of patients and clearly, the marrow involvement by Hodgkin's disease should not be a contraindication of going ahead and doing these patients. Repeated leukaphereses and storage of peripheral blood stem cells certainly lead to rapid engraftment following the CBV therapy. As a matter of fact, patients reconstituted with peripheral stem cells leave the hospital generally around 3½ weeks, when the average time of hospitalization for the marrow patients has been closer to 5 weeks.

DR. ARMITAGE: That also has been our experience, a much more rapid recovery.

DR. T. PHILIP: Dr. Carella, can you comment on the toxicity of CBV1 versus CBV2 and, specifically, about toxic deaths in both of your protocols?

DR. A. CARELLA: I have six patients who have received CBV2 treatment. The complete remission with the second protocol is very high because five out of six are in CR. The toxicity is identical between the two protocols. The six patients who received CBV2 had not received radiotherapy to the mediastinum and the lungs, and I have not observed an increase in lung fibrosis or cardiac toxicity after a higher dose of BCNU.

DR. DICKE: One question for Dr. Carella. What is the difference in response rate between CBV1 and CBV2?

DR. CARELLA: I have said before that I now have only six patients who received CBV2, and five out of six of these patients obtained a CR. The follow-up is limited, and I don't know the future.

DR. G. CORNWELL: Dr. Carella, could I just get a clarification on your conclusion? You had 45 patients of which about 25 went into CR. Is it correct that 15 of those 25 had a relapse?

DR. CARELLA: Yes. I want to comment that the Hodgkin's disease patients treated in our unit are probably the worst in the world because they always receive second-, third-, and fourth-line chemotherapies and also radiotherapy on the mediastinum and a second course of radiotherapy on the new localizations before ABMT.

DR. VRIESENDORP: I have a question for Dr. Wolff about his involved-field radiation therapy before transplantation. Can he give us a total dose, impression of the toxicity to the spinal cord, lung, or other dose-limiting tissues?

DR. WOLFF: Involved-field radiation was a total of 20 Gy given for about 10-14 days before transplantation. Initially, we worked out a dose, with the radiobiologist, in which you could give it to the midline without spinal cord complications. But, unfortunately, with mediastinal radiation, there really appeared to be a higher incidence of interstitial pneumonitis, so we now do not give mediastinal involved-field radiation—not because of cardiac complications but because of interstitial pneumonitis. That is the only restraint we have to the involved field right now.

DR. ARMITAGE: In conclusion, it is increasingly clear that Hodgkin's disease is one of the more responsive diseases to high-dose therapy and it sounds like a number of investigators are moving to try to use this modality earlier. But even patients with refractory disease are occasionally long-term disease-free survivors.

IIB. Non-Hodgkin's Lymphoma

Recent Results With Salvage Chemotherapy for Recurrent Lymphoma

Fernando Cabanillas, William S. Velasquez, Sundar Jagannath, Fredrick B. Hagemeister, Peter McLaughlin, and Forrest Swan, Jr.

For several years, we have used various drug combinations based on ifosfamide and etoposide to treat patients with recurrent lymphoma (1-3). In 1986 we reported the results of another salvage regimen based on cisplatin and high-dose cytarabine that has also given us encouraging results (4). In order to put into perspective the achievements of bone marrow transplantation for recurrent lymphoma, it is important to understand what can be obtained with conventional-dose chemotherapy regimens. In this chapter we will summarize the results of the two salvage chemotherapy regimens we have most recently used for lymphoma.

The synergism observed between cisplatin and high-dose cytarabine in the LoVo adenocarcinoma of the colon cell line recently led us to initiate a trial with the combination of Decadron (dexamethasone), high-dose cytarabine, and cisplatin that we have named "DHAP" (4). On days 1-4 we administered 40 mg of dexamethasone daily for 4 days. Cisplatin was administered day 1 (100 mg/m^2), and cytarabine (2 g/m^2) was administered on day 2 over 2 hours every 12 hours for two doses. Eighty-three patients with lymphoma that was recurrent,

refractory, or both were treated. Response rates (Table 1) were similar in the indolent and aggressive lymphomas.

Of several prognostic factors analyzed, three have shown a significant relationship either with overall response rate or complete remission rate (Table 2). The most striking correlation was observed in tumor burden, as defined by Jagannath *et al.* (5).

The response rates with this DHAP regimen and those with our previous MIME (mitoguazone, ifosfamide, methotrexate, etoposide) regimen in aggressive lymphomas were almost identical, but for the indolent lymphomas, the DHAP regimen has been able to produce a higher complete remission rate (Table 3). Whether these improved results in the indolent lymphomas are owed to the synergism between cisplatin and cytarabine or to the use of high-dose dexamethasone cannot be determined in this study.

Bone marrow transplantation regimens for lymphoma vary not only in the different chemotherapy and radiotherapy used but also in the patient selection

Table 1. Response of Recurrent Lymphoma to Salvage Chemotherapy With DHAP

Cell Type	No.	CR (%)	CR + PR (%)
Indolent	28	36	61
Aggressive	55	33	58

Abbreviations: DHAP, dexamethasone, cytarabine, and cisplatin; CR, complete response; PR, partial response.

Table 2. Response to DHAP According to Prognostic Factors

Pretreatment Variable	No.	CR (%)	*P*	CR + PR (%)	*P*
CR to frontline Rx	39	15 (38)		28 (72)	
			.12		.02
<CR to frontline Rx	44	13 (30)		22 (50)	
LDH level <225 U/l	19	11 (58)		13 (68)	
			.01		.15
LDH level >225 U/l	64	17 (27)		37 (58)	
Low tumor burden	58	27 (47)		41 (71)	
			<.001		.002
High tumor burden	25	1 (4)		9 (36)	

Abbreviations: DHAP, dexamethasone, cytarabine, and cisplatin; CR, complete response; PR, partial response; LDH, lactate dehydrogenase.

Table 3. Comparison of Response Rates of DHAP and MIME

	DHAP			MIME		
Cell Type	No.	CR (%)	CR + PR (%)	No.	CR (%)	CR + PR (%)
Aggressive	55	33	58	123	32	64
DLCL	41	29	56	94	33	62
Others	14	43	64	29	28	71
Indolent	28	36	61	85	12	54
Transformed	13	38	62	31	10	61
FSCC (NPDL)	8	50	88	27	11	63
Others	7	14	43	27	15	37
Total	83	34	60	208	24	60

Abbreviations: DHAP, dexamethasone, cytarabine, and cisplatin; MIME, mitoguazone, ifosfamide, methotrexate, and etoposide; CR, complete response; PR, partial response; DLCL, diffuse large cell lymphoma; FSCC (NPDL), follicular small cleaved cell lymphoma (nodular poorly differentiated lymphoma).

criteria utilized. Most of these trials have been carried out in patients under 60 years of age, who have no evidence of marrow involvement and are of good performance status. Some studies have also required the achievement of a complete response to frontline therapy. These selection criteria have not allowed an easy comparison with standard-dose chemotherapy regimens, which usually include all patients, irrespective of their pretreatment prognostic features. Using these criteria necessarily results in a much more favorable group of patients. In order to illustrate this, we have retrospectively identified from our recently completed trial with MIME 25 patients with diffuse large cell lymphoma who meet these criteria. The response rate for this selected group of patients is shown in Table 4. Undoubtedly, a very high complete remission rate

Table 4. MIME as Salvage Therapy for Non-Hodgkin's Lymphoma

Patient Characteristics	No.	CR (%)	PR (%)	No. in CCR at 2 Years
All	94	34	28	4
<60 years BM not involved	52	42	25	4
<60 years BM not involved; prior CR	25	72	16	2[a]

Abbreviations: MIME, mitoguazone, ifosfamide, methotrexate, etoposide; CR, complete response; PR, partial response; CCR, continued complete response; BM, bone marrow.

[a]*Two other patients are also alive with disease.*

can be achieved when all these criteria are applied. When the survival of these selected patients was examined, however, only 16% of that group were found to be alive at 4 years (Fig 1). Most of these remissions were not durable, and as a consequence, most of these patients have died of their disease.

When interpreting the results of bone marrow transplantation, it is important to keep in mind that a modest fraction can be cured with standard-dose chemotherapy. The long-term results of high-dose chemotherapy regimens with bone marrow transplantation for recurrent lymphoma have similarly shown in most cases modest long-term survival rates in large cell lymphoma. We now have to examine the potential of combining the best standard-dose chemotherapy regimen followed immediately by high-dose

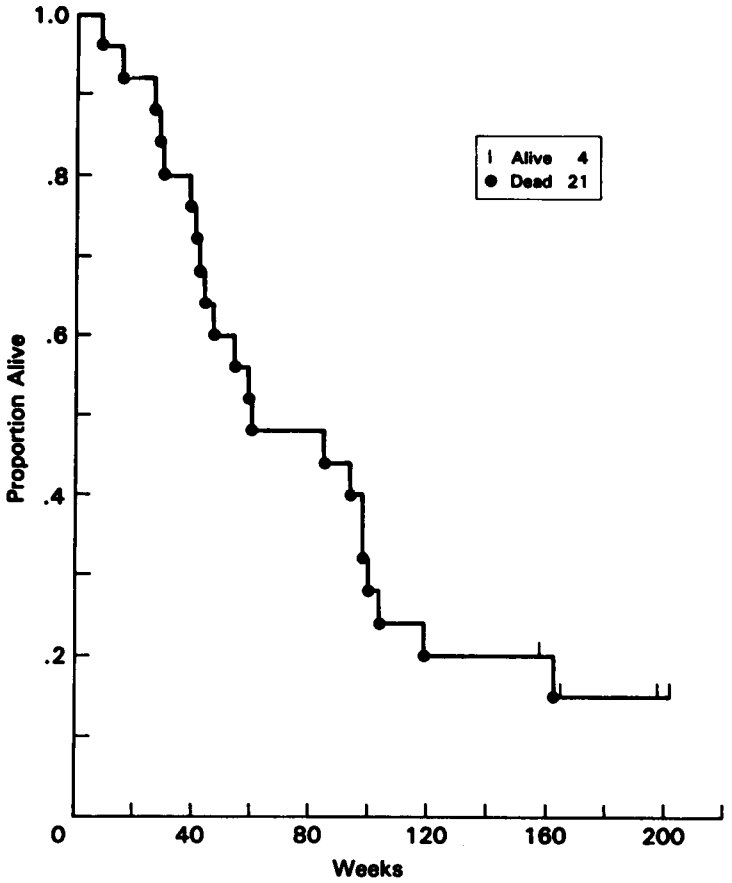

Figure 1. *Results of MIME (mitoguazone, ifosfamide, methotrexate, and etoposide) therapy in large cell lymphoma in relapse. Survival was related to being less than 60 years of age, having no marrow disease, and achieving complete remission previously. Four of 25 (16%) were alive at 4 years.*

chemotherapy plus radiation and autologous bone marrow transplantation. It is conceivable that by combining these approaches a larger fraction might be cured.

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Autologous Bone Marrow Transplantation in Burkitt's Lymphoma: 50 Cases in the Lyons Protocol

T. Philip, P. Biron, I. Philip, D. Frappaz, R. Pinkerton, G. Souillet, J. L. Bernard, J. P. Laporte, N. Philippe, M. DeTerlizzi, F. Demeocq, J. F. Dufilhaud, B. Kremens, F. Bonetti, and M. Favrot

Despite the acute sensitivity of Burkitt's lymphoma to both chemotherapy and radiotherapy, the outcome in advanced or disseminated disease was, until recently, very poor (1-3). In relapsed patients, long-term survival was almost unknown. Although predominantly a tumor of childhood (40-60% of patients with non-Hodgkin's lymphoma [NHL] are under 14 years of age) (3,4), a small number of adults will have disease that demonstrates the characteristic diffuse, small noncleaved cellular morphology and behaves in a similar clinical fashion (5). Encouraging results with allogeneic transplantation in high-risk and relapsed leukemias and the likelihood that Burkitt's lymphoma would demonstrate a dose-response effect with intensive chemotherapy made this tumor an obvious candidate for treatment with massive chemotherapy and autologous bone marrow transplantation (ABMT).

The first studies by Appelbaum *et al.* (6,7) showed clearly that a dose-response relationship existed and this was subsequently confirmed by a number of investigators studying a variety of chemotherapeutic regimens

(8-10). A remarkably high response rate was observed in patients who had relapsed after conventional regimens and were resistant to remission reinduction at standard doses (9). The long-term survival and probable cure of a small number of patients from the early series and the subsequent demonstration of cures in selected cases leave no doubt about the efficacy of this modality of treatment in Burkitt's lymphoma.

A number of questions remain unanswered, however, in relation to the indications for ABMT in Burkitt's lymphoma; namely, 1) What is the optimum ablative regimen and should it include total body irradiation (TBI)? 2) Which patients should be considered for ABMT, and in particular, should patients in first complete remission (CR) be considered? and 3) Is purging of the marrow necessary, and can it be done effectively?

For a variety of biologic reasons Burkitt's lymphoma has shown itself to be a particularly appropriate model for the study of these questions, most of which also apply to using ABMT to treat other tumors. In this review we consider these problems within the context of our group's experience of over 50 autografts performed in Burkitt's lymphoma over the past 5 years.

NATURE OF MASSIVE THERAPY

New chemotherapeutic agents, usually introduced into treatment after the completion of phase I and II studies, have demonstrated significant effectiveness in relapsed or resistant disease. New combinations of conventional agents, with the exception of those based on kinetic or in vitro synergism, are often constructed rather empirically on the assumption that the cumulative antitumor effect will outweigh any enhanced toxicity. Such an approach, using combinations of conventional agents in relatively high doses, has produced impressive early results in both acute myeloid leukemia (AML) (11) and acute lymphoblastic leukemia (ALL) (12). A similar approach has, in general, been taken in ABMT, though some of the agents used in high-dose combinations have been individually studied, such as etoposide (VP-16-213) (13), cyclophosphamide (14), carmustine (15), and melphalan (16). Such assessment is, however, often difficult in view of the small number of patients involved; moreover, clinicians are somewhat reluctant to subject patients to the prolonged hospitalization inevitable with ABMT unless the chances of long-term benefit are maximized. Thus, combination regimens, which seemed more likely to provide the possibility of durable response, have been used to test the efficacy of this new therapeutic approach.

The most commonly used regimens are shown in Table 1. The original Appelbaum BACT (BCNU [carmustine], ara-C [cytarabine], cyclophosphamide, and 6-thioguanine) was subsequently further intensified with a three-fold increase in the dose of carmustine (8,9). The BEAM regimen (BCNU [carmustine], etoposide, ara-C [cytarabine], and melphalan) incorporated two agents that in phase I and II studies were effective at high doses and were

Table 1. Massive Therapy Regimens, With and Without Total Body Irradiation, Used in Burkitt's Lymphoma

Chemotherapy Regimen	Investigators
Without total body irradiation	
BACT (Appelbaum)	Appelbaum *et al.* (7)
BACT (Institut Gustave-Roussy)	Philip *et al.* (9)
BEAM	Biron *et al.*[a]
Cyclophosphamide + carmustine	*Barbasch et al.* (17)
Cyclophosphamide	Philip (5)
Cyclophosphamide + doxorubicin + vinblastine + cytarabine + methotrexate	Ekert *et al.* (18)
With total body irradiation	
Cyclophosphamide + TBI	Phillips *et al.* (19)
BACT + TBI	Philip (5)
Cyclophosphamide + vinblastine + TBI	Gale *et al.* (20) Douer *et al.* (21)
Cyclophosphamide + vinblastine + doxorubicin + TBI	Baumgartner *et al.* (10)
Cyclophosphamide + doxorubicin + TBI	Kaizer *et al.* (22)

Abbreviations: BACT, carmustine, cytarabine, cyclophosphamide, 6-thioguanine; BEAM, carmustine, etoposide, cytarabine, melphalan; TBI, total body irradiation.

[a]*See Biron* et al., *"A Phase II Study of a New Cytoreductive Conditioning Regimen With Autologous Bone Marrow Transplantation for Lymphomas: The BEAM Protocol," in this volume.*

introduced in an attempt to reduce the toxicity of the BACT combination (see Biron *et al.*, "A Phase II Study of a New Cytoreductive Conditioning Regimen With Autologous Bone Marrow Transplantation for Lymphomas: The BEAM Protocol," in this volume).

The use of TBI remains somewhat controversial. Its place in conditioning regimens in leukemia is unquestioned by most, though not all (23), and some of the massive therapy regimens are undoubtedly not marrow ablative (9). However, in the case of Burkitt's lymphoma, TBI may not be necessary, and graft acceptance is not a problem with ABMT. There is little doubt that adding TBI enhances the toxicity of the procedure, being linked with interstitial pneumonitis and encephalopathies (24); moreover, in young children the long-term sequelae must be taken into consideration (25). The question of localized boost x-ray therapy either pre-or post-ABMT rarely arises in Burkitt's lymphoma, unlike it can in T-cell lymphoma in which a residual mediastinal mass may be a problem. In rare cases, however, atypical sites, such as bone, may be treated in this way. In the case of CNS involvement, it is probably mandatory to irradiate the cranium at least if TBI is not being used. Relying on

intrathecal therapy alone is, as in the case of CNS disease in ALL (26), probably inadequate.

In patients with initial CNS involvement, especially those in whom there is also marrow infiltration or B-cell ALL, the outcome of massive therapy with chemotherapy alone has been relatively disappointing. There is, therefore, a need to evaluate the addition of irradiation in such cases, possibly with a subsequent randomization against BEAM alone.

The use of double autologous grafts has been investigated (27), but because of the rapid cell turnover time of Burkitt's lymphoma, only a single graft is possible in most cases. However, for patients with resistant relapse, we are currently studying the use of BEAM followed after 7-10 days with cyclophosphamide, TBI, and reinfusion of marrow.

We review here our 6 years' experience with massive therapy and ABMT for Burkitt's lymphoma. When we initiated this program, overall survival in Lyons by conventional chemotherapy was 42%, and CNS relapse was the major problem (2,3). In addition, with regard to ABMT, a purging procedure was not ready for clinical use. Now overall survival in Lyons with a conventional regimen is 77% (28), and CNS relapse is a rare event in Burkitt's lymphoma (<5%) (29). A purging procedure is now available and was used for 29 of the 50 patients (30-32; see also Favrot *et al.*, "Comparative Efficiency of an Immunomagnetic Purging Procedure and a Rabbit Complement Lysis for Eliminating Burkitt's Lymphoma Cells From Bone Marrow," in this volume). The increase in survival rate after conventional chemotherapy was observed in parallel with the progress of massive therapy regimens, causing a problem in interpreting the value of the latter.

Despite these difficulties in analysis we think that firm conclusions can now be drawn from the initial experience and that massive therapy is still indicated in 20% of Burkitt's lymphoma cases. The objective of this report is to use our background to define clearly the present indications for such a procedure in Burkitt's lymphoma.

PATIENTS AND METHODS

Patients

A total of 52 courses in 50 patients are reviewed. Forty-one courses were performed in the Lyons-Saint-Etienne group (26 at Centre Leon Berard, 10 at Hopital Debrousse, and 5 at Saint-Etienne). Two patients are from Besancon, and the others from Nantes, Marseilles, Nancy, Bordeaux, and Paris. All patients were children, except five who were adults (and four of these were younger than 35 years old), and included in a pediatric protocol at initial presentation. Median age was 10 years (range, 3-49 years). Twenty-four were stage III, 21 stage IV (12 had disease in the CNS—some in the bone marrow as well—and 9 had disease only in the bone marrow). Five were initially stage I or II. All had received doxorubicin-containing regimens prior to ABMT. The

median interval between diagnosis and ABMT was 6 months (range, 1-11 months). At the time of ABMT, 24 patients (after 26 courses) were in relapse but still responding to rescue protocol ("sensitive relapse") (33,34). Nine patients were in resistant relapse or (six patients) had progressive disease (three patients). Three were in partial remission after first induction therapy, and fourteen were in first CR (six experienced a long delay before achieving CR, seven had initial CNS disease, and one had L_3 Burkitt's leukemia). All relapses occurred on therapy. The median time patients were observed after graft was 819 days (range, 35-2,169 days) (up to almost 6 years).

Methods

Parents or patients were always fully informed of the protocol risks, and they gave formal consent in the presence of at least two physicians. The protocol was reviewed and accepted by the Comite d'Ethique des Hospices Civils de Lyons et de l'Universite Claude Bernard. The cooperative group France Autogreffe de Moelle Osseuse also reviewed and accepted the protocol. Thirty-one bone marrow harvests were performed either after relapse (31) or during disease progression (6). Marrow harvesting and freezing procedures were performed as previously reported (9,34). In 2 of 52 courses no ABMT was performed after massive BACT therapy, as previously reported and discussed (9). Patients were always isolated in a sterile room according to standardized gut decontamination, bacteriologic, and transfusion policies (9).

Monoclonal antibody and complement were used to purge marrow collected from 29 patients. Indications for and the practical aspects of such procedures have been previously reported (30-32,35,36; see also the above-named chapter by Favrot *et al.* in this volume). At time of relapse patients had been treated with the best rescue protocol available. If disease was progressing or only stable on this rescue protocol, a patient's status was defined as resistant relapse. The status of all others was defined as sensitive relapse. At the time of CNS relapse, intermediate-dose cytarabine, high-dose methotrexate and etoposide, and methotrexate and cytarabine by lumbar puncture (twice weekly until the cerebrospinal fluid was free of tumor cells) were used to induce remission. All patients with CNS relapse underwent cranial radiotherapy (18-24 Gy) if they had not had it previously. No patient received maintenance therapy after ABMT.

The massive regimens used in this study are shown in Table 2. The Appelbaum BACT protocol was used in three courses, the Institut Gustave-Roussy modified BACT protocol (8) in 15, and the BEAM protocol (see the above-named chapter by Biron *et al.* in this volume) in 22. One patient received cyclophosphamide alone (60 mg/kg × 5). Details of the drug infusion protocol have been previously published (9,34,36).

Complete remission, partial remission (PR), nonresponse, and progressive disease were defined at day 30 post-ABMT according to World

Table 2. Massive Chemotherapy Regimens Used in This Study

Regimen		Days						
		1	2	3	4	5	6	7
BACT (Appelbaum) (3 courses)								
Carmustine	200 mg/m²	*						
Cytarabine	200 mg/m²		*	*	*	*		
Cyclophosphamide	50 mg/kg		*	*	*	*		
6-Thioguanine	200 mg/m²		*	*	*	*		
ABMT								*
BACT (Institut Gustave-Roussy) (15 courses)								
Carmustine	200 mg/m²	*	*	*				
Cytarabine	200 mg/m²		*	*	*	*		
Cyclophosphamide	50 mg/kg		*	*	*	*		
6-Thioguanine	200 mg/m²		*	*	*	*		
ABMT							*	
BEAM (22 courses)								
Carmustine	300 mg/m²	*						
Cytarabine	200 mg/m²		*	*	*	*		
Melphalan	140 mg/m²						*	
Etoposide	200 mg/m²		*	*	*	*		
ABMT								*
BEAC (5 courses)								
Carmustine	300 mg/m²	*						
Etoposide	200 mg/m²		*	*	*	*		
			*	*	*	*		
Cytarabine	200 mg/m²		*	*	*	*		
			*	*	*	*		
Cyclophosphamide	35 mg/kg		*	*	*	*		
Mesna	50 mg/kg		*	*	*	*		
ABMT								*

Note: One patient received high-dose cyclophosphamide only; three patients received busulfan and cyclophosphamide; and three patients received various other cyclophosphamide-containing regimens.

Abbreviation: ABMT, autologous bone marrow transplantation.

Health Organization recommendations (37). Complications were defined as early when observed before day 90 and as late when observed after day 90.

RESULTS

As shown in Figure 1 only nine patients who were in resistant relapse had progressive disease at time of massive therapy. We observed only two PRs, and all nine patients died before day 54 post-ABMT. In patients with sensitive relapse (33,34) results were good, as expected, with an overall survival rate for patients with no evidence of disease (NED) of 58% despite three therapy-related deaths in CR in this group (Fig 1). Median observation time for the survivors is 565 days post-ABMT. All disease-related deaths were observed before day 90. Only three patients were submitted to massive therapy when in PR after initial induction therapy. All three are alive with NED at 960+, 1,235+, and 1,955+ days post-ABMT. Fourteen patients were grafted in first CR either because of a long delay to CR or for consolidation in those with initial CNS involvement or L_3 leukemia. Of those, eight are alive with NED (57%), including five of six with a long delay to CR but only two of seven with initial CNS disease. The patient with L_3 leukemia relapsed on day 35 and died on day 73 post-ABMT.

Twenty-eight patients were grafted either because they had isolated CNS

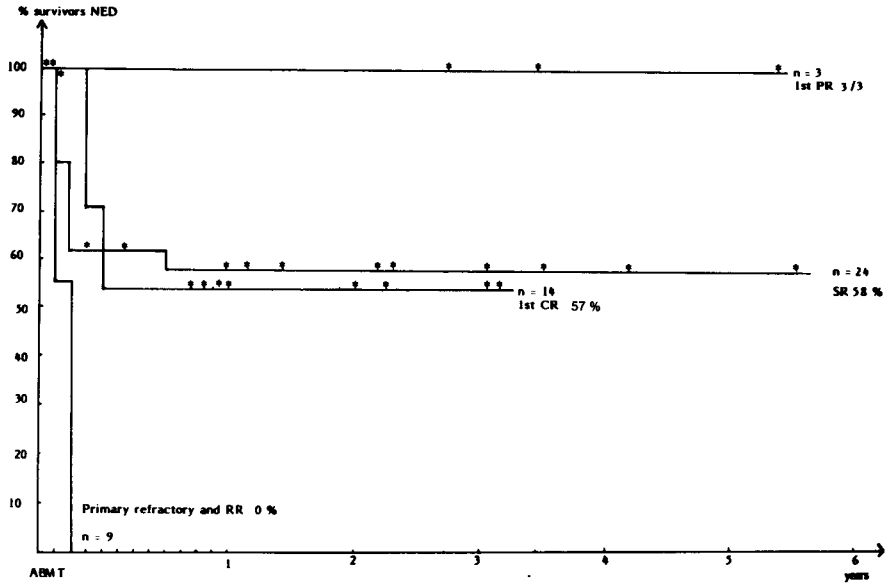

Figure 1. *Disease-free survival (1980-1986) of 50 patients with Burkitt's lymphoma according to status at autologous bone marrow transplantation (NED, no evidence of disease; PR, partial remission; SR, sensitive relapse; CR, complete remission; RR, resistant relapse).*

relapses (19 patients) or because after initial CNS involvement they achieved only PR or CR (9 patients). No clear difference is observed between the results for those experiencing CNS relapse and the results for those who had initial CNS involvement (47% compared with 55% NED survivors). Patients with initial CNS involvement do very poorly with conventional therapy (29). Five of nine are alive with NED 1,235+, 960+, 767+, 353+, and 270+ days post-ABMT. No major difference was observed between patients grafted with marrow harvested in first CR and the others.

During the 52 courses of intensive therapy morbidity was observed in 13 (25%). In 5 of 52 courses (10%), patients experienced pneumonitis (two cases of candida pneumonitis, one case of idiopathic pneumonitis, and two cases of cytomegalovirus pneumonitis). All patients recovered completely, including two patients who subsequently died of other complications. Cystitis was observed in two cases, despite mesna therapy (mesna prophylaxis was used in all cases), and was transient in both. Cardiomyopathy occurred in one case but regressed with digitalis and furosemide therapy. Leukoencephalopathy was observed in one patient, who is alive but with neurological sequelae. One case of hepatitis was also transient.

Six patients died of therapy-related complications in CR—one of myocardiopathy, one of acute pulmonary edema, two of candida sepsis, one of veno-occlusive disease, and one of an unexplained brain complication—producing a 12% mortality rate for these 52 courses of massive therapy in 50 patients.

As shown in Figure 2 the overall survival rate with NED for the 50 patients is 50%. The median observation time post-ABMT is 27 months. If patients who underwent transplantation in the first CR are excluded, the overall survival rate is 47% (17 of 36 patients). In such a group (24 relapsed on therapy, nine had progressive disease, and three had PRs), survival is very unlikely with a conventional chemotherapy regimen (3).

Comparison of patients receiving the purged marrow (n = 29) and those receiving unpurged marrow (n = 21) is difficult because of the predominance of initial CNS disease in the patients receiving the purged marrow. However, as shown in Figure 3, if the analysis is limited to the patients in sensitive relapse at time of ABMT, the overall survival rates with NED for the two groups are comparable (61.5% compared with 54.5%). Another way to look at this small group is to compare the four who relapsed in the group of 11 receiving unpurged marrow and the two who relapsed in the group of 13 patients grafted with purged marrow. All relapses were early relapses. The four relapses in group 1 were observed first in the marrow, whereas only one relapse in group 2 was clearly a marrow relapse. In the group of patients grafted in first CR of initial bone marrow involvement, four relapsed with disease in bone marrow despite purging, but marrow was shown in all cases to be normal either by a cytological examination or a cell liquid culture assay (35,38).

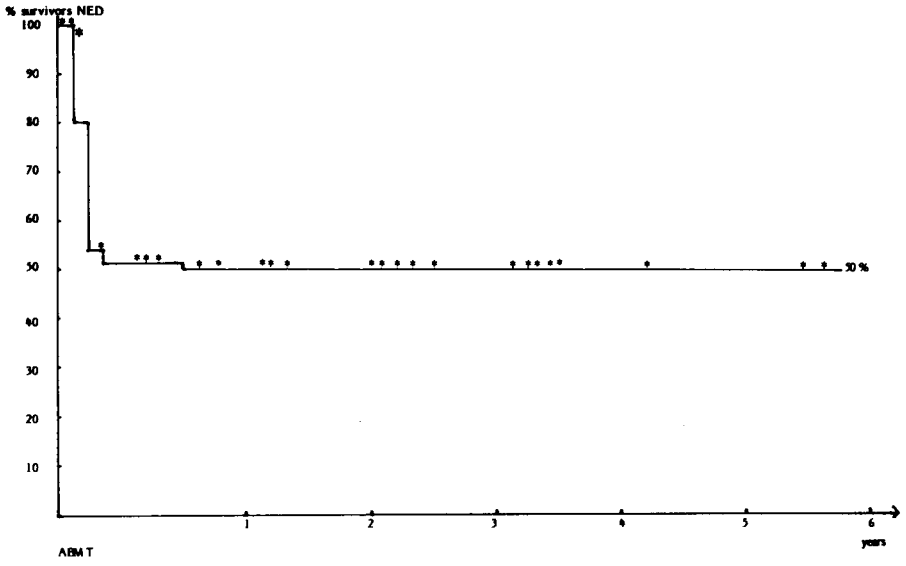

Figure 2. *Overall disease-free survival (1980–1986) (counting toxic death as a failure of therapy) of 50 patients with Burkitt's lymphoma (NED, no evidence of disease).*

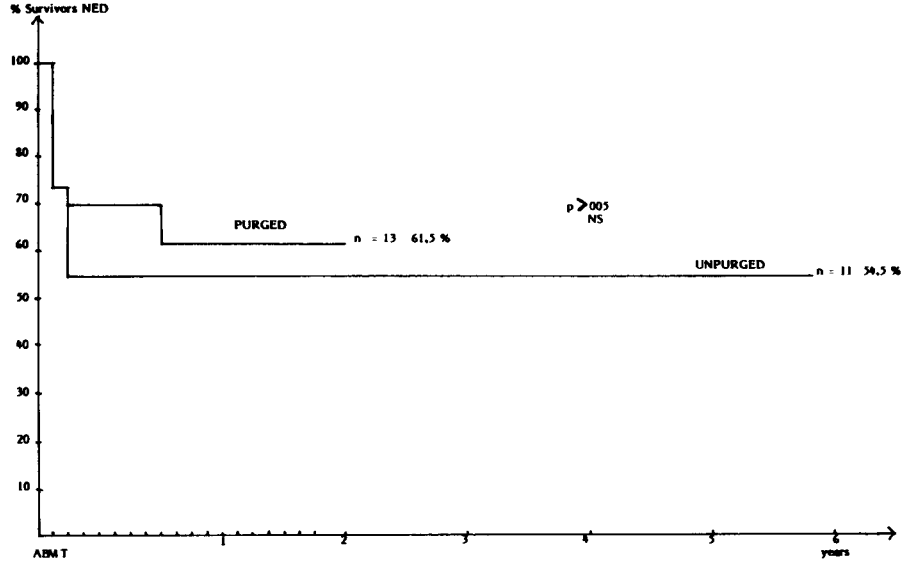

Figure 3. *A comparison of 24 patients with Burkitt's lymphoma who were in sensitive relapse and received purged or unpurged marrow (1980–1986).*

DISCUSSION

During this 6 years of using massive therapy and ABMT, we have been involved in the French SFOP protocol of conventional chemotherapy, which has increased the cure rate for Burkitt's lymphoma from 42% to 77% (2,3,5,29). At present, 100% of our patients with Burkitt's lymphoma are included at diagnosis in the conventional regimen, but 20% still remain candidates for ABMT. Based on an analysis of the progress achieved over recent years with massive therapy and the new conventional regimen, we think it is possible to draw several conclusions.

Patients with Burkitt's lymphoma who have progressive disease, relapses, or PR after initial induction therapy are still in a group of patients whose survival is very rare with conventional therapy (5,29). In the group of like patients we report here, 17 of 36 survive with NED (47%). This is clearly considerable progress for patients with Burkitt's lymphoma. The percentage of therapy-related deaths in this group (12%) is not very different from that associated with most modern conventional chemotherapy (29,39,40). It is also of interest to consider all patients treated in Lyons during this period who were potential candidates for ABMT. In our group six such patients out of 41 were not included because of early death after relapse (two), resistant relapse on progression under rescue protocol (one), bone marrow involvement at relapse (one), or progressive disease early after reinduction of remission (two). If we considered this total experience of Burkitt's lymphoma, the overall survival rate between 1980 and 1985 in Lyons for patients considered candidates for ABMT in Burkitt's lymphoma is then 40%. The selections for ABMT of 41 patients out of 47 with clear indications does not modify the conclusion. In our previous experience, such a group had no survivors (3). As previously shown, patients with Burkitt's lymphoma who stay in CR more than 8 months can be considered cured (3), and 70% of our survivors belong to this group.

The patients with a long delay to CR were clearly patients with very poor prognoses (2-4), but five of six such patients who were grafted are long-term survivors. However, with the new conventional regimen, this characteristic has disappeared and is not now an indication for ABMT in our group.

Results of treating patients with ABMT after a relapse in the CNS or cerebrospinal fluid are encouraging: 9 of 19 are long-term survivors. The question of whether patients experiencing these particular relapses can be cured by conventional regimens (as patients with ALL can be) must now be addressed.

Our very preliminary experience of massive therapy as consolidation for initial CNS involvement in first CR is quite encouraging. The numbers are small, but the survival of five of nine is better than the 25% survival produced with a conventional regimen (2).

The question of the possible adverse effects of massive therapy followed by ABMT must be considered. Two cases are of particular interest. Both had

initial CNS and abdominal Burkitt's lymphoma involvement, more than 80% marrow involvement, but a normal WBC count. Both reached a quick CR under the induction protocol. Each was given 20 Gy to the CNS before ABMT, and both received BACT (Institut Gustave-Roussy) or BEAM when in well-defined CR and received histologically normal marrow purged by monoclonal antibodies and complement. Central nervous system relapses occurred very early (day 30 and day 37) after a quick recovery following massive therapy that did not produce major complications. In one case the marrow was clearly normal at the time of relapse, and immunodepression because of massive therapy in the explosive relapse is questionable. In the other case the relapse occurred at the same time in the marrow as it did in the CSF, and it is possible that Burkitt's lymphoma cells contaminated them because in vitro data showed that in this case the addition of RFB_7 or B_1 would have been necessary to effectively purge the marrow (see the above-named chapter by Favrot *et al.* and the chapter by I. Philip *et al.*, "Using a Liquid Cell Culture Assay to Measure In Vitro Elimination of Burkitt's Cells From Bone Marrow," both in this volume). Unfortunately, we were not able to use these antibodies for ex vivo treatment at that time.

We have previously shown in vitro that Burkitt's lymphoma cells could grow in a liquid culture system from cytologically and histologically normal marrow (35,38,41). On the basis of this test we showed that marrow apparently normal at the time of harvest grew lymphoma cells in culture (35,38,41; see also the above-named chapter by I. Philip *et al.* in this volume). However, whether cells that grow in liquid culture are capable of reestablishing a malignancy when reintroduced into the human is unknown. We, nevertheless, selected a cocktail of three monoclonal antibodies that would react with virtually all Burkitt's lymphoma and developed a purging procedure using complement lysis (32,42; see also the above-named chapter by Favrot *et al.* in this volume). We believe, however, that ultimately a number of different techniques will have to be associated for in vitro purging to be successful (43; see also V. Combaret *et al.*, "Eliminating Burkitt's Cells From Excess Bone Marrow With an Immunomagnetic Purging Procedure," and the above-named chapter by Favrot *et al.*, both in this volume). The feasibility of purging is clearly demonstrated here in our patients. The continued occurrence of marrow relapse despite purging might lead to the conclusion that such procedures have little to contribute. However, we have laboratory evidence that some purging procedures were incomplete (32,35; see also Favrot *et al.* and I. Philip *et al.*, both in this volume). Only case-by-case analysis, using a clonogenic or liquid culture assay, can in the future identify the need and determine the efficacy of the purging procedure. Our preliminary conclusion is that purging techniques still require perfection at the laboratory level and that their worth should not be judged on the basis of incomplete procedures.

The indications for ABMT in Burkitt's lymphoma are, in our opinion,

restricted to 20% of patients who should be divided in two groups according to therapy response. Massive therapy and ABMT are currently the best treatment for Burkitt's lymphoma in PR after initial induction therapy or in responding relapse after rescue protocols. The only question that remains unanswered is whether the high efficacy of second-line rescue protocols will still be observed when relapses follow more aggressive initial therapy. Massive therapy and ABMT are still experimental treatment for patients with Burkitt's lymphoma who have initial CNS involvement, patients for whom results remain disappointing with conventional regimens (29).

Massive therapy, such as BACT or BEAM, are clearly not able to cure patients with progressive disease (33). For this group of patients new phase II studies are urgently needed and should be set up as a multicenter cooperative trial. These studies could be based on conventional chemotherapy regimens tested with ABMT to determine if a dose-effect relationship can improve the results. New massive therapy combinations, including high-dose cisplatin, melphalan, ifosfamide, carmustine, cytarabine, and high-dose methotrexate, should be explored. Combinations of various alkylating agents as proposed by the Baltimore group may be a useful avenue to explore (23). The role of TBI remains unclear in Burkitt's lymphoma, despite poor results reported in other lymphomas (24). However, it is clear that such phase II studies will be the basis of any future progress in Burkitt's lymphoma, either in conventional or massive therapy regimens.

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Biology and Therapy of Multiple Myeloma

Bart Barlogie and Raymond Alexanian

Multiple myeloma is a neoplasm of monoclonal well-differentiated B cells, and its associated clinical symptoms result from bone destruction, anemia, hypercalcemia, and renal failure. Melphalan and prednisone have been the standard therapy for 20 years, providing initial responses in 50-60% of patients. However, there are few complete remissions, and the median survival time is only 2-3 years. Patients presenting with a small tumor mass and achieving marked cytoreduction account for the majority of the 5-10% of patients who survive up to 10 years, although they, too, ultimately succumb to progressing disease.

CELLULAR BIOLOGY

At The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, we have studied the cellular biology of multiple myeloma in an effort to understand better the considerable heterogeneity in the clinical disease course, thus facilitating the selection of patients whose prognosis is poor for novel therapeutic strategies. Most of these studies use flow cytometry, a convenient method for quantitative tumor-cell description.

Myeloma plasma cells typically exhibit a hyperdiploid DNA content about 10-15% higher than that of normal diploid cells (1). As a reflection of the plasma cells' commitment to protein production, their RNA content is usually

high, about four- to six-fold greater than that of peripheral blood lymphocytes (2). In most patients, DNA and RNA features remain stable throughout the clinical disease course, although ploidy changes and a decrease in plasma-cell RNA content have been observed in association with dedifferentiation of the myeloma. The biochemical hallmark of myeloma plasma cells is the presence of a monoclonal immunoglobulin in their cytoplasm, which can be evaluated flow cytometrically with the use of antiheavy- and antilight-chain reagents. Approximately 15% of well over 100 separate cases of myeloma examined showed coexpression of kappa and lambda light chains by the same aneuploid tumor cells; interestingly, immunoglobulin G (IgG) lambda was secreted in all instances (3). Combined DNA, RNA, and Clg analysis can be employed to define the about 10% of patients who have diploid myeloma with a low RNA content or nonsecretory and nonproducing variants.

The nature of the myeloma stem cell has remained elusive, although recent reports have suggested the presence of a common acute lymphoblastic leukemic antigen (CALLA)-positive precursor population in the peripheral blood and bone marrow (4,5). A panel of antibodies has been used to study the myeloma phenotype expression along the B-cell differentiation pathway and the expression of B2 microglobulin. These studies have revealed that aneuploid tumor cells expressed CALLA in about 40% and B4 in 15% of cases; the plasma cell-associated antigen R1-3 was present in 70%. B2 microglobulin in 80%, and Clg in all patients. Interestingly, CALLA and B4 were frequently coexpressed along with the mature B-cell markers R1-3 and Clg, indicating the presence of an unusual tumor cell phenotype without a normal B-cell counterpart.

Although tumor cytogenetics is playing an increasingly dominant role in biologic and clinical studies of leukemia and lymphoma, cytogenetic studies of myeloma are still sparse. This is because mature plasma cells, which constitute the majority of tumor cells present in patients with myeloma, have a low proliferative propensity. Using standard metaphase karyotyping, we observed, among 150 patients, a quarter with cytogenetic aberrations. Among patients with DNA aneuploidy and greater than 10% marrow plasmacytosis, this incidence increased to 40%. Chromosomal anomalies consisted mainly of numeric changes: the addition of odd-numbered chromosomes, involving particularly chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, and losses involving chromosomes 8, 13, 16, and 22. Structural aberration involved chromosomes 1, 6, 8, 11, and 14. We found an association between specific karyotype patterns and immunoglobulin phenotypes: only light-chain disease was associated strongly with hypodiploid karyotypes; IgA with deletions of chromosome 8 or 18 and translocations of chromosome 8; and IgG with odd-numbered chromosome gains, particularly 7 and 21 (6). The observation of t(8;14) and t(11;14) translocations led us to examine whether c-*myc* and *bcl*-1 oncogenes were involved in this well-differentiated B-cell neoplasm. *Myc*-DNA rearrangement was observed in

only 1 of 100 patients studied, but increased *myc*-RNA expression was noted in about 25% of patients, including 1 with an abnormal transcript size. *Bcl*-1 was rearranged in 5% of 100 patients evaluated (8). These studies were further complemented by analysis of *myc*-protein expression using flow cytometry with DNA counterstaining, which demonstrated *myc*-protein expression preferentially in aneuploid tumor cells (9).

To summarize our biologic studies: we found hyperdiploid abnormalities in over 80% of patients. Often these were accompanied by complex morphologic chromosome anomalies, which were associated with IgA myeloma in the case of lymphoma-like translocation and with only Bence Jones proteinuria in cases of hypodiploidy. As in some B-cell lymphomas, the observations of CALLA and B4 expression and of translocations involving the immunoglobulin heavy-chain gene locus on chromosome 14 suggest the involvement of a pre-B precursor cell in the neoplastic disease process, whereas the coexpression of early and well-differentiated B-cell phenotypes is an example of differentiation infidelity. Clinically, drug resistance was associated with a low plasma-cell RNA content and hypodiploid DNA stem lines (10).

THERAPY

The relatively dismal outlook for patients with established resistance to melphalan and prednisone results from the lack of effective salvage therapy. Alkylating-agent combinations, interferon, and prednisone pulses induce short remissions in about 15% of such patients. These results have been substantially improved through use of the VAD (vincristine [Oncovin], Adriamycin [doxorubicin], dexamethasone) regimen and of high-dose melphalan.

VAD

In order to determine the relative therapeutic contribution of dexamethasone to the success of VAD (11), we compared dexamethasone pulsing and VAD in two successive clinical trials of patients refractory to standard melphalan and prednisone and also, in 25% of patients, resistant to Adriamycin (12). Responses to dexamethasone or VAD were defined by greater than 75% tumor cytoreduction and occurred in about 60% of patients with relapsing myeloma who received VAD. Dexamethasone alone induced remission in 25% of patients regardless of prior responsiveness, and a third of patients with primary resistance responded to VAD. There was a steep incremental increase in response rate as the duration of primary resistance decreased, from 16% for patients with resistance for greater than 1 year to 46% for those with primary refractory disease for less than 6 months (combined dexamethasone and VAD groups). Responses occurred rapidly: median tumor halving times were 1.4 months for dexamethasone and 0.7

months for VAD. VAD cytoreduction was typically more complete, inducing a high proportion of greater than 90% tumor cytoreduction, whereas dexamethasone responses seldom exceeded the 75% mark. Objective responses included prompt clearing of marrow plasmacytosis within 1 to 2 months, often dramatic pain relief within days of beginning therapy, and rapid improvement of anemia, hypercalcemia, and renal failure where present. Myelosuppression was infrequent. Once patients became refractory to dexamethasone alone, few responded upon cross-over to the full VAD regimen. Multivariate regression analysis revealed that the duration of primary therapeutic refractoriness was the single most important adverse feature associated with response to dexamethasone or VAD; in addition, only 5% of patients with only light-chain disease responded. Therapy entered as a third independent variable: the activity of dexamethasone was inferior to that of VAD. On the other hand, high serum B2 microglobulin levels greater than 6 mg/l as a reflection of high tumor mass, renal failure, or both, were the only significant variables associated with short survival. Toxicity of dexamethasone and VAD regimens—fever and infection in 20% of patients and a 7% mortality—was mainly related to glucocorticoid-associated immunosuppression. The median response duration, similar for dexamethasone and VAD, was 1 year. Thus the dose and schedule modification of the VAD regimen compared to a previous VAP combination resulted in greater antitumor activity that, however, benefitted chiefly those patients who did not have primary drug resistance.

High-Dose Melphalan

Based on encouraging pilot data in both untreated and previously treated patients, we evaluated high-dose melphalan against VAD-refractory myeloma (13). Once we established the marked toxicity from melphalan at a dose of 100 mg/m^2—among 15 treated patients the mortality was 35%—we gave subsequent patients either a lower dose of 90, 70, or 50 mg/m^2 according to age and renal function, or administered a higher dose of 140 mg/m^2, but supported the patients by infusing autologous remission marrow (14). Both dose reduction and autologous marrow support greatly reduced the early mortality. Responses occurred in 14 of 29 patients treated, but the median survival was 3 months for the 22 patients receiving less than or equal to 100 mg/m^2 of melphalan and 6 months for the 7 patients receiving 140 mg/m^2 in conjunction with bone marrow support.

Total Body Irradiation

Once we had established the better tolerance of a higher dose equivalent of melphalan when given in conjunction with autologous bone marrow support, we added total body irradiation (TBI) in an effort to augment the antitumor effect (15). To date, four patients with VAD-refractory myeloma have received melphalan (140 mg/m^2) and TBI (850 cGy) in 5 fractions. All

responded dramatically: their median tumor-halving time was only 0.3 months, and the maximum tumor cytoreduction was greater than 90%. Of particular interest was the response in a patient with refractory primary myeloma whose marrow autograft contained 30% tumor cells that did not compromise the net tumor cytoreduction or duration of disease control. In fact, all four patients continue in remission after 3-15 months (median, >8 months), which is superior to the median of 4 months for the 14 responders to melphalan alone.

Our preliminary data, then, indicate that high-dose melphalan can be combined effectively and relatively safely with TBI when the patient is supported by ABMT, even in the presence of considerable marrow plasmacytosis. The absence of major nonmyelosuppressive side effects makes this treatment attractive for managing a disease that typically affects a more elderly patient population. The low proliferative propensity of myeloma plasma cells and perhaps a small tumor stem cell compartment may permit durable remissions, even when all tumor cells have not been removed from marrow autografts by immunologic or cytotoxic means.

SUMMARY

Major advances in the understanding of myeloma biology and therapy have been accomplished over the past 5 years. In an effort to alter fundamentally the natural history of myeloma in high-risk patients, those presenting with intermediate and large tumor mass are now offered high-dose melphalan and TBI supported by autologous bone marrow transplantation to consolidate VAD-induced remissions. For patients with a small tumor burden, we are currently evaluating a combination of interferon and dexamethasone, investigating whether this combination might be synergistic. We hope that dexamethasone may ameliorate interferon-related side effects and permit administration of higher and perhaps more effective doses of this biologic response-modifying agent.

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Bone Marrow Transplantation in Relapsed Diffuse Large Cell Lymphoma

James O. Armitage

High-dose chemotherapy plus autologous bone marrow transplantation (ABMT) is becoming a frequently utilized treatment for patients with a variety of malignancies (1). A recent survey of investigators working in the field of ABMT showed that most frequently treated disease was non-Hodgkin's lymphoma, which made up 32% of all transplant cases (1). The proportion of patients with non-Hodgkin's lymphoma undergoing ABMT and having diffuse large cell histology has varied considerably from one series to another (Table 1). In series containing predominantly adults, diffuse large cell lymphoma seems to make up a larger proportion of patients, and most series of patients treated with ABMT for non-Hodgkin's lymphoma have included primarily patients with relapsed or refractory disease (Table 2). However, increasing numbers of patients are being treated earlier in their disease.

RESULTS OF ABMT

Results of high-dose therapy and ABMT in patients with relapsed diffuse large cell lymphoma are presented in Table 3. This table includes series in which it was possible to determine outcome by histological type. The results in these patients show a composite complete remission rate of 51% and an

Table 1. Frequency of Diffuse Large Cell Lymphoma in Patients With Non-Hodgkin's Lymphoma Undergoing Autologous Bone Marrow Transplantation

Group	No. of Patients with ABMT for NHL	No. with de novo DLCL
St. Louis, Vancouver, Cleveland, Nashville, Dallas	70	40 (57%)
University of Nebraska	53	39 (74%)
EBMTG	50	10 (20%)
Minnesota	23	6 (26%)

Abbreviations: ABMT, autologous bone marrow transplantation; NHL, non-Hodgkin's lymphoma; DLCL, diffuse large cell lymphoma; EBMTG, European Bone Marrow Transplantation Group.

Table 2. Bone Marrow Transplantation in Patients With Non-Hodgkin's Lymphoma

Group	Histological Findings	No. of Patients	No. of Patients in Relapse or with Refractory Disease
Seattle	Intermediate and high grade	82	80 (98%)
EBMTG	Intermediate and high grade	50	34 (68%)
University of Nebraska	Diffuse large cell	41	40 (98%)
Dana-Farber	Low, intermediate, and high grade	39	35 (90%)
MSKCC	Diffuse large cell	24	11 (46%)

Abbreviations: EBMTG, European Bone Marrow Transplantation Group; MSKCC, Memorial Sloan-Kettering Cancer Center.

early death rate of 26%. Twenty-five of the 90 patients (27%) continue in remission for 3+-45+ months with the median remission duration in excess of 1 year. These results do not vary significantly from those from studies of relapsed, aggressive non-Hodgkin's lymphomas of mixed histological types. Approximately 20% of patients with relapsed diffuse large cell lymphoma will be long-term survivors when treated with high-dose therapy and ABMT. These results are not greatly different from those seen with allogeneic transplantation in patients with relapsed acute leukemia (2).

Table 3. Autologous Bone Marrow Transplantation in Relapsed Diffuse Large Cell Lymphoma

Group	Treatment	No. of Patients	Complete Remission	Early Death	Alive and Well (mo)
University of Nebraska and M. D. Anderson	Chemotherapy + TBI	47	21 (45%)	12 (25%)	12 (7+–45+)
St. Louis, Vancouver, Cleveland, Nashville, Dallas	CY + TBI	13	6 (46%)	—	2 (38+, 19+)
Iowa	Chemotherapy + local XRT	11	7 (64%)	4 (36%)	6 (3+–30+)
MSKCC	CY + TBI	11	—	3 (27%)	5 (3+–24+)
EBMTG	Chemotherapy	8	6 (75%)	1 (12%)	0

Abbreviations: TBI, total body irradiation; CY, cyclophosphamide; XRT, radiotherapy; MSKCC, Memorial Sloan-Kettering Cancer Center; EBMTG, European Bone Marrow Transplantation Group.

PROGNOSTIC FACTORS FOR ABMT

In general, whenever any treatment is applied to patients with malignancy, all are not equally likely to benefit. Identifying factors that predict outcome allows a more intelligent application of the treatment. In patients with diffuse large cell lymphoma undergoing autologous transplantation, potential prognostic factors can be lumped into those related to the therapy employed, those related to the patient, and those related to the disease.

Treatment-related variables that might affect treatment outcome in patients with relapsed lymphoma include the regimen employed and the source of stem cells utilized to restore hematopoiesis. A wide variety of treatment regimens has been utilized to date. It is not clear that any one of these regimens is clearly superior to the others. In a large, retrospective international study of patients with aggressive non-Hodgkin's lymphomas undergoing high-dose therapy and ABMT, the treatment regimen and whether or not total body irradiation was utilized in the regimen did not affect outcome (T. Philip *et al.,* unpublished data). In a large series of patients treated with high-dose therapy and bone marrow transplantation at Seattle, the source of marrow (whether allogeneic, syngeneic, or autologous) did not have a significant effect on treatment outcome (F. R. Appelbaum, unpublished data). Similarly, it is difficult to identify any impact of marrow purging on treatment outcome. In patients treated by the European Bone Marrow Transplantation Group, there was no definite difference in outcome between patients who received purged or unpurged marrows (3). That no one treatment regimen has been shown to be definitely superior or that marrow purging has not been shown to infer an advantage does not mean that one of these approaches is not superior. It more likely reflects a great variation in patient and disease-related prognostic factors that make comparisons difficult between series.

It has quickly become apparent to most investigators in this area that patient-related prognostic factors have a significant impact on outcome. Most groups will not treat patients beyond 60 years of age with high-dose therapy and ABMT. At the University of Nebraska, we have treated four such patients with three in-hospital deaths and none of the patients surviving in continuous remission. Similarly, patients with low performance status are generally thought to be poor candidates for high-dose therapy. We have treated nine patients with Karnofsky performance scores of ≤ 70 (i.e., not totally ambulatory). Seven of these patients (78%) died in the hospital, and none are long-term disease-free survivors. Although the number of patients in each group is small, we believe the results do strongly suggest that very old patients or those with a poor performance status are poor candidates for this treatment approach.

The most important disease-related prognostic factors that have been identified for ABMT in diffuse large cell lymphoma can be classed into two categories. The first of these is tumor responsiveness. Several series have

now demonstrated that patients with tumors that still respond to chemotherapy after relapse are much more likely to achieve long-term disease-free survival than are patients with resistant relapse or primarily refractory disease. In fact, patients with lymphomas who have never achieved a complete remission appear to be poor candidates for this treatment approach, with no long-term survivors in two fairly large series (T. Philip, unpublished data; 4). The second group of disease-related prognostic factors are those that reflect tumor bulk. In a large retrospective international study (T. Philip, unpublished data) of patients with relapsed aggressive non-Hodgkin's lymphomas, there were no long-term survivors in patients with tumors greater than 10 cm in diameter or lactate dehydrogenase levels of greater than twice normal at the time of transplantation. However, these factors were related to tumor responsiveness, and in a multivariate analysis were not found to have independent prognostic significance.

CONCLUSIONS

When only good-risk patients (i.e., age < 60 years, high performance status, and responsive disease after complete remission or near-complete remission with salvage chemotherapy at traditional doses) are treated, the results with high-dose therapy and ABMT are excellent. In 54 such patients treated at the Dana-Farber Cancer Institute (35 patients) (4) or as part of a retrospective international study (19 patients) (T. Philip, unpublished data), all of the patients were in complete remission at the completion of therapy, and the 2-year disease-free survival was 55-60%. However, if high-dose therapy and ABMT are confined to such patients, only a minimal number of the total group of patients with diffuse large cell lymphoma will benefit. If one accepts the estimates for the proportion of patients with diffuse large cell lymphoma relapsing from complete remission (25%), those being younger than 60 years of age (40%), those having tumor that is chemotherapy sensitive at relapse (50%), and those not having other health problems who would ordinarily be excluded from transplantation (75%), only 4% of all patients with diffuse large cell lymphoma would be candidates for this therapy. For ABMT to have an important impact on the management of diffuse large cell lymphoma, the treatment must be available to more patients. The most likely way to accomplish this will be to treat patients early in the course of their disease. When they can be identified, patients at particularly high risk of relapse should undergo transplantation in first complete remission. Patients who respond slowly to chemotherapy given in traditional doses (i.e., not in complete remission by the third treatment cycle) and patients who do not achieve an initial complete remission should be considered for transplantation immediately while their tumors are still responsive to therapy. Finally, good-risk patients who achieve a complete remission should undergo transplantation at relapse and not after having

undergone multiple other salvage regimens that fail. Utilized in this manner, high-dose therapy and ABMT would offer cure to a significant proportion of the approximately 50% of patients not currently cured with standard chemotherapeutic approaches.

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Treatment of Malignant Lymphoma at First Relapse or Second Remission With Marrow Transplantation

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Between July 1970 and January 1985, 100 patients with recurrent malignant lymphoma were treated in Seattle with cyclophosphamide, total body irradiation (TBI), and marrow transplantation (1). The actuarial probability of disease-free survival 5 years from transplantation was 22%, and the actuarial probability of relapse after transplantation was 60%. A proportional hazards regression analysis showed that two factors were significantly associated with an improved disease-free survival. Patients who underwent transplantation earlier in their disease course (untreated first relapse or second remission) had a greater likelihood of attaining disease-free survival than patients who underwent transplantation in resistant relapse. In addition, patients who had not received prior chest radiotherapy had a greater probability of survival than those who had. Neither disease histological findings (Hodgkin's disease, high-grade lymphoma, or intermediate-grade lymphoma) nor type of marrow (syngeneic, allogeneic, or autologous) significantly influenced disease-free survival.

Based on these results, several phase 2 trials have been initiated in Seattle. "Good-risk" patients (patients in untreated first relapse or second remission)

are being treated with cyclophosphamide (60 mg/kg/day for 2 days), TBI (2 Gy/day for 6 days), and marrow transplantation in order to define the probability of disease-free survival in this group of patients and to measure the influence of disease histological characteristics and marrow source on outcome. The early results of this study are described here. Patients with resistant disease and those who have received prior chest radiotherapy are being treated on other pilot studies.

METHODS

Among the first 100 patients who underwent transplantation for lymphoma in Seattle, 24 were in untreated first relapse or second remission. From January 1985 to March 1986, 13 additional good-risk patients were treated. Thus, information on 37 consecutive patients treated with cyclophosphamide, TBI, and marrow transplantation and followed for at least 6 months is available. Seventeen patients were classed as having intermediate-grade lymphoma; fifteen, high-grade lymphoma; four, Hodgkin's lymphoma; and one, low-grade lymphoma.

In general, marrow was selected according to the following order of preference: 1) syngeneic marrow, 2) marrow from a donor with identical human leukocyte antigen or from a family member with a single antigen mismatched, 3) autologous remission marrow, or 4) marrow from a family member with two antigens mismatched. Twenty-one patients received allogeneic matched marrow; twelve, autologous marrow; three, syngeneic; and one, allogeneic marrow that was mismatched. Recipients of allogeneic marrow were treated after transplantation with either methotrexate (15 cases) or methotrexate plus cyclosporine (7 cases). Recipients of autologous and syngeneic marrow received no posttransplantation chemotherapy. Eight of the 12 autologous marrow recipients received marrow untreated in vitro and 4 had marrow treated with an anti-B1 monoclonal antibody plus complement or with a panel of anti-T-cell antibodies plus complement.

RESULTS

Eighteen of the 37 patients are alive in remission from 6 to 90 months after transplantation. The Kaplan-Meier probability of disease-free survival at 2 years is 42%. Reasons for treatment failure include disease recurrence in 11 patients, bacterial infection in 2, and 1 case each of fatal hemorrhage, cytomegalovirus pneumonia, veno-occlusive disease of the liver, idiopathic interstitial pneumonia, renal failure, and graft-versus-host disease. The Kaplan-Meier probability of disease relapse is 41%. No conclusions can yet be reached about an influence of disease histology or marrow source on outcome.

DISCUSSION

The results of this study should be viewed as preliminary. More patients and longer follow-up are needed to define accurately the curability of malignant lymphoma with marrow transplantation and to identify the influences, if any, of disease histology and marrow source on outcome. Further, there has not yet been a formal comparison of marrow transplantation to other forms of salvage therapy. However, the results seen in these 37 patients are consistent with those obtained by others and are considerably better than what we and others have observed in patients with resistant disease (16% disease-free survival at 3 years) (2-5). These results also are better than what has been obtained with most salvage chemotherapy programs (6). Therefore, marrow transplantation at first relapse or second remission is probably the treatment of choice for patients with disseminated malignant lymphoma who relapse after obtaining an initial complete remission.

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Optimum Timing of Autologous Bone Marrow Transplantation for Patients With Large B-Cell Lymphoma

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Recent combination chemotherapy protocols have improved the survival rates for patients with non-Hodgkin's lymphoma. Extended long-term disease-free survival was first reported with MOPP (mechlorethamine, Oncovin [vincristine], procarbazine, prednisone) or C-MOPP (MOPP plus cyclophosphamide) (1). Subsequently, most protocols have used regimens that contain Adriamycin (doxorubicin), Cytoxan (cyclophosphamide), and vincristine (CHOP, CHOP-Bleo, BACOP, ACOMLA). These protocols have produced a complete remission (CR) incidence of 40-70%, but a significant number of patients relapse (2-6). Newer regimens, including ProMACE-MOPP (7), COP-BLAM (8,9), M-BACOD (10), m-BACOD (11), and MACOP-B (methotrexate + Leucovorin rescue, Adriamycin [doxorubicin]) (12), have

produced CRs in the range of 60-80%, with a projected 5-year survival rate of 50-75% (7-12). Longer follow-up and standardized analysis of the patient's prognostic factors will be necessary before the results of these trials can be properly compared and evaluated. It is particularly important to know what proportion of patients with favorable or unfavorable prognostic features were included in each series and their respective outcomes. The problem of conducting such an analysis of prognostic features is further confounded by the lack of agreement on what are the most important prognostic features. A standardized system is greatly needed. An analysis of prognostic factors at Memorial Sloan-Kettering Cancer Center (MSKCC) revealed that patients with large B-cell lymphoma who had bulky mediastinal disease, abdominal disease, or both (5,6,13-15) and an elevated serum lactic dehydrogenase (LDH) level at presentation were destined to do poorly and that only 20% could anticipate a 2-year disease-free survival.

Because of the poor results with conventional therapies, in 1981 a clinical trial was initiated to evaluate the role of intensive chemotherapy followed by autologous bone marrow transplantation (ABMT) for patients with poor prognostic features. Several other investigators have also found that patients with large B-cell lymphomas who have high levels of serum LDH and bulky mediastinal disease, abdominal disease, or both are destined to do poorly on the conventional protocols (4,5,16,17), and similar prognostic features tend to produce similar results even on the newer treatment protocols (18).

The following features were identified as being associated with poor prognosis in our study: large B-cell lymphoma, bulky mediastinal and abdominal tumor masses with dimensions greater than 8 x 8 cm, or a serum LDH level greater than 500 U/ml. For this group of patients, the results of ABMT performed in first remission are compared with those from a historical control group with similar prognostic features who were treated by conventional chemotherapy. Comparisons are also made prospectively to ABMT performed after continuing chemotherapy failed or after relapse.

Previous trials involving ABMT were usually performed on patients whose conventional chemotherapy had failed, usually with only 20-30% of the patients achieving long-term survival. The survival benefit was mainly enjoyed by younger patients with undifferentiated Burkitt's lymphoma (19-23). Most protocols use hematoablative dosages of cyclophosphamide prior to total body irradiation (TBI) (19-23). At MSKCC the use of hyperfractionated TBI (HF-TBI) followed by cyclophosphamide reduced the incidence of interstitial pneumonitis in patients undergoing allogeneic bone marrow transplantation (BMT) (24,25). The dose of HF-TBI and cyclophosphamide used is the same as in our allogeneic BMT program for acute leukemia (25-27), and considering our previous encouraging results in leukemia, we elected to use the same myeloablative therapy for patients with lymphoma. These patients were rescued with an autograft instead of allograft. In this chapter we describe our results with 31 patients with non-Hodgkin's lymphoma who received ABMT.

PATIENTS AND METHODS

Patients with large B-cell lymphoma received transplants from March 1981 to December 1985, and no data collected after June 1, 1986, is included in the analysis. The patients were evaluated at Memorial Hospital and were treated using protocols approved by the institutional review board (IRB). Staging work-up included confirmation of pathological diagnosis, and the highest pretreatment serum LDH level recorded is listed in Tables 1 and 2. Tumor dimensions were measured at surgery, x-ray evaluation, or both and are also given in Tables 1 and 2. Bone marrow aspirate and biopsy specimens were evaluated for cellularity, morphological presence of lymphoma, growth of colony-forming units granulocyte-macrophage (CFUs-GM) or erythrocyte burst-forming units (BFUs-E), and immunoglobulin surface markers. Bone marrow was considered involved if greater than 5% lymphoma cells were detected. Because it is difficult to distinguish between small numbers of lymphoma cells and normal lymphoid precursors without using special procedures, any patient with less than 5% blasts that appeared lymphoid by cell-surface marker analysis, morphology, or both was considered to have probable bone marrow involvement. The patient's disease status and bone marrow were reevaluated after completion of induction therapy.

All previously untreated patients with poor prognostic features were induced using the L-17M protocol (28), requiring cyclophosphamide on day 1 and day 35. Vincristine was given intravenously on days 1, 8, 15, 22, and 29. Prednisone was given by mouth from day 1 through day 35, and then administration was tapered with Bactrim (trimethoprim and sulfamethoxazole) prophylaxis. Doxorubicin was given intravenously on days 16, 17, 18, and 35. Intrathecal methotrexate was given four times during the induction phase. Bone marrow was harvested after hematologic recovery from the last part of induction therapy. Bone marrow harvest was performed with the patient under general anesthesia, and heparinized bone marrow was enriched for mononuclear cells by unit gravity sedimentation in the presence of 0.8% hydroxyethyl starch (HES). Bone marrow with no suggestion of involvement by lymphoma was cryopreserved in 6% HES, 5% dimethyl sulfoxide (DMSO), 4% human albumin at 4-5 $\times$ 10^7 cells/ml (29). Patients with probable bone marrow involvement at presentation had their marrow divided into two aliquots. Sixty percent of their marrow was purged with 4-hydroperoxycyclophosphamide (4-HC) (60-120 μM) (30-34) for 30 minutes at 37°C and cryopreserved and used first for hematopoietic reconstitution. The other 40% was cryopreserved to be used as backup for the purged bone marrow. Patients then received one cycle of daunomycin, ara-C (cytarabine), and 6-thioguanine (DAT) chemotherapy (28), which allowed adequate time to evaluate the cryopreserved bone marrow's viability, cell-surface markers, and CFU-GM and BFU-E growth. The initial protocol called for randomization of patients to arm 1 (receive transplant following induction) or to arm 2 (continue on consolidation and maintenance phase of L-17M and consider

Table 1. Characteristics of Patients Undergoing Autologous Bone Marrow Transplantation With Poor Prognosis Large B-Cell Lymphoma

Patient No.	Age/Sex	Site in cm	Bone Marrow		Initial Stage	LDH Level		L-17M Response[a]	Survival (mo)	
			Initial	ABMT		Initial	ABMT		Initial Rx	DFS
ASCT at First Remission-S/P L-17M Induction										
1	16/M	M-11 x 13	—	—	IV	1077	201	PR	62.8+	60.1+
2	24/F	M-12 x 13	—	—	IV	623	213	CR	58.2+	54.1+
3	38/M	M-11 x 12	—	—	IV	426	177	CR	54.4+	50.2+
4	32/F	M-12 x 14	—	—	II	834	159	PR	52.1+	49.3+
5	37/M	M-13 x 12	—	—	II	310	217	CR	48.9+	45.6+
6	27/M	M-10 x 14	—	—	III	528	307	CR	48.4+	45.1+
7	30/F	M-17 x 16	—	—	III	564	202	PR	44.5+	39.4+
8	23/M	M-18 x 14	P	—	IIe	501	296	PR	40.4+	37.8+
9	32/F	M-13 x 14	—	—	IV	645	217	PR	33.0+	29.0+
10	41/M	R-10 x 10	P	—	III	867	274	CR	31.1+	27.0+
11	20/F	M-17 x 16	—	—	III	1341	199	PR	24.1+	20.0+
12	28/F	M- 8 x 9	P	—	IIIe	801	195	CR	11.8	3.4
13	17/M	Bone/R- 9 x 5	P	—	IV	669	225	PR	9.2	5.4
14	20/F	M-11 x 15	—	—	IV	634	242	PR	11.7	2.3
					Median	*639*	*215*		*42.4*	*38.6*
ASCT at L-17M Fail/Relapse										
15	29/M	M- 8 x 8	P	—	IV	140	458	CR(11.3)	29.1	10.0
16	30/M	M-17 x 15	P	—	IV	753	277	PR (3)	17.0	5.2
17	24/F	M- 8 x 10	—	—	III	240	284	F	9.8	1.7
18	29/M	M-16 x 14	—	—	IV	379	192	F	6.7	0.7
19	24/M	M/Bone 12 x 2	—	—	IV	180	341	CR (2.0)	8.0	0.9
20	25/F	M- 8 x 9	P	—	IV	178	273	PR (1.4)	6.0	0.6
					Median	*210*	*280*		*7.8*	*1.3*

Abbreviations: ASCT, autologous stem cell transplantation; M, mediastinum; R, retroperitoneum; P, probable bone marrow involvement; ABMT, autologous bone marrow transplantation; LDH, lactic dehydrogenase; PR, partial remission; CR, complete remission; F, failure; DFS, disease-free survival.

[a]*Duration of response in months is in parentheses.*

Table 2. Autologous Bone Marrow Transplantation as Salvage Therapy

Patient No.	Age/ Sex	Histo-pathologic Type	Primary Site in cm	Bone Marrow		Initial Stage	LDH Level		Max. Response Initial Rx[a]	Survival (mo)	
				Initial	ABMT		Initial	ABMT		Initial Rx	ABMT[b]
21	20/M	DLC	M-10 x 12	P	—	II	230	401	PR (6.6, C,R)	62.9+	53.5+
22	28/F	DLC	M-10 x 12	P	—	IV	265	157	PR (5.5, C,R,L)	56.0+	42.1+
23	34/M	NML/DLC	R	—	—	IV	192	126	PR (3.7, B)	41.5+	25.1+
24	19/M	DLC	Mult. bone	P	—	IV	335	184	F (11.4, N,R,L)	40.9+	29.5+
25	35/F	DLC	M	P	—	II	299	157	CR (5, C,L)	30.0	16.8
26	40/M	NPDL/DLC	R-15 x 13	—	—	IV	216	156	PR (4.1, I,N)	24.5	13.4
27	34/F	DLC	B-12 x 10	P	P	IV	364	236	CR (8.5, N,L)	21.4	2.6
28	38/F	DLC	M	—	—	II	1260	349	PR (6.60, N,L)	15.4	7.6
29	20/F	DLC	B-8 x 8	P	—	IV	566	343	F (7.8, L)	17.1	2.3
30	36/F	DPDL	B-8 x 6	P	—	IV	276	247	CR (2.0, N)	11.0	1.4
31	24/F	DLC	M-11 x 13	—	—	II	155	927	F (5.9, C,N,L)	6.2	0.3
						Median				*21.8*	*13.40*

Abbreviations: DLC, diffuse large cell; NML, nodular mixed lymphoma; NPDL, nodular poorly differentiated lymphoma; DPDL, diffuse poorly differentiated lymphoma; M, mediastinum; R, retroperitoneum; B, breast; P, probable bone marrow involvement; ABMT, autologous bone marrow transplantation; LDH, lactic dehydrogenase; PR, partial remission; CR, complete remission; F, failure.

[a] *Usually CHOP (C) or NHL-7(N) or L-17M (L) or RT (R) or COP-BLAM (B) or IL-2 (I).*

[b] *Disease-free survival following ABMT.*

ABMT only at relapse or treatment failure). However, most patients refused randomization and preferred to choose arm 1 or arm 2, and the protocol was therefore revised, IRB approval obtained, and patients were then given a choice of arm 1 or arm 2.

Fourteen patients elected to have ABMT while they were in CR (no evidence of disease by physical examination or roentgenographic analysis) or partial remission (PR) (greater than 50% reduction of tumor mass but residual mass detected by physical examination or roentgenographic analysis). Thirteen patients elected to continue on the L-17M protocol, among them five patients who remain in CR at 62+, 44+, 28+, and 18+ months (two patients). Eight patients had progressive disease or relapsed on the L-17M protocol (arm 2). Six of these eight patients were able to receive a transplant, and the other two died of rapid disease progression. The initial response to L-17M and the number of months these patients remained in CR is given in Table 1. All six patients who underwent transplantation in relapse showed significant improvement, but all six patients have died. Eleven patients with non-Hodgkin's lymphoma who had relapsed on other treatment also underwent transplantation. Their clinical course and treatment prior to considering ABMT are summarized in Table 2.

Patients were evaluated for potential dental problems before transplantation. Dental cavities and infected teeth were treated. Plastic dental molds were made for possible use in the event of gum bleeding and subsequent fluoride treatment of teeth. Patients with a residual tumor mass after induction chemotherapy received radiation boosts to the area of bulky disease (usually 3 Gy/day for 4 days). Patients were then admitted to a single room for transplantation. One week of prophylactic Bactrim double-strength by mouth twice a day was given before ABMT. All patients with previous herpes simplex infection or high viral titers of herpes simplex also received acyclovir intravenously (35). Hyperfractionated TBI (total dose 13.2 Gy) was given in 11 fractions over 4 days (24,25). Intravenous hydration was started, and patients received cyclophosphamide for 2 days at 60 mg/kg/day. The patient's cardiac and urinary functions were carefully monitored, and after 48 hours the patient's cryopreserved marrow was thawed in a water bath at the bedside and rapidly reinfused without any treatment. Hydration was maintained until hemoglobinuria subsided (the few RBCs present in the cryopreserved marrow are lysed during the thawing procedure). Moderate-to-severe mucositis was managed with frequent mouth care and antifungal medications.

Patients were placed on total parenteral nutrition until they were thought able to eat. All blood products were irradiated with 30 Gy to prevent transfused lymphocyte-mediated graft-versus-host disease (36). Patients usually required a 5- to 6-week hospital stay and were then followed as outpatients. Prophylaxis for *Pseudomonas carinii* was used. Double-strength Bactrim (twice daily by mouth) was begun 50 days after ABMT and given for 90 days with frequent monitoring of the complete blood count.

RESULTS

Twenty previously untreated patients with large B-cell lymphoma and a poor prognosis underwent ABMT. The patient characteristics are described in Table 1. Fourteen patients received ABMT while they were in CR or PR (group 1) and six patients received ABMT after relapse or after induction therapy failed (group 2). The number of patients in the two groups is small, and the two groups are prognostically comparable for age, stage, and tumor mass; however, the patients who relapsed on L-17M therapy had a lower median serum LDH level at initial presentation than did patients in group 1 (210 U/ml compared with 639 U/ml). Survival from the time of initial diagnosis and from the time of ABMT for the two groups of patients is shown in Table 1.

Of the 14 patients in group 1, 11 are disease free (median follow-up, 28+ months). The projected survival for this group is shown by Kaplan-Meier analysis in Figure 1 and is compared with the historical control group of 70 patients with similar presentation (B-cell lymphoma, bulky mediastinal or

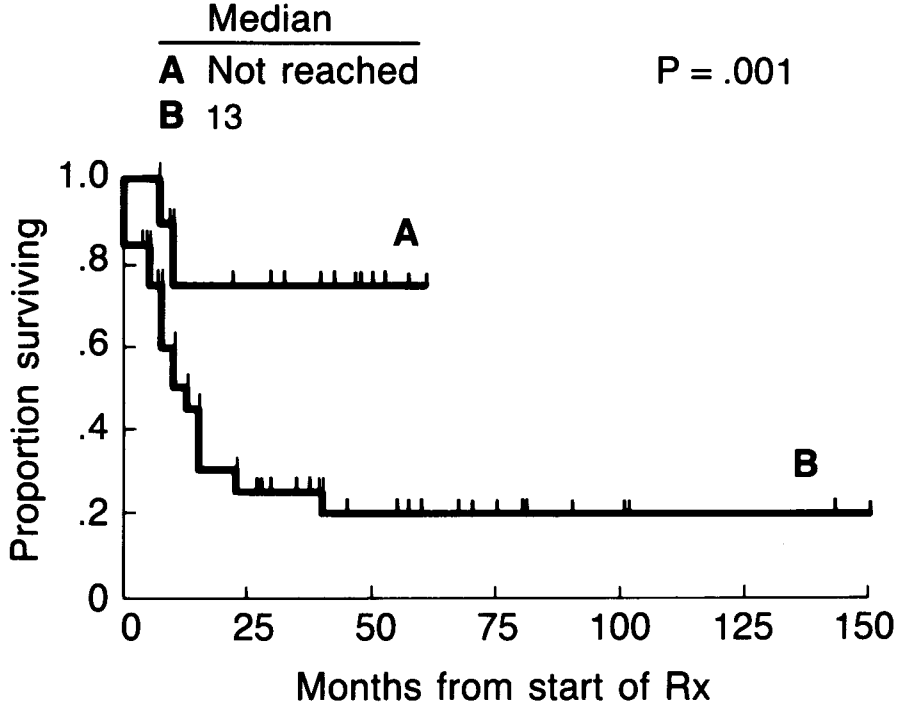

Figure 1. *Projected survival of patients with large B-cell lymphoma with poor prognosis. Those patients undergoing the Memorial Sloan-Kettering protocol described herein* (A) *are compared with a historical control group of 70 patients treated with conventional chemotherapy* (B) (P = *.001).*

abdominal disease, or serum LDH level >500 U/ml) treated on previous MSKCC protocols. Patients in the historical control group received CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) combination chemotherapy or its variants (6,14,15). The median survival for the historical control group was 12.8 months (because the majority of patients are alive in group 1, the median survival for group 1 cannot be determined at this time). There have been no late complications (1 year postdiagnosis) in this group of patients. The results of ABMT performed in relapse are shown in Table 1 (group 2); all six patients have died, two of pulmonary hemorrhage and peritransplant complications, one of fungal infection, and one died of complications related to chronic thrombotic episodes experienced before ABMT. Two patients had lymphoma progression and died at 10 and 5.2 months after transplantation.

Eleven additional patients were referred to us for ABMT after other protocols had failed. Eight of these patients had trials on two or more drug combinations before referral for ABMT. Bone marrow was harvested, and depending on the patient's clinical status, one or two cycles of CHOP or other combination chemotherapy were given in attempts to reduce disease prior to ABMT. All patients responded to intensive therapy followed by ABMT, but one patient died soon after transplantation of complications caused by superior vena cava obstruction, which was present before ABMT. Six patients have relapsed and died 1.4-25.5 months after ABMT (Table 2), and the other four patients are alive 17.6+-45.6+ months after ABMT (Table 2).

Among the 31 patients undergoing transplantation, 14 had signs of marrow involvement at presentation (Tables 1 and 2), and these patients received 4-HC-purged bone marrow. Eight of these patients relapsed with disease in an area where it had been previously detected, including the bone marrow (three of the eight). Seventeen patients received unpurged bone marrow; four relapsed with disease found in previous sites or sites contiguous to them. One patient (no. 29) had marrow involvement with lymphoma at autopsy. These patients received TBI and chemotherapy doses known to cause aplasia in allogeneic BMT (24-26). The number of marrow mononuclear cells infused per kilogram of body weight is shown in Table 3, as are the number of days before the WBC count increased to over 1000/mm^3, the absolute neutrophil count increased to over 500/mm^3, and the platelets increased to over 50,000/mm^3.

DISCUSSION

There have been several previous reports describing the long-term results of intensive chemotherapy followed by ABMT for patients with non-Hodgkin's lymphoma (19-23). Even though most studies have demonstrated a significant CR rate, only a small fraction of patients in previous trials attained a long-term benefit from the aggressive treatment. In recent ABMT trials,

Table 3. Cell Dose, Hematopoietic Recovery, and Complications of Autologous Bone Marrow Transplantation

Patient No.	Cell Dose ($\times 10^8$/kg)	Pre-ABMT Preparation	4-HC Rx (μM)	Viability of Marrow (%)	Days to 1,000/mm^3 WBCs	Days to 500/mm^3 Neutrophils	Days to 50,000/mm^3 Platelets	Late Complications	Cause of Death
ABMT After CR or PR on L-17M									
1	3.8	R	—	71	10	12	18	RM, S, NT	—
2	3.4	—	—	99	12	8	13	—	—
3	3.95	RT	—	99	12	11	18	Skin cancer	—
4	5.52	R	—	90	16	15	18	—	—
5	2.89	R	—	85	12	13	24	HZ resolved	—
6	3.07	R	—	99	13	15	26	—	—
7	4.25	R	—	88	15	16	18	HZ resolved	—
8	2.08	R	100	95	22	32	33	HZ resolved	—
9	4.0	R	—	97	19	20	23	—	—
10	1.58	R	120	94	14	14	31	—	—
11	1.78	R	—	65	12	12	18	RM, S, NT	—
12	3.6	—	—	96	12	12	23	—	RL (M)
13	2.2	R	100	96	12	13	31	—	RL (M, BM)
14	3.68	R	120	93	12	13	22	—	RL (M)
ABMT After Relapse on L-17M									
15	2.89	—	100	92	11	12	24	—	RL (M)
16	4.42	R	100	91	13	13	17	—	RL (L, M)
17	2.47	R	—	98	12	15	+	—	Thrombosis
18	3.27	R	—[a]	—	—	—	—	—	—
19	3.42	—	—	70	13	13	40	—	HS (D)
20	2.4	R	100	57	16	14	16	—	P
ABMT After Relapse on Other Protocols									
21	1.51	PRD, RT	60	75	18	17	30	HZ resolved	—
22	2.60	DAT, RT	100	80	29	40	121	—	—

Table 3. Cell Dose, Hematopoietic Recovery, and Complications of Autologous Bone Marrow Transplantation (Continued)

Patient No.	Cell Dose ($\times 10^8$/kg)	Pre-ABMT Preparation	4-HC Rx (μM)	Viability of Marrow (%)	Days to 1,000/mm^3 WBCs	Days to 500/mm^3 Neutrophils	Days to 50,000/mm^3 Platelets	Late Complications	Cause of Death
ABMT After CR or PR on L-17M									
23	3.7	CHOP, R	—	97	11	12	23	—	—
24	2.18	R	120	95	20	23	41	—	—
25	1.86	—	120	94	18	27	29	—	—
26	2.40	R	—	92	16	18	25	—	RL (M, LN)
27	3.45	R	100	93	15	17	24	—	RL (LN, ABD)
28	1.62	R	—	91	+	+	+	—	RL (B, BM)
29	4.86	RT	100	92	42	42	59	—	RL (ABD, LN, B, BM)
30	0.98	RT	120	95	25	29	30	—	RL (B, LN, BM)
31	3.9	RT	—	73	+	9	+	—	RL (B, LN, BM, SVC)

Abbreviations: ABMT, autologous bone marrow transplantation; 4-HC, 4-hydroperoxycyclophosphamide; CR, complete remission; PR, partial remission; R, radiotherapy boost to the site of bulky disease; RM, residual mass; S, skin; NT, tissue necrosis with no identifiable source of infection; RT, radiotherapy; HZ, herpes zoster; RL, relapse; M, mediastinum; BM, bone marrow; L, lung; HS, herpes simplex; D, death; P, pulmonary hemorrhage; PRD, prednisone; DAT, daunomycin, ara-C (cytarabine), 6-thioguanine; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; LN, lymph nodes; ABD, abdomen; B, breast; SVC, superior vena cava.

[a] *This patient died early of pulmonary hemorrhage.*

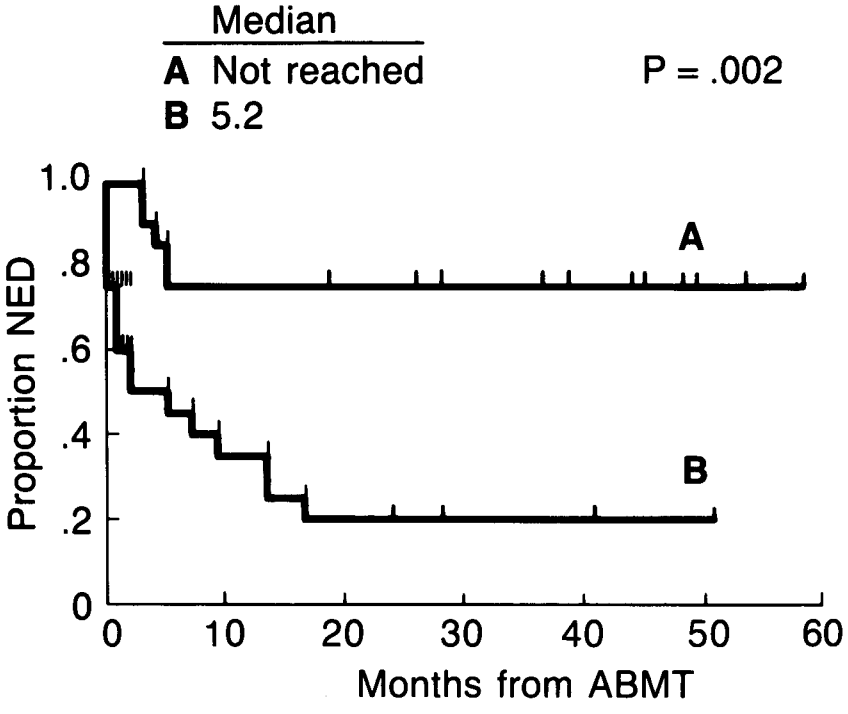

Figure 2. *Patients who undergo autologous bone marrow transplantation (ABMT) after induction therapy* (A) *are compared with those who undergo ABMT at relapse* (B) (P = *.002).*

there has been an improvement in length of survival for patients with non-Hodgkin's lymphoma because appropriate patients were selected and various agents were used to purge the marrow (35-42). In this report, patients with large B-cell lymphoma were selected on the basis of having the most unfavorable prognostic features we had previously identified (14,15). Patients with these features have done poorly when treated with conventional chemotherapy protocols or after ABMT when it is performed after relapse; only about 20% of the patients in either of these last two categories survived over 2 years (Fig 2). We have demonstrated an improvement in the survival of these patients when appropriately timed aggressive chemotherapy is followed by ABMT performed in the first CR or PR.

There are several factors to consider in evaluating the ABMT trials, and the results from each trial may be different because of the alteration in clinical management. The overall success of the ABMT protocols depends on the best management of many factors.

Role of Intensive Chemotherapy

Clinical trials of intensive chemotherapy are most promising in diseases in which an improved response rate can be expected with escalating doses of cytotoxic drugs, radiation, or both. One approach is to treat diseases that have a high response rate but also a high relapse rate. Such diseases are acute myeloblastic leukemia (AML), breast cancer, small cell cancer of the lung, neuroblastoma, and non-Hodgkin's lymphoma when patients have poor prognostic features. Drugs that have been found effective for each malignancy can then be considered for dose escalation trials. As would be expected from animal models, there is some evidence that higher doses of chemotherapy give better results than lower doses in AML (43), non-Hodgkin's lymphoma (44), Hodgkin's disease (45), and breast cancer (46). In dose escalation trials, the nonhematopoietic toxicities must also be evaluated and appropriate dose adjustments made to lower the morbidity and mortality from the procedure (25-27,47). Because of the reasons stated above, we elected to use large B-cell lymphoma as a model to evaluate the timing for intensive chemotherapy trials.

In 1980, when designing this protocol, we had been unsuccessful with four previous protocols in curing more than 20% of patients with poor prognostic features, and toxicity to the normal hematopoietic stem cells was also a concern. We had been encouraged by the remarkable success with our previous L-2 and LSA-L2 protocols (28,48) in pediatric lymphoma patients, and a variant of the L-2 and LSA-L2 protocols (i.e., L-17M) had shown improved results in adults with acute lymphoblastic leukemia (28). In our preliminary trials, the L-17M induction regimen caused minimal toxicity to the bone marrow in lymphoma patients, so we elected to use it for induction treatment for this protocol. Pretransplantation cytoreduction with hematoablative doses of HF-TBI and cyclophosphamide was chosen because of its known therapeutic benefit in allogeneic BMT (24,25). The drug doses chosen in our study were the same as the ones used for allogeneic BMT at this institution and are known to result in the need for hematopoietic rescue (25-27).

Method and Timing of Hematopoietic Stem Cell Harvesting

Bone marrow was usually harvested 16-40 days after the last dose of chemotherapy, when the blood counts had recovered from their nadir. The effects of treatment with various drugs prior to bone marrow harvest have been evaluated in an animal model system (49,50), but there is still a need for more information on how different drugs and drug combinations used for induction therapy before marrow harvest affect human progenitor cells. Most investigators try to transplant 2×10^8 mononuclear marrow cells per kilogram body weight, and the bone marrow is usually cryopreserved in liquid nitrogen in 10% DMSO using controlled-rate freezing (38-42,51). Upon thawing, the bone marrow has a tendency to aggregate (25,26,46). Marrow cryopreserved

in a mixture of 5% DMSO, 6% HES, and 4% human albumin can be easily cryopreserved by placing it directly into a -80°C freezer and subsequently transferring it into the liquid nitrogen tank (29). After rapid thawing, the marrow is immediately infused into the patient without any additional treatment. We obtained suitable engraftment in all our patients who survived the immediate posttransplantation period in this study. The cell dose and viability of the cells infused is detailed in Table 3. One patient's platelet count took 121 days to increase to over 50,000/mm^3, but none of the patients has required any blood product support after the first 40 days.

Role of Marrow Purging

The therapeutic benefit of bone marrow purging in the animal model systems is well established (30) and is easy to prove because of the availability of inbred strains of animals and the ability to work with defined numbers of malignant cells. Significant data are also available demonstrating the ability to purge human marrow in vitro (33,34,52,53), but the benefit in clinical trials is hard to prove because of the difficulty in detecting a minimal number (<1%) of neoplastic cells in the bone marrow. Perhaps newer techniques, such as preferential tumor cell growth (54,55), molecular probes, or specific antibodies against the neoplastic hematopoietic cells will help detect small numbers of lymphoma cells and also aid in developing better methods for purging. Another approach to proving the clinical usefulness of purging is to compare outcome of patients who receive purged marrow and that of those who receive unpurged bone marrow, but such studies require large numbers of patients and are subject to statistical uncertainties because of variability in prognostic factors.

Among the various chemotherapy drugs we evaluated, we found 4-HC to be the best for purging marrow of B-cell lymphoma (34) and acute myelogenous leukemia cells (33) in vitro. Under the optimum conditions developed, 4-HC was found to give a four- to five-log reduction of the neoplastic cells with only moderate damage to the normal bone marrow progenitors (32-34). We therefore decided to evaluate 4-HC for clinical phase I purging trials. So far the number of patients entered into this ongoing clinical trial is small, but we have found that the patients have had no problem with bone marrow engraftment after purging with 120 μM of 4-HC for 30 minutes. Clinical trials using 4-HC-purged marrow for patients with acute leukemia have also shown encouraging results (31,37).

Our results demonstrate that intensive chemotherapy and TBI followed by ABMT is well tolerated in the patient population treated. The therapeutic advantage is most significant for the patients who receive ABMT after induction therapy when they are in a CR or PR; 11 out of 14 patients in this group remain disease free with a median follow-up of 31+ months. This group of patients has done markedly better than our historical control group of patients with the same unfavorable prognostic features. More intensive

induction therapies were avoided because we were trying to avoid severe damage to the normal stem cells (49,50) prior to bone marrow harvest. Patients who continued on L-17M therapy after the induction therapy did not do as well as the patients who received ABMT in CR or PR, and only 5 of 13 are still alive. It is not possible to evaluate separately the therapeutic effectiveness of each component of the therapy (i.e., compare induction therapy with HF-TBI plus cyclophosphamide and ABMT rescue) in the protocol employed. About 40% of patients had CRs after L-17M induction, and some of those having PRs went on to CRs after ABMT. However, it is difficult to evaluate the completeness of remission in patients with large mediastinal or retroperitoneal masses, and some apparent CRs may be hard to confirm and others may be of short duration. Conversely, some PRs may in reality be shown to be CRs after surgical restaging. Only two patients (nos. 1 and 11) in this group had surgical restaging after ABMT. Both had large persistent mediastinal masses after ABMT, but surgical resection of these masses revealed only necrotic tissue, fibrosis, and no residual tumor. Both patients remain disease free 52 and 12 months after surgery. The results for ABMT when performed immediately after induction of a CR or PR are much better than when ABMT is performed in relapse. We therefore feel that timing of intensive chemotherapy followed by ABMT is of critical importance.

Marrow transplantation was well tolerated by patients who received ABMT after the induction therapy employed in our protocol. The major side effects are listed in Table 3. As mentioned above, patients chose whether to receive ABMT in CR or PR (group 1) or at relapse (group 2). The patient's decision was usually based on two factors: 1) the time involved (patients in group 1 received intensive therapy for approximately 6 months and no maintenance chemotherapy, whereas patients in group 2 received 2-2½ years of treatment with frequent hospital visits) and 2) their referring physicians' assessment of the therapeutic benefit of ABMT compared with that of continuing chemotherapy.

Although initial results are encouraging, modification of the regimen we used may prove necessary with more experience and follow-up. It may be possible to develop better methods of induction and ablative treatment as well as improved ex vivo purging methods. The results of ABMT also need to be compared to those achieved with the best drug combination protocols (7-13) that do not involve ABMT, and we have begun a new clinical trial to do that, comparing ABMT with MACOP-B (12) in treating patients with poor prognostic features.

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Treatment Strategies for Advanced Aggressive Non-Hodgkin's Lymphoma

R. Schots, P. Biron, C. Bailly, F. Mornex, and T. Philip

Before 1965, diffuse histiocytic lymphoma as defined by Rappaport (1) was a rapidly fatal disease. Only patients with stage I disease treated with radiotherapy had prolonged survival (2). During the 1970s, a significant proportion of patients with advanced diffuse histiocytic lymphoma could be cured by combination chemotherapy (3,4). CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) (5-11) probably remains the most commonly used drug regimen for this type of lymphoma. During the past decade, further progress has been achieved. Several more intensive chemotherapy regimens have produced better response rates and improved long-term survival rates (12-16). High-dose chemotherapy followed by autologous bone marrow transplantation (ABMT) has been performed in patients with non-Hodgkin's lymphoma in various conditions and at different stages of disease, and results have been reviewed (17). With regard to these new possibilities, we attempted to identify different treatment strategies for patients with diffuse, advanced non-Hodgkin's lymphoma. For this purpose, results from a single center (Centre Leon Berard in Lyons, France) were compared with those found in the literature.

PATIENTS AND METHODS

From May 1983 to May 1986, 35 consecutive patients, 16-83 years old (median, 53 years), were treated with an intensive induction regimen. None of them had had previous chemotherapy. The patients were histologically classified according to the Working Formulation Classification (18). Evaluation included at least blood examination, lymphangiography, ultrasonography, and computed tomography, two iliac crest bone marrow aspiration biopsies, and cytological examination of the cerebrospinal fluid. Histology and staging are summarized in Table 1. Two thirds of the patients had stage III or IV disease, and all patients with stage II disease had bulky or extensive extranodal lymphoma. Of the 14 patients with stage IV disease, nine had bone marrow involvement and two had CNS involvement.

Thirty patients were treated with methotrexate, bleomycin, Adriamycin (doxorubicin), Oncovin (vincristine), and dexamethasone (M-BACOD) (12) (200 mg/m^2 of methotrexate if patient was > 60 years); five had Burkitt's lymphoma and received an intensive regimen, currently used in Centre Leon Berard for pediatric patients (LMB 01-02) (20). The four patients with T-cell lymphoblastic lymphoma had a modified LSA_2L_2 (21) maintenance treatment.

Table 1. Patient Characteristics

Characteristic	No.	%
Number	35	100
Sex		
Male	19	54
Female	16	46
Stage (Ann Arbor)[a]		
II	12	34
III	9	20
IV	14	46
Histological subtype[b]		
Intermediate grade	22	63
Diffuse mixed	11	31
Diffuse large cell	7	20
Diffuse small cleaved	4	12
High grade	11	32
Immunoblastic	2	6
Lymphoblastic	4	12
Burkitt	5	14
Unclassified		
Pleomorph T cell	2	6

[a]*See Carbone* et al. *(19).*
[b]*See Rosenberg* et al. *(18).*

Survival was calculated from the date of diagnosis and remission duration from the date of first objective complete remission.

RESULTS

Of the 35 patients, 33 were evaluable for response. Of those, 20 (61%) reached a complete remission (CR), 6 (18%) had a partial response (PR), and 7 (21%) had primary refractory disease (PRD). Eighteen (90%) of the patients who achieved CR remain in continuous complete remission (median follow-up, 12 months; range, 1-38 months). Of the six patients who achieved PR, two received treatment on an alternative conventional-dose regimen including mitoguazone, ifosfamide, methotrexate, and etoposide (MIME) (22), and three received high-dose chemotherapy and ABMT. A fourth patient was also grafted but had progressive disease before high-dose chemotherapy was started. Five (83%) of the patients who achieved PR survive disease-free with a minimal observation time of 10 months. Of the seven patients with progressive disease, only one (14%) survives. He achieved CR after MIME and boost radiotherapy. At this time, 11 patients have died: six had PRD, one had PR, and four died early. Of those four, two died in CR and two were not evaluable for response. Two of them died suddenly, probably without relation to therapy, and two died of toxicity (one with sepsis and one with bilateral pneumonitis and cerebral hemorrhage). We show in Figure 1 survival according to response to initial treatment. Twenty-three (88%) of the responding patients remain disease free after a median observation time of 14 months. Overall survival is 69% (Fig 2) after a median observation time of 18 months. Survival according to age clearly

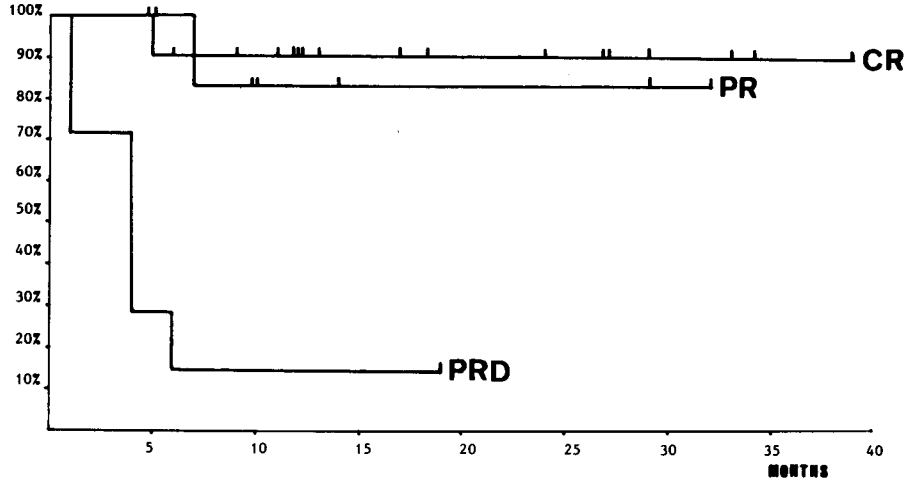

Figure 1. *Survival according to response to initial treatment (n = 33) (CR, complete remission; PR, partial remission; PRD, primary refractory disease).*

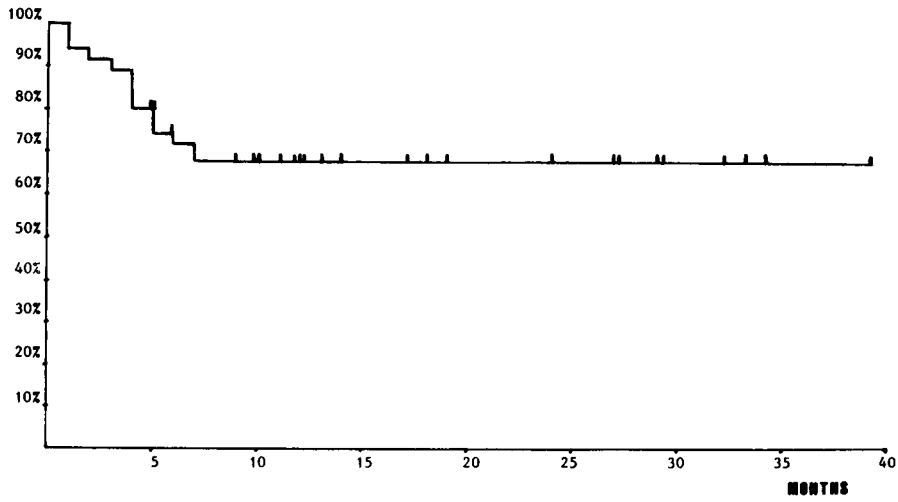

Figure 2. *Overall survival (n = 35).*

demonstrates a trend toward poor prognosis for patients older than 65 years. Only one survives (at 29 months), and all others died of progressive disease. Five of the six patients who died had significant dose reductions in order to limit treatment-related toxicity.

DISCUSSION

Based on the CHOP regimen (5-11), several other "first-generation" regimens (4,23-34) are capable of producing long-term survival in advanced aggressive non-Hodgkin's lymphoma. With those regimens, CR is reached in 40-60% of patients, and relapse occurs in 25-50%. The cure rate is estimated to be 30-40%. During the past decade, further progress has been achieved. Several more intensive chemotherapy regimens (Table 2) have produced better response rates and improved long-term survivorship. Adding methotrexate reduced CNS relapse significantly (12). Initial intensification (13,15,16) produced higher CR rates; sequential chemotherapy and late intensification (13-15) resulted in prolonged duration of remission. More aggressive treatment is limited by toxicity (Table 2) and raises treatment-related morbidity and mortality. Patients may have a prognosis so poor it justifies using a potentially toxic treatment. Several of these prognostic factors recognizable at diagnosis have been identified but not completely confirmed (35-38). Even among subgroups with poor prognoses heterogeneity prevents data from being comparable. In contrast, everybody agrees that response to initial treatment has a constant and predominant prognostic significance.

Patients who respond completely carry a good prognosis. Complete remission rates have improved with the newer, more intensive regimens (Table

Table 2. Intensive Treatment Regimens

Regimen	CR	Relapses	PR	Survival	Severe Toxicity	Deaths Owed to Toxicity
MBACOD Skarin *et al.* (12)	73/95 (77%)	19 (26%)	14 (15%)	59%	Infections (6%) Mucositis (10%)	6%
LNH-80 Cabanillas *et al.* (14)	84/97 (87%)	18 (21%)	9 (9%)	65%	GIT (22%) Infections (26%) Renal toxicity (8%)	6%
Coiffier *et al.* (15)	46/56 (82%)	9 (20%)	4 (5%)	71%	Cardiotoxicity (7%) Fatal sepsis (5%)	5%
ProMACE-MOPP Fisher *et al.* (13)	55/74 (74%)	10 (18%)	16 (22%)	65%	Mucositis (16%) Fatal sepsis (11%)	14%
MACOP-B Klimo and Connors (16)	51/61 (84%)	4 (8%)	10 (16%)	75%	Mucositis (25%)	None

Abbreviations: CR, complete remission; PR, partial remission; MBACOD, methotrexate, bleomycin, Adriamycin (doxorubicin), cyclophosphamide, Oncovin (vincristine), dexamethasone; GIT, gastrointestinal; ProMACE-MOPP, methotrexate, doxorubicin, cyclophosphamide, etoposide, prednisone plus mechlorethamine, Oncovin (vincristine), procarbazine, prednisone; MACOP-B, methotrexate, Adriamycin (doxorubicin), cyclophosphamide, Oncovin (vincristine), prednisone, bleomycin.

Note: LNH-80 is a protocol number assigned at Centre Leon Berard. Three phases taken from abstract of [15] are as follows: 1) induction with three courses of an intensified CHOP-Bleo regimen (cyclophosphamide, doxorubicin, vindesine, methylprednisolone, bleomycin); 2) consolidation with cytarabine, followed by high-dose methotrexate and folinic acid rescue, then asparaginase; and 3) final intensification with two courses of CVAP-Bleo regimen (cyclophosphamide, teniposide, cytarabine, methylprednisolone, bleomycin).

2). Moreover, relapses beyond 24 months are rare. If we look at the data of several representative series (9,12-16,35,39,40), we see that only 7 of 88 patients (8%) relapsed after 24 months (two had nodular histological findings at relapse) (12).

In a large series of patients treated with CHOP (5), no new responses were observed following the third course of therapy, and only 8% of the responding patients went from a PR to a CR. Switching to an alternative drug combination may achieve additional complete remissions. With the ProMACE-MOPP regimen (methotrexate, Adriamycin [doxorubicin], cyclophosphamide, etoposide, prednisone plus mechlorethamine, Oncovin [vincristine], procarbazine, prednisone) (13), 27% of patients—some of them having evidence of tumor progression—reached CR after switching to MOPP. In Cabanillas and coworkers' series (14), eight who had partial responses after three CHOP courses entered CR after switching to a second (four patients) and even a third (four patients) drug combination, representing 9% of all CR patients.

Most patients who are partial responders have a greater than 90% tumor reduction or clinical remission with microscopic residual disease. Even in those conditions, rapidly progressive disease develops and survival is generally short. Continuation of the same six-drug combination after three to four courses permits additional achievement of CR in about one third of the patients finally reaching CR; however, remission duration is significantly shorter in those patients (41). From these observations it can be concluded that patients with PR after three to four courses of treatment undoubtedly carry a poor prognosis. Continuation of the same regimen is probably not very useful. Finally, patients with PRD invariably have a poor prognosis. Median survival is generally less than 10 months.

Regimens containing ifosfamide and etoposide (22) are capable of producing durable remissions in a significant number of patients who relapse after successful first-line treatment. Results are poorer in patients with PRs or PRD; only about 10% achieve CR. Logically, patients who do not obtain CR or relapse after intensive initial therapy are less likely to be rescued. The precise role of conventional salvage therapy with regard to the newer, more intensive regimens needs further clarification.

ABMT has been performed in patients with non-Hodgkin's lymphoma in various conditions. Results have been reviewed (17), and the optimum timing of ABMT discussed. It appears that relapsed patients who are still responsive to conventional-dose chemotherapy benefit most by ABMT. This finding has recently been confirmed by a multicenter retrospective study (T. Philip *et al.*, unpublished data) pooling data on 100 patients with relapsed or resistant non-Hodgkin's lymphoma who underwent high-dose chemotherapy and ABMT. It was shown that almost 40% of patients who achieved the so-called sensitive relapse may reach a 2-year disease-free survival. A prospective randomized study is planned to compare these results with those from a study of conventional-dose salvage regimens.

Some encouraging data from studies of small numbers of patients (42-46) suggest a possible role for ABMT for patients who respond partially to initial treatment. Unfortunately, in spite of relatively high response rates, high-dose chemotherapy and ABMT do not improve survival rates for patients with PRD. To retrospectively evaluate the possible role of ABMT in PR, we collected data on ten consecutive PR patients who were treated at Centre Leon Berard (Table 3). All patients had diffuse advanced non-Hodgkin's lymphoma (six with bulky stage III disease and three with stage IV disease, including two with CNS involvement) and were initially treated by a relatively intensive regimen (M-BACOD or LMB 01-02) (20). Only patients with no evidence of tumor progression during the induction phase are included. Only one patient died with progressive disease (at 7 months). Seven of eight patients who received high-dose chemotherapy and ABMT are alive and well with a median follow-up of 24 months (range, 10-49 months). The one patient who died also underwent ABMT but disease progressed before high-dose chemotherapy was started. Two patients received only MIME (22) and remain disease free at 10+ and 30+ months. Of course, these are data on only 10 patients, and the mixture of adult and pediatric patients, as well as the high proportion of patients with Burkitt's lymphoma (3 of the 10 patients) may be criticized. Although preliminary, the results of the Centre Leon Berard strategy for the treatment of advanced aggressive non-Hodgkin's lymphoma prompt the following comments: First, note that five of six of the PR patients achieved CR after switching to an

Table 3. Salvage Treatment After Partial Remission

Patient (age/sex)	Salvage Treatment	Response	Evolution (months of follow-up)
3/M	BACT (IGR)	CR	CCR (34)
4/F	BEAM	CR	CCR (10)
7/M	BEAM/TBI	CR	CCR (12)
11/M	Busulfan Cyclophosphamide	CR	CCR (19)
36/M	BEAM	CR	CCR (24)
52/M	—	PD	Died (7)
52/F	BEAM	CR	CCR (10)
57/M	BACT (IGR)	CR	CCR (49)
61/M	MIME	CR	CCR (10)
70/M	MIME	CR	CCR (30)

Abbreviations: BACT, BCNU (carmustine), ara-C (cytarabine), cyclophosphamide, 6-thioguanine (Institut Gustave-Roussy); CR, complete remission; CCR, continuous complete remission; BEAM, BCNU (carmustine), etoposide, ara-C (cytarabine), melphalan; TBI, total body irradiation; MIME, mitoguazone, ifosfamide, methotrexate, etoposide.

alternative regimen, and three of the complete responders received high-dose chemotherapy and ABMT. This procedure may be of interest in PR patients, even after relatively intensive initial treatment. Second, 88% of the responding patients (those with CRs or PRs) finally attain CR and remain disease free with a median follow-up of 14 months. If 20% relapse (accepted rate), a long-term survival rate of around 70% is to be expected. Third, only PRD patients carried a poor prognosis, and most of them were more than 65 years old. Apparently patients of this disease status and age cannot tolerate a full-dose regimen to obtain good response.

CONCLUSIONS AND TREATMENT STRATEGIES

1. Intensive induction therapy probably improves survival in advanced aggressive non-Hodgkin's lymphoma.
2. For at least 50% of the patients, exposure to relatively toxic initial therapy is not necessary to achieve long-term survival.
3. Patients with poor prognosis should be selected early according to response to initial therapy.
4. High-dose chemotherapy with ABMT as consolidation of first CR after intensive initial treatment is difficult to justify.
5. High-dose chemotherapy with ABMT is promising as salvage treatment for those with PRs and those in relapse. Its value in comparison with conventional-dose salvage regimens remains to be investigated.

With the above conclusions in mind, two possible strategies for advanced aggressive non-Hodgkin's lymphoma can be discussed. First, consider a strategy dominated by intensive initial treatment in which a significant number of patients will be lost to toxicity or suffer high treatment-related morbidity. The better results of those regimens should be confirmed by randomized studies. The possibility of salvage after PR or relapse in this strategy is probably limited but remains to be investigated. Second, consider a strategy dominated by the increased possibilities of salvaging patients who had PR or relapsed after a less toxic traditional first-line treatment (Fig 3). Most patients in PR and up to 40% of

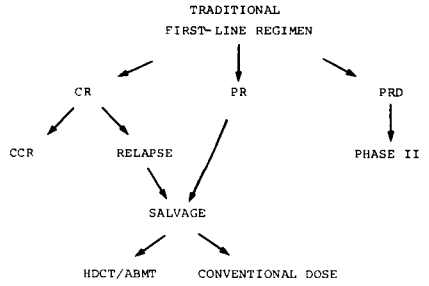

Figure 3. *Flow chart of possible outcomes after traditional first-line therapy (CR, complete remission; PR, partial remission; PRD, progressive refractory disease; CCR, continuous complete remission; HDCT/ABMT, high-dose chemotherapy and autologous bone marrow transplantation).*

patients in relapse are possibly salvaged by high-dose chemotherapy with ABMT. However, the value of such chemotherapy and ABMT as a salvage treatment in comparison with alternative conventional-dose regimens has yet to be determined. Finally, for PRD patients the salvage possibilities are limited: their therapy options should be examined in phase II studies.

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Non-Hodgkin's Lymphoma

S. Gulati and F. Cabanillas, Chairmen

DR. C. GORIN: I would first like to ask Dr. B. Barlogie about the quality of the marrow harvested for transplantation. My suspicion would be that this marrow is very poor since the marrow of his patients was heavily treated in the past. Second, I would like to know the degree of plasma cell contamination. Third, do you consider treating the marrow in vitro before infusion, or do you consider collection of peripheral blood stem cells?

DR. B. BARLOGIE: In response to the third question—first, I think we like to establish a baseline, no purging initially. This is based on the limited experience in this advanced stage of the disease, particularly in the one patient who had 30% tumor cells. She remains in bone marrow remission 14 months after harvest. The serum protein is still declining and is less than 0.5 g%. The quality of the marrow was initially poor. Many of these patients had a long-term disease history, from 2 to more than 6 years. One of the patients who received an allogeneic transplant is one of our longer survivors, with a history of more than 10 years. The VAD regimen, which is not very marrow toxic at all, is usually the induction regimen given before harvesting. In the group of patients who did not receive total body irradiation, but just melphalan, the marrow quality was much poorer than in the more recent patients.

With regard to the treatment strategy, I believe that when one looks at the prognostic factors in terms of achieving good remission with high dose

melphalan-type regimens, we found that the duration of prior unresponsiveness was the most important variable. Factors that were very important, such as very unfavorable DNA and RNA features, became less significant, so we had sufficient evidence that these two regimens were non-cross-resistant and that is why we are combining them now.

DR. D. BUCKNER: I would like to ask Dr. S. Gulati a question about the patients in first relapse whom he performed transplants on. Were these truly first-relapse patients? Did you try to induce a second remission? Because that's really in variance with our results, we found that early relapse in lymphoma, acute myelogenous leukemia, and other diseases a favorable time for transplantation.

DR. S. GULATI: Yes, initially we would take any relapse and the first three or four patients with bulky disease would receive transplants. Subsequently, and especially now, we have a revised protocol in which we try to control the disease by other combination chemotherapies. So, for example, if a patient comes to us after an unsuccessful regimen of CHOP we try to reinduce him with other combination chemotherapies before we perform a transplant. By performing the transplant upfront, the success rate is 10-15%. When given upfront, we feel that two transplants might be necessary. Using the same regimen after reinduction, the success rate is 30-40%. The numbers are too small to draw definite conclusions.

DR. G. CORNWELL: Dr. Barlogie, of the five patients you have treated with the most aggressive therapy, you had three who were partial responders. Has there been enough time to see what those three patients are going to do with their disease?

DR. BARLOGIE: The one patient who had 30% plasma cells and the elderly gentleman with 6-8% plasma cells both continue to show a progressive decline in the myeloprotein. This is hovering around 0.3-0.7 g% and the hemoglobin continues to rise gradually but continuously. They are not in complete remission. The only complete remissions were observed in conjunction with allogeneic transplantation.

DR. T. PHILIP: A question for you, Dr. Cabanillas. You have 52 patients who have lymphoma and are being treated with DHAP, and you report a disease-free 2-year survival rate of 15%. Is this 15% of the patients who achieved complete remission or of the total 52 patients?

DR. F. CABANILLAS: That is 15% of the group of patients who had the favorable characteristics that I showed: no bone marrow involvement, less than 60 years old and having had a prior complete response to frontline therapy. So those are patients who are relatively similar to the ones who will have autologous bone marrow transplantations; that is the reason they were selected. So that is 15% of the whole group of patients.

DR. PHILIP: Then the reference of conventional chemotherapy is 15% at 4 years?

DR. CABANILLAS: Correct.

DR. F. BRITO-BABAPULLE: Dr. Philip, the conventional way of staging patients with Burkitt's lymphoma is to stage A, B, C, and D, depending on whether they have abdominal or extra-abdominal disease. The reason for doing this is because extra-abdominal disease has a good prognosis. Now you stage them differently. Could I ask whether your group of patients who did not respond had abdominal or extra-abdominal disease? And secondly, in your review of your current treatment for Burkitt's lymphoma, I notice you didn't mention the studies which you published in *Blood* in 1984, in which you reported a complete remission of 80% in Burkitt's and other poor-prognosis large cell lymphomas.

DR. PHILIP: There are a lot of questions. The first one is to address the lymphoma classification. There is a Ziegler classification (A, B, C, D), which is commonly used in Africa but not in Europe and the United States. The Murphy classification is generally used in the United States, and it considers stage III as large abdominal masses, while stage IV is the same kind of tumor, with bone marrow or CNS involvement. In my opinion, the Ziegler classification is interesting. We published in the *British Journal of Haematology* that, within stage III, you can make the division between the good and the bad stage III. The good one corresponds with Ziegler's stage C, the one with only one abdominal mass, whereas the one with several masses, stage D, has the bad prognosis of Murphy's stage III. Secondly, it was not my objective to make a complete review of the treatment of Burkitt's lymphoma. The survival rate, which is 75%, has been published by several groups. The difference between pediatric and adult Burkitt's lymphomas is not significant. Therefore, the indication of bone marrow transplantation in Burkitt's lymphoma is limited.

DR. CABANILLAS: We have time for one more question.

DR. A. HAGENBEEK: I would like to ask something about the message delivered by Dr. Philip on the intensive induction chemotherapy regimens in intermediate- and high-grade lymphomas. It might be of interest to those of you who are not aware of those data that since 1982, Bonadonna has been running a randomized prospective trial in patients with high-grade lymphomas (stages III and IV) comparing conventional CHOP with ProMACE. The studies have been going on now for 4 years and there is not one single difference, neither in remission rate nor in disease-free survival.

DR. CABANILLAS: Thank you. I think we are going to end this session and go on to the next one, which will be chaired by Drs. Philip and Spitzer.

IIC. International Randomized Study in Relapsed Adult Intermediate- and High-Grade Lymphoma

An International Randomized Study of Relapsed Diffuse Intermediate- and High-Grade Lymphoma in Adults

T. Philip, J. Armitage, G. Spitzer, F. Cabanillas, W. Velasquez, S. Jagannath, T. Hagenbeek, P. Biron, J. Cahn, G. Zagars, P. Carole, and F. Chauvin

The major objective of this international study of diffuse intermediate- and high-grade lymphoma is to determine the rate of disease-free survival and duration of response for a group of adults who are in relapse. Patients with non-Hodgkin's lymphoma will be selected for the study on the basis of chemosensitivity to the DHAP (dexamethasone, cytarabine, cisplatin) salvage regimen. Patients will be randomized to undergo an effective conventional rescue protocol followed by involved-field radiotherapy or to receive involved-field radiotherapy plus high-dose chemotherapy with autologous bone marrow transplantation (ABMT). A secondary objective of the study is to determine the crude survival in both groups and to confirm that the strategy with ABMT does not lead to more than 15% mortality.

BACKGROUND FOR THE PRESENT PROPOSAL

A retrospective study of data from the France Autogreffe Study group and London group was performed in July 1983 (1-3). From these data, as

shown in Figure 1, some conclusions could be drawn on the following points. First, patients in resistant relapse (RR) (i.e., disease still progressing at time of ABMT) will usually not be cured by massive therapy. However, responses were observed in 73% of the cases. But as shown in Figure 1, only 1 of 16 patients is still alive 3 years post-ABMT (6%). Second, patients in nonresistant relapse (NRR) (i.e., all other patients, excluding those with stable disease and those with a minor response on rescue protocol) had a long-term survival rate of approximately 50%. Our initial data were drawn from 15 of 19 patients who had relapsed on therapy (Fig 1). (It was clearly a group with a very poor prognosis.) No difference in outcome was observed between patients achieving partial remission (PR) or complete remission (CR) prior to ABMT if CR was obtained after ABMT. This work prompted the conclusion that patients in relapse have to be separated into two groups: those in RR (patients with progressive or stable disease or those with a minor response on salvage chemotherapy) and those in NRR or sensitive relapse (SR) (patients achieving partial or complete response within the first two courses of salvage chemotherapy).

Two major criticisms have been leveled at this study. The first is that this retrospective analysis included both adults (two thirds) and children (one third). The second is that there was a high proportion of childhood non-Hodgkin's lymphoma of the Burkitt type (12 of 42 had Burkitt's lymphoma). Of the remaining 30 patients, 15 had intermediate-grade and 15 had high-grade non-Burkitt's lymphoma. As shown in Figures 2 and 3, a study by

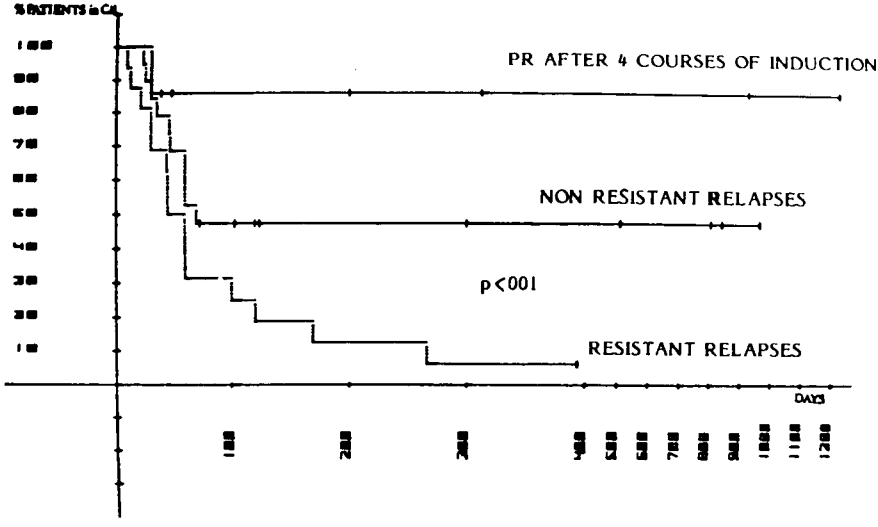


Figure 1. *Disease-free survival for 42 patients with non-Hodgkin's malignant lymphoma (CR, complete remission; PR, partial remission). Reprinted, by permission, from Philip* et al. *(25).*

Goldstone (4) later concluded that no statistically significant difference was observed in outcome between adults and children (Fig 2) or between patients with Burkitt's and those with non-Burkitt's lymphoma (Fig 3). The concept of RR and NRR has been subsequently confirmed in 1) a review of 42 cases in France of Burkitt's lymphoma (Fig 4) (5); 2) a review of 42 cases of adult diffuse lymphoma from France and England (Fig 5) (3); and 3) a review of 39 patients with non-Hodgkin's lymphoma from Houston and Omaha (Fig 6; Table 1) (6). In addition, in 1986 data from bone marrow transplantation centers in Europe and the United States were pooled to determine the outcome of ABMT in adult patients with relapsed diffuse intermediate- or high-grade non-Hodgkin's lymphoma (excluding those with Burkitt's lymphoma) and to identify the prognostic significance of response to therapy preceding the bone marrow transplant procedure. One hundred patients were treated with high-dose chemotherapy alone (61 patients) or high-dose chemotherapy plus total body irradiation (TBI) (39 patients). The median age was 35 years and the median Karnofsky performance score was 80%. Thirty-four patients had disease that was primarily refractory to chemotherapy (they never achieved CR) and had progressive disease. Sixty-six patients achieved CR with primary chemotherapy but later relapsed. After receiving further (salvage) chemotherapy at traditional doses, 22 patients had no response or disease progression (i.e., RR), and 44 patients had PRs or CRs after salvage chemotherapy (SR).

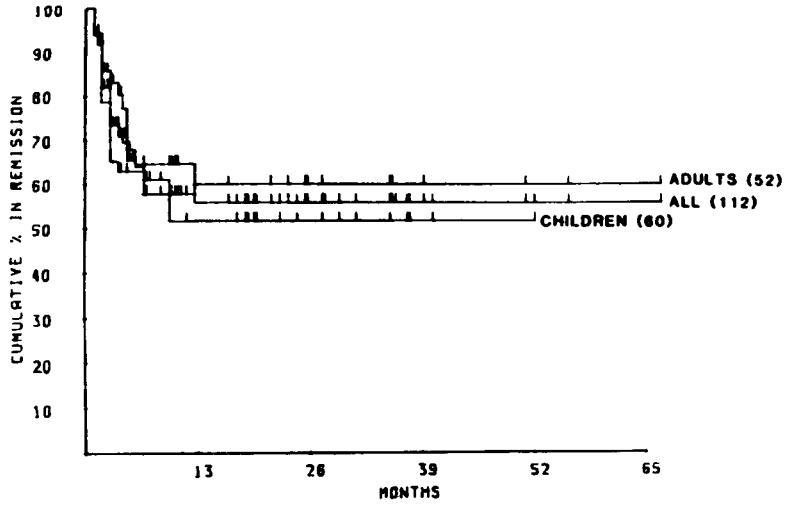


Figure 2. *Disease-free survival of children and adults with non-Hodgkin's lymphoma who underwent autologous bone marrow transplantation in a European Bone Marrow Transplantation Group trial (data compiled January 1984). Reprinted, by permission, from Goldstone (4).*

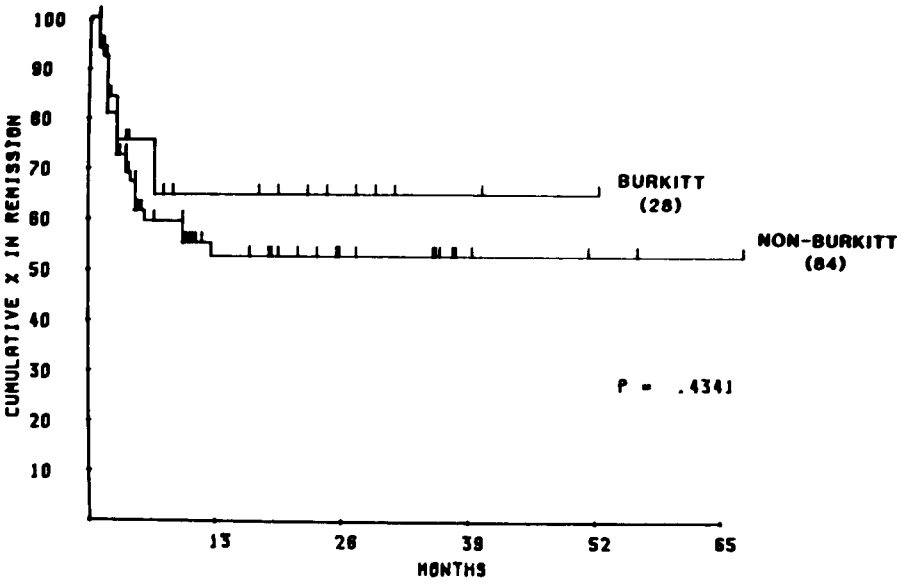


Figure 3. *Disease-free survival of patients with Burkitt's and non-Burkitt's lymphoma (data compiled January 1984). Analysis showed there was no statistically significant difference between the two groups.*

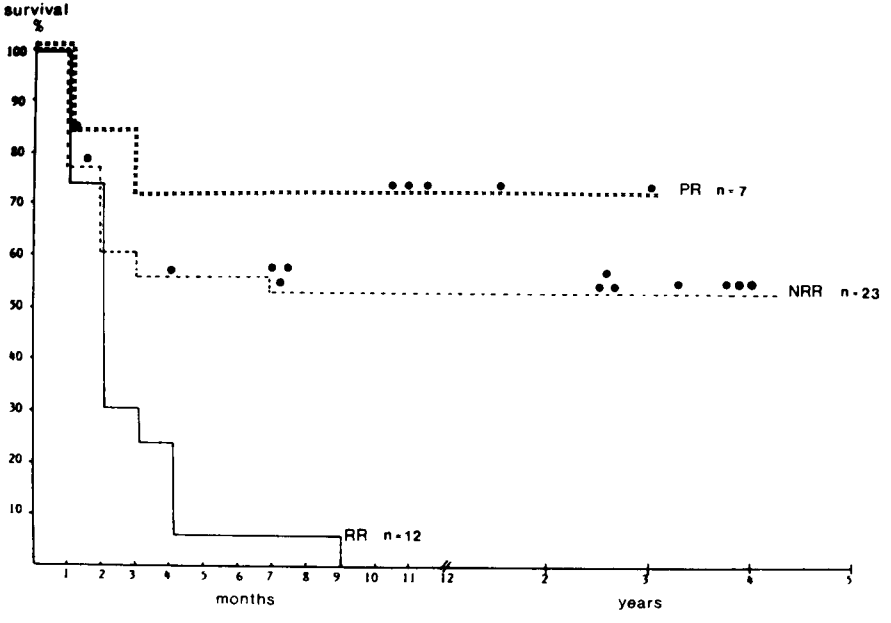


Figure 4. *Survival of 42 children with Burkitt's lymphoma in a 1985 French study (PR, partial remission; NRR, nonresistant relapse; RR, resistant relapse).*

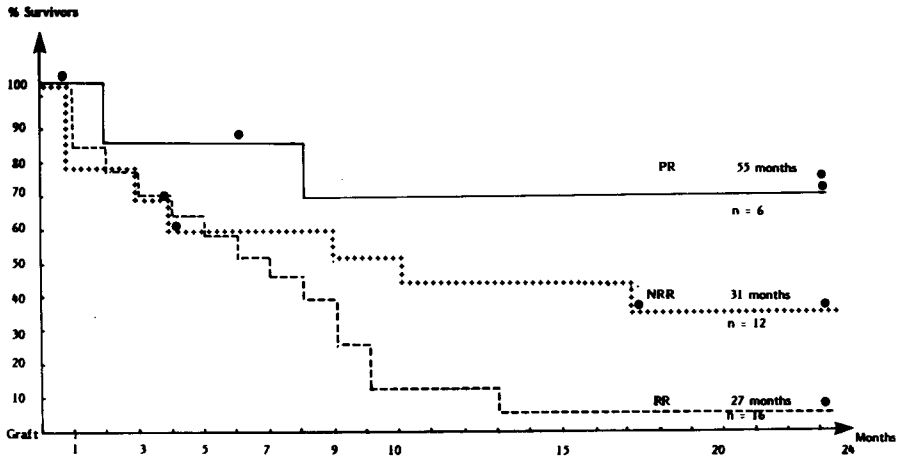


Figure 5. *Disease-free survival of adult patients with diffuse non-Hodgkin's malignant lymphoma who were treated in relapse with autologous bone marrow transplantation (PR, partial remission; NRR, nonresistant relapse; RR, resistant relapse).*

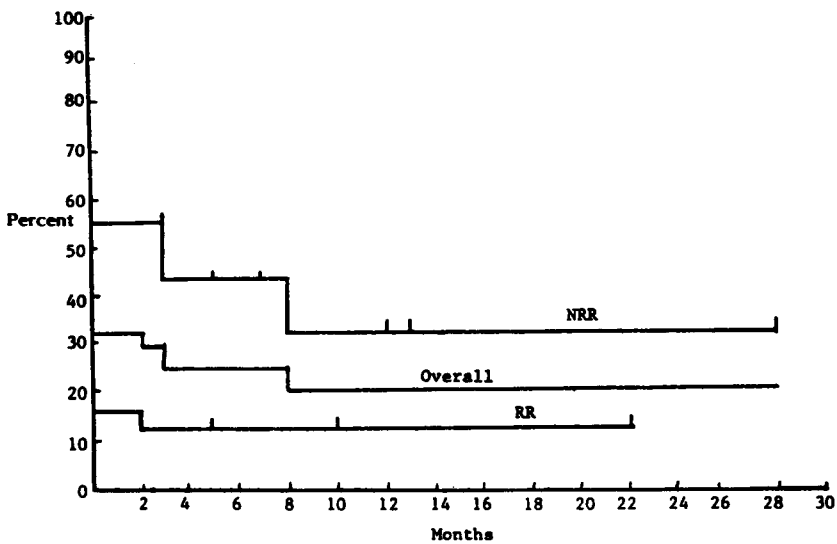


Figure 6. *Complete remission survival by treatment response for 39 adult patients treated in the United States (NRR, nonresistant relapse; RR, resistant relapse) (J. Armitage* et al. *and G. Spitzer* et al., *1985, unpublished data).*

Table 1. Treatment Response

Classification	No. of Patients	Complete Tumor Response		Complete Remission	
		No.	%	No.	%
NRR	16	13	81	9	56
RR	23	4	17	4	17
Total	39	17	44	13	33

Source: J. Armitage et al. *and G. Spitzer* et al., *1985, unpublished data.*

Note: Investigators compared these two groups (those in nonresistant relapse with those in resistant relapse) on complete tumor response (Yates $\chi = 13.22$; $P < .002$) *and complete remission (Yates* $\chi = 4.82$; $P = .03$).

Abbreviations: NRR, nonresistant relapse; RR, resistant relapse.

The actuarial 2-year disease-free survival for the entire group was 20%, with the last death at 31 months (median observation time, 33 months) (Fig 7). Disease-free survival was significantly related to previous response to chemotherapy. The 2-year disease-free survival was 14% in the RR group and 38% in the SR group (Fig 8). Patients who never achieved CR would not survive 2 years. Patients who had achieved a CR in initial chemotherapy had a superior disease-free survival rate after ABMT when compared with patients who never achieved CR (Fig 9). Patients with SR had a better disease-free survival rate than did patients with RR. Outcome was not affected by treatment regimen or histological grade. Whether relapse occurred on or off therapy was also not of significance, but the probability of being in SR was significantly higher for those who relapsed while off therapy. In conclusion, it appears that prior response to chemotherapy is an important prognostic variable in patients with intermediate- or high-grade non-Hodgkin's lymphoma undergoing ABMT. These results explain why patients with RR or no previous CR must be excluded from the randomized study.

The question of whether cures can be obtained with conventional salvage regimens without ABMT remains unanswered. There are reports of occasional long-term survivors after relapses treated with MIME (mitoguazone, ifosfamide, methotrexate, etoposide) or DHAP (7-10; W. S. Velasquez, M.D., 1986, personal communication). A randomized study is now urgently needed to compare both treatment modalities. Several conclusions can, however, be drawn from the literature.

The response rate of high-dose therapy and ABMT (72% of patients with CR or PR) in the group of patients who never achieved CR and whose disease is progressing on salvage therapy is a clear argument in favor of the dose-effect relationship in non-Hodgkin's lymphoma and in favor of investigating ABMT procedures (6,11). The significantly better survival rate for patients still responding to conventional rescue prior to ABMT raises the possibility

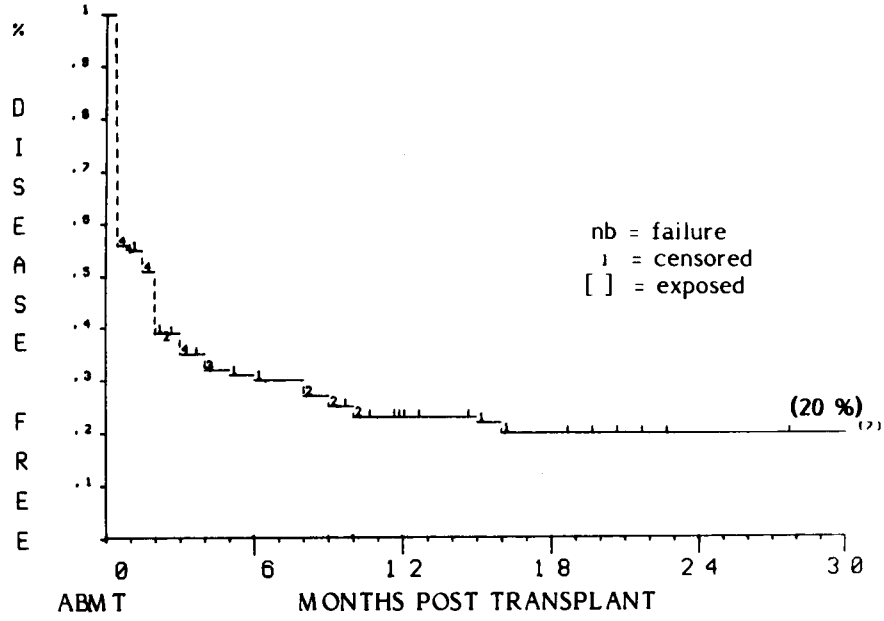


Figure 7. *Actuarial 2-year disease-free survival for 100 patients whose data were pooled from bone marrow transplantation centers in Europe and the United States.*

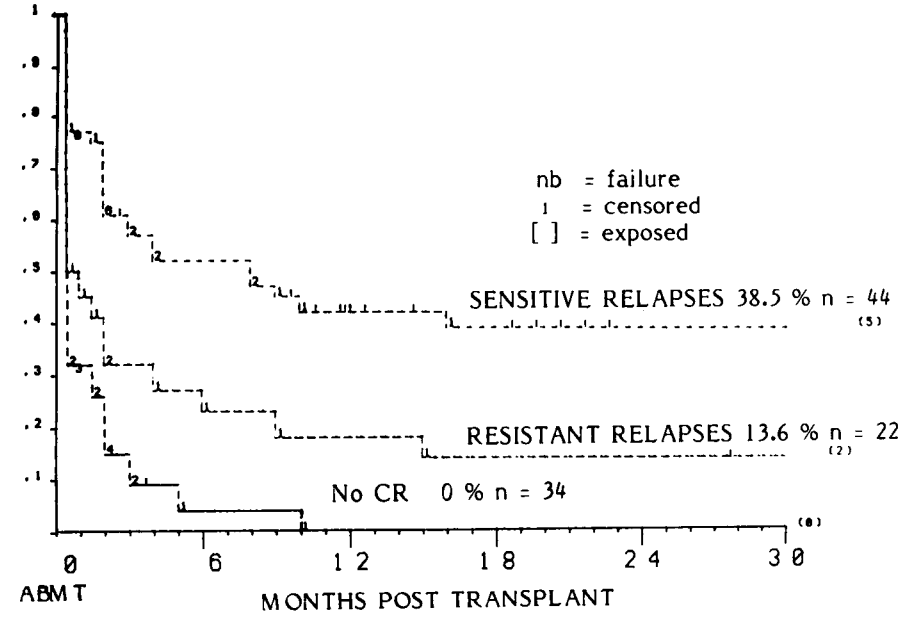


Figure 8. *Actuarial 2-year disease-free survival according to response to initial or salvage chemotherapy for 100 patients whose data were pooled from bone marrow transplantation centers in Europe and the United States (CR, complete remission).*

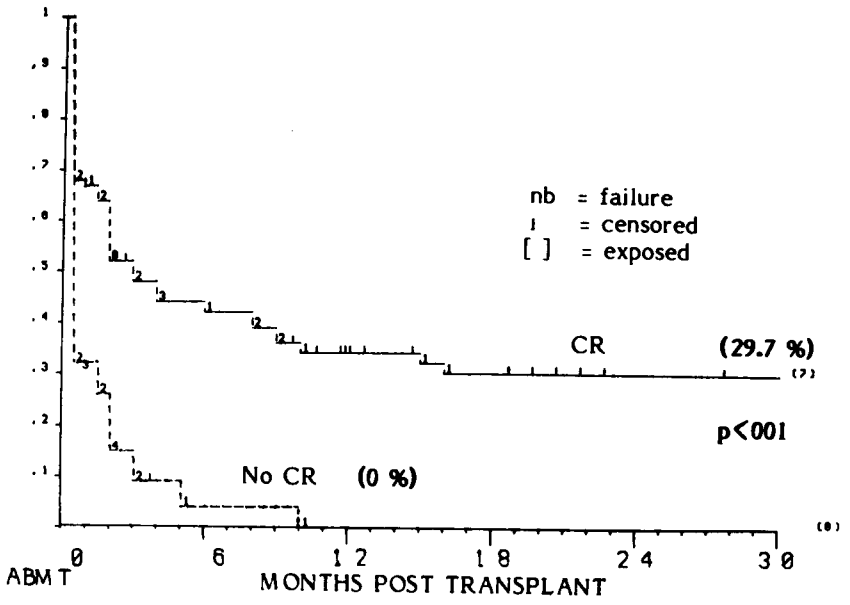


Figure 9. *Outcome after autologous bone marrow transplantation (ABMT) by whether or not patients had reached complete remission (CR) with initial therapy for 100 patients whose data were pooled from bone marrow transplantation centers in Europe and the United States.*

that conventional rescue and high-dose therapy with ABMT could be complementary procedures. Their combination in treating a group of relapsed patients who previously reached CR may have the synergism to push the expected survival rate to at least 40% (12-33). The difference between MIME or DHAP, which produce an overall expected survival in relapsing patients of approximately 5%, and ABMT (20-30% survival rate) cannot, however, be used in support of transplantation because of selection bias (patients whose performance status is generally good, who have no marrow or CNS involvement, and who have minimal disease are generally selected for transplantation programs). As an example, the 44 of 66 responding patients in our review of the literature (67%) indicate a large selection bias, considering the maximum expected response rate is 40-50% with MIME in such a group of patients. Several questions are still unclear and cannot be answered on the basis of the world literature review: these are the role of TBI, the role of involved-field radiotherapy before or after ABMT, the indication for purging procedures (34-36), and whether allogeneic marrow should be used if available. In our study 39 patients received a regimen containing TBI, whereas 61 did not. The two groups are comparable in the number of primary refractory cases, histological findings, and bulky disease. The actuarial survival at 2 years is 20% in both groups. In the group with primary refractory

disease and RR, response rates are nearly identical with and without TBI (68% compared with 72%). Also, 75% of the relapses were isolated and occurred primarily at the site of initial lymphoma involvement prior to the salvage therapy, indicating that local control is a major factor. Involved-field radiotherapy (15-20 Gy) has been advocated by Phillips *et al.* (37,38), following a nonrandomized comparative study favoring this strategy. This question will be considered in our randomized study (see below).

In this series, patients with marrow involvement were excluded, and marrow relapses were not a major problem following ABMT. It is impossible to distinguish relapse owed to failure to eradicate the tumor from relapse owed to reinfused tumor cells. However, experience with allogeneic bone marrow transplantation (BMT) suggests that failure of the preparative regimen is responsible for most relapses (39-43). Encouraging results have been reported with marrow purging in adult patients with B-cell non-Hodgkin's lymphoma (44,45), and we would advocate that this issue be evaluated in a future comparative study. (If regimens without TBI are used, it is possible that spontaneous marrow recovery could occur; therefore, intensification therapy without ABMT is also a possible alternative.) However, in this first international collaborative study, no attempt will be made to purge marrow prior to ABMT.

In summary, our group's background work confirms that patients who did not achieve CR on first-line therapy are rarely curable with current high-dose therapy regimens. In patients who relapse after a first CR, a significant cure rate can be achieved. Patients still responding to conventional doses of chemotherapy have an outcome superior to that of patients whose tumors are resistant to rescue protocols (38% compared with 14% 2-year disease-free survival). Despite strong suggestions from the literature that high-dose therapy with ABMT is superior to conventional modalities of treatment for relapsed non-Hodgkin's lymphoma, a randomized study to clearly determine whether ABMT adds to survival in a group of potentially curable patients is needed because of the various selection criteria that until now clearly favored the ABMT patients. The conclusions of these studies are the following:

1. Massive therapy and ABMT should not be tested in a randomized study of patients who never reach CR (Fig 8).
2. All patients whose disease has a diffuse histology, either intermediate or high grade, at first diagnosis are eligible (cases of transformation from nodular histology are excluded).
3. With the exclusion from the study of patients who never reach a CR in the course of their disease, the group in SR is the most suitable for comparing chemotherapy alone with the ABMT strategy.
4. Purging marrow is not a major issue for this group of patients. The study will concern only selected patients with normal marrow, and no purging procedures will be allowed in the ABMT arm of the study.

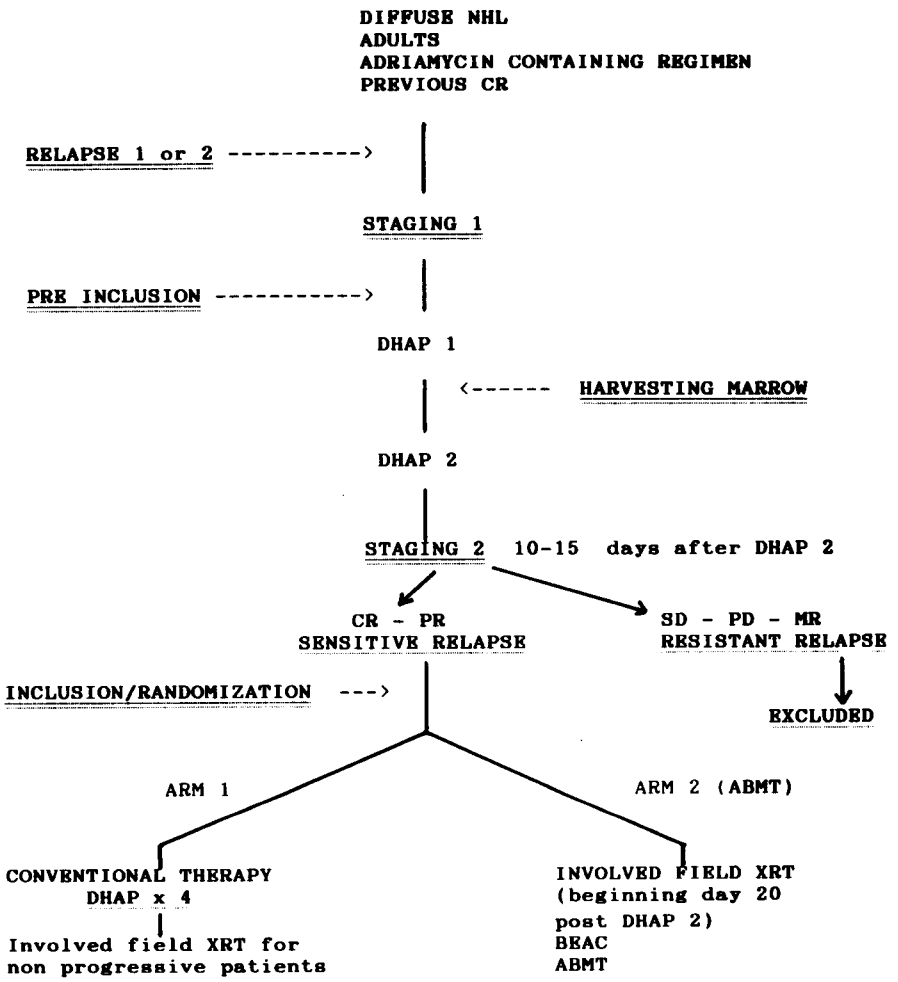
5. As shown in the figures named above, an early plateau was reached by most of the patients who achieved continuous CR; therefore, no maintenance therapy will be given after ABMT. Pre-ABMT involved-field radiotherapy on an "iceberg" of initial localization of the relapses will be recommended because 75% of the relapses are caused by recurrence of local disease.
6. In the chemotherapy arm of this study and in order to avoid any delay in the DHAP regimen timing, involved-field radiotherapy will be recommended at the end of the program for patients whose disease is not progressing (see Fig 10).
7. It is our intention that patients from our group or those referred to our group at time of relapse (they should have reached CR previously) with intermediate- or high-grade adult non-Hodgkin's lymphoma, first, will be classified as being in RR or SR after two courses of DHAP; second, will be randomized, if in SR, to a conventional rescue protocol or ABMT. Third, this protocol will concern all adult patients with diffuse non-Hodgkin's lymphoma who are in relapse. No case of Burkitt's lymphoma will be included.

Chemotherapy Salvage Regimens

In 1981, based on prior experience with two ifosfamide-etoposide combinations (one with methotrexate and the other with doxorubicin), MIME was devised at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston (8,9). Mitoguazone was included as part of the regimen because of the encouraging results of two independent phase II studies conducted by the Southwest Oncology Group and the Memorial Sloan-Kettering Cancer Center (46,47). A major advantage of this drug is that it does not cause myelosuppression and therefore can be combined at almost full dose with other drugs.

MIME Results

Of the 208 patients in the U.T. M. D. Anderson Hospital MIME study, 206 had received treatment with doxorubicin-containing regimens. In order to determine whether the MIME regimen could be cross-resistant with frontline combination chemotherapies, the response to MIME was compared with response to frontline therapy. Those who responded best to MIME were those who had responded to frontline treatment but who had relapsed more than 6 months after the frontline therapy had been completed. The CR rate of those who were only able to achieve PR on frontline therapy was low (12%), but the overall response rate was 64%, with a 33% response rate in patients with diffuse non-Hodgkin's lymphoma. Those who had responded but relapsed while still undergoing active frontline therapy showed an intermediate response rate. Patients whose disease had not responded to frontline therapy had the lowest CR rate as well as the lowest overall response rate (10,48). We



1) Bone marrow harvesting prior to randomization, for all patients.

2) Regional radiotherapy both arms : 26 Gy in 2 w. prior to BEAC-ABMT ; 40-45 Gy in 5 w. after 6 DHAP.

3) Relapsers in either arm may be offered alternative treatment including High dose treatment + ABMT.

Figure 10. *Study design (NHL, non-Hodgkin's lymphoma; CR, complete remission; DHAP, dexamethasone, cytarabine, cisplatin; PR, partial remission; SD, stable disease; PD, progressive disease; MR, disease relapse in marrow; XRT, x-ray therapy; BEAC, BCNU [carmustine], etoposide, ara-C [cytarabine], cyclophosphamide; ABMT, autologous bone marrow transplantation).*

show in Figure 11 the survival curves of the MIME patients according to their histological subtype. These curves are almost identical to those from previous experience with IMVP-16-213 (ifosfamide, methotrexate, etoposide) and AIVP-16-213 (doxorubicin, ifosfamide, etoposide) for both the aggressive and the indolent disease types.

We used MIME to treat diffuse large cell lymphoma in patients less than 60 years of age who had no bone marrow involvement: 52 patients have been treated who meet these criteria. Twenty-two achieved a CR (42%), 13 achieved a PR (overall response rate, 67%), and 17 did not respond. There are three long-term survivors at 46+, 26+, and 12+ months. The median survival of this group is 40 weeks (Fig 11).

The toxicity for patients on MIME was acceptable in most instances. However, despite good results—the best ever reported in relapse of non-Hodgkin's lymphoma—and low toxicity, mitoguazone and ifosfamide, which were available for this study, are no longer available for use in the United States. Furthermore, since the completion of the MIME studies, many patients now receive etoposide as primary therapy. A program with nonexperimental drugs, possibly drugs non-cross-resistant to primary therapy having response rates equal to those associated with MIME would be desirable for reinduction therapy.

DHAP Results

Previous experience with either cisplatin or high-dose cytarabine shows response rates of approximately 20%, practically all of the patients being in PR

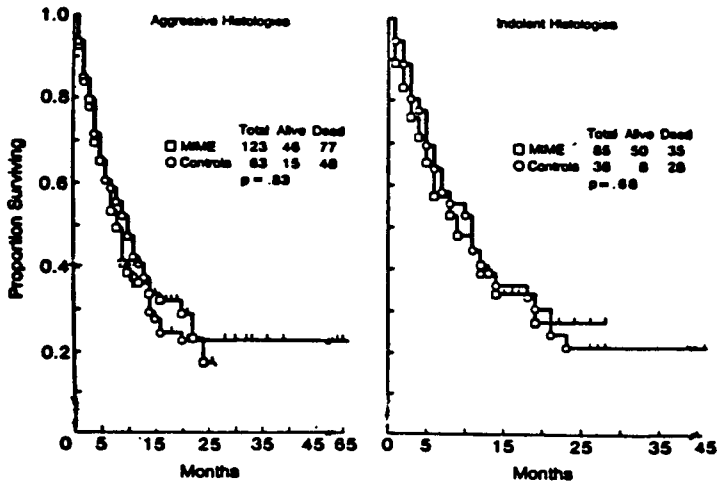


Figure 11. *Survival of patients treated with the MIME (mitoguazone, ifosfamide, methotrexate, etoposide) salvage regimen according to histological subtype compared with that of controls treated with other ifosfamide-etoposide combination regimens.*

only and realizing no significant improvement in length of survival (49-53). This degree of activity is similar to that of other potentially active single agents in relapsed large cell lymphoma.

Recent in vitro studies suggest that combining cytarabine with cisplatin may have a marked synergistic effect. These studies were conducted on LoVo cells, an established human colon carcinoma line (54). When combined with other antitumor drugs, cisplatin displayed superadditive effects with hydroxyurea, bleomycin, cis-retinoic acid, BCNU (carmustine), Adriamycin (doxorubicin), mitomycin C, and an unexpectedly strong synergistic effect was noted with cytarabine. Although cytarabine displayed no cytotoxicity on LoVo cells when administered alone, when combined with cisplatin it induced a several-fold increase in lethality over that attained by cisplatin alone. The expected-to-observed survival ratios, for a cisplatin concentration of 5 μg/ml ranged from 1.3 (cytarabine of 100 μg/ml) to 12.9 (cytarabine of 1,000 μg/ml), providing a considerably higher performance index than that observed for cisplatin combined with other drugs tested on the same cell line. This result is not particularly surprising because it conforms with previous observations on L1210 leukemia cells; however, what is remarkable is that although murine leukemia cells are exquisitely sensitive to cytarabine, even when it is administered as a single agent, the drug is totally ineffective against cultured human cells, even at concentrations of 10,000 μg/ml. This supports the conclusion that the marked potentiation observed for the combination originates from a modification of the nature of the DNA cross-links induced by cisplatin in the presence of large intracellular concentrations of cytarabine. Perhaps the simultaneous presence of both agents induces the formation of multimeric platinum pyrimidine complexes in a structure similar to the "dimers of dimers" described by Lock *et al.* (55) for cisplatin-DNA adducts.

The immediate practical impact of these studies is the rationale they provide for combining cisplatin and cytarabine in clinical trials. Should the phenomenon discovered for in vitro cells be shared by in vivo tumors, combining cisplatin and cytarabine could evolve into an effective form of treatment. An enhanced therapeutic index is an additional advantage for this combination because, though the antitumor activity of the paired drugs can be expected to increase, audiological and renal toxicity, unrelated to DNA damage, should remain unchanged.

The simultaneous administration of these two agents and dexamethasone in a pilot study did demonstrate the potential benefit of the combination for patients with progressive lymphomas with prior resistance to doxorubicin. Four of fourteen patients have achieved CR, and six other patients achieved PR. Pathological diagnosis included all types of lymphoma, with a predominance of diffuse large cell lymphoma (one patient with chronic lymphocytic leukemia and undifferentiated lymphoma also responded). Renal toxicity from cisplatin has been reported in up to 36% of patients. Toxicity is dose

related and may also be related to the duration of infusion and the use of hydration and mannitol (56). Myelosuppression from cytarabine is less severe with shorter infusions than it is with 24-hour infusions or longer ones.

Tolerance so far has been acceptable, and the regimen can be administered on an outpatient basis for young patients. Careful monitoring of fluid intake and renal status will be performed during this study to keep urine output at a minimum of 150 ml/hour during the cisplatin infusion.

Further expansion of these data has shown that the combination of cisplatin and cytarabine in high doses has produced a 57% response rate in the first 67 patients with diffuse lymphoma treated, of whom more than half (30% of the total number of patients) are in CR. Seventeen patients with diffuse large cell lymphoma who were less than 60 years of age with no bone marrow or CNS involvement and good Karnofsky performance scores have been treated at U.T. M. D. Anderson Hospital on dexamethasone, high-dose cytarabine, and cisplatin. Of those, 35% achieved a CR and 41% responded. Fifty percent of the CR patients are still disease free (median follow-up, 9 months). Nine comparable patients have been treated at Omaha University, and six responded, four having CRs. The response rate for the 25 patients (Houston and Omaha) is 50%.

Radiotherapy Results

The rationale for including irradiation in an autologous transplant program is the known radiosensitivity of lymphomas. Radiobiologic studies corroborate the clinical findings of significant radiosensitivity for this lymphoma type. One study on large cell lymphoma of B-lymphocyte origin showed that in vitro the radiation cell survival curve had a small shoulder (n = 1.2) and a steep slope (D_0 = 1.4 Gy) (57). From this it follows that the radiotherapy schedule proposed in this study (see below) should result in 6-7 logs of additional cell killing (57-60). Previous experience with large cell lymphoma in general (61) and with massive therapy in particular (W. Velasquez, M.D., personal communication, 1986) has shown that initial sites of bulky disease tend to be the sites of relapse and progression. Our review of 100 cases in adults (see Armitage, "Bone Marrow Transplantation in Relapsed Diffuse Large Cell Lymphoma," in this volume) show that of 100 patients treated, 64 relapsed, and of these 48 (75%) had recurrences at sites of initial bulky disease. This suggests that additional cytoreduction of bulky disease is necessary. Because TBI was not able to overcome this problem in our studies, because radiation is primarily a local-regional modality, and because the probability of local control increases as dose increases, it is logical to investigate in conjunction with the chemotherapy program the effectiveness of involved-field (boost) radiation therapy (XRT) to sites of bulky disease.

Radiotherapy

Treatment in one arm of the study will integrate XRT and BEAC (BCNU [carmustine], etoposide, ara-C [cytarabine], cyclophosphamide). To limit potential nonhematopoietic toxicities, we will employ a reduced XRT dose. Assuming that cell survival statistics apply to the clinical situations included in this study, we believe the proposed XRT schedule (26 Gy in 20 fractions of 1.30 Gy each that will be delivered twice daily over 2 weeks) will result in approximately 7 logs of cell killing. Small doses will be employed to take advantage of the relatively small shoulder on lymphoma cell survival curves and should improve the therapeutic ratio relative to mucosal epithelial cells, which generally have radiation survival curves with broad shoulders (62). To allow for adequate normal tissue repair of sublethal damage, we will allow at least 4 hours to elapse between each of the two daily treatments (62).

Bulky disease sites in this study are defined as sites of relapse in which a mass measures 5 cm or greater prior to institution of DHAP therapy. In extranodal head and neck sites, bulky disease is defined as any tumor falling into a T3 or T4 category according to the International Union Against Cancer or the American Joint Committee criteria for epithelial cancers. Sites of bulky relapse that have received prior irradiation to doses greater than 30.0 Gy or to doses that would result in exceeding commonly accepted tolerances (63) if XRT in our protocol were added will not receive involved-field XRT. Regardless of disease status at relapse, the following sites will *not* receive systematic XRT: bone marrow, lungs, heart, liver, and kidneys. It is admissible to incidentally include up to 20% of the total volume of the lungs, heart, liver, and kidneys during involved-field XRT to adjacent structures. The definition of an XRT involved field is based on the Ann Arbor concept of a nodal region (64) and is elaborated on in the section dealing with treatment methods.

Continued DHAP

The considerations given previously relative to the potential benefits of involved-field XRT also apply to conventional chemotherapy of intermediate- and high-grade lymphomas, and accordingly XRT will also be included in this arm of the study. Since it is unlikely that XRT could be integrated early into the DHAP-only arm without compromising the timing or dosage of chemotherapy or both, XRT will be delivered after completion of six courses of DHAP. Involved-field XRT will consist of conventional once-daily treatment of 1.50-2.00 Gy to a total of 35.0-40.0 Gy delivered to sites of initial bulky disease as defined previously. However, patients showing disease progression before the commencement of XRT will be regarded as patients whose treatment failed, and their disease will be managed according to the discretion of the investigator. The approach of DHAP-XRT is regarded as the best conventional treatment, and against it XRT-BEAC-ABMT will be compared.

BEAC

The review of 100 cases of relapsed or progressive disease with no adults with non-Hodgkin's lymphoma achieving CR has shown no significant difference in the response and survival rates for the 39 patients who received TBI compared with the 61 who received only chemotherapy (Fig 12). In this study of 100 cases, 11 of 39 patients died of toxicity in the group receiving TBI, whereas only 10 of 61 died of toxicity in the group receiving no TBI. It appears that TBI did not significantly increase efficacy but did increase toxicity.

The BEAM protocol (BCNU [carmustine], etoposide, ara-C [cytarabine], melphalan) was initiated in France in 1983 with the objective of reducing the high number of toxic deaths related to BACT (BCNU [carmustine], ara-C [cytarabine], cyclophosphamide, 6-thioguanine) protocol treatment (see Biron *et al.*, "A Phase II Study of a New Cytoreductive Conditioning Regimen With Autologous Bone Marrow Transplantation for Lymphomas: The BEAM Protocol," in this volume). Since 1984, 51 patients have been treated with BEAM in the France Autogreffe Study group; 7 patients with Hodgkin's disease and 44 with non-Hodgkin's lymphoma were included. Among the latter, 28 were in relapse (9 RRs and 19 SRs). Five were in first PR, and 11 were in first CR. The response rate was 73%, which was comparable to that in previous BACT experience. As expected, toxicity was reduced, the toxicity-related death rate being 9.8% (5 of 57 patients) (see the above-named chapter by Biron *et al.* in this volume). The BEAC protocol proposed for this study

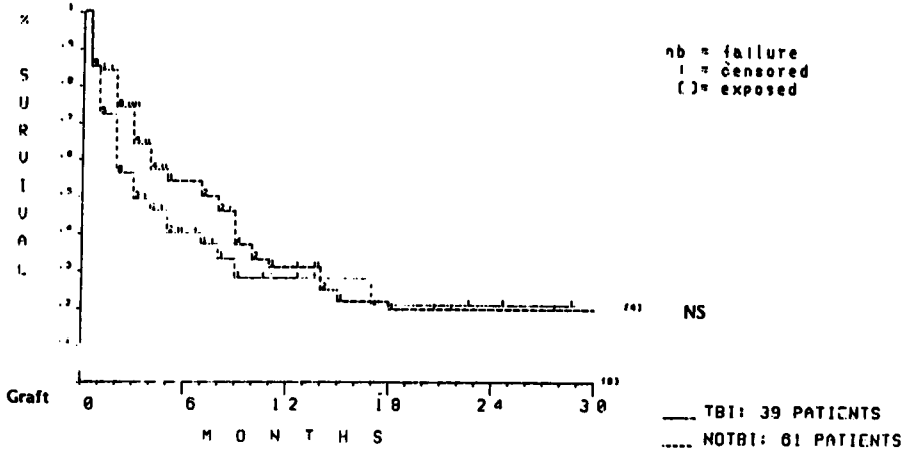


Figure 12. *Comparison of survival in 100 patients with relapsed or progressive disease, 39 of whom underwent total body irradiation (TBI) and 61 of whom did not. The difference between the two groups in the percentage surviving was not significant (NS); however, because 11 of the 39 patients treated with TBI and only 10 of the group of 61 not treated died of toxicity, it appears that TBI did not significantly increase efficacy but did increase toxicity.*

replicates the BEAM regimen with only one modification. Because melphalan is still an experimental drug in the United States, we decided to substitute the classic cyclophosphamide (35 mg/kg × 4).

THE PROJECTED INTERNATIONAL STUDY: A SUMMARY

The projected international study will be a phase III randomized study of involved-field XRT, high-dose chemotherapy consolidation (BEAC), and ABMT therapy compared with maintenance chemotherapy and involved-field XRT in patients totally responding to DHAP therapy at the time of first or second relapse (in or off frontline therapy) (Fig 10). The objectives are to compare intensive therapy with or without ABMT rescue in patients with SRs, to show at least a 20% improvement in disease-free survival rates with ABMT 2 years after randomization, and to evaluate the toxicity and mortality associated with each approach.

Patient eligibility standards will be the following: 1) patients must have relapsed diffuse intermediate- and high-grade lymphomas and must be adults who are less than 60 years old; 2) patients must have been previously treated with a doxorubicin-containing regimen or COMLA (Cytoxan, vincristine, methotrexate, Leucovorin rescue, ara-C); 3) patients must not have CNS or bone marrow involvement at relapse; 4) only patients who have previously reached a first CR on the induction regimen (i.e., a true relapse) are eligible for this protocol; and 5) only patients with a first or second relapse will be admitted to the study group.

The international study is expected to enroll 142 patients who will be randomized into two groups of 71 each. A 3-year inclusion period is scheduled for reaching this sample size. Statistical parameters will be the following (two-sided test): $\alpha = 5\%$ and $\beta = 20\%$.

There will be two phases—the preinclusion and inclusion phases—to the treatment plan. In the preinclusion phase (with all patients admitted with an intention to treat), two courses of DHAP will be administered (see Table 2), and then patients whose disease responds to therapy even in relapse (SRs) will be randomized into two groups. After randomization, one group will receive involved-field XRT, high-dose BEAC chemotherapy (Table 3), and

Table 2. DHAP Protocol

Agent	Dose	Day	Administration
Dexamethasone	40 mg	1-4	15-min bolus i.v.
Cisplatin	100 mg/m²	1	24-hr continuous infusion i.v.
Cytarabine	4 g/m²	2	2 g/m² 3-hr i.v. every 12 hr × 2

Table 3. BEAC Protocol

Drug	Day 1	2	3	4	5	6	7
Carmustine—300 mg/m²/24 hr (30-min i.v.)	x						
Etoposide—200 mg/m²/24 hr		x	x	x	x		
(100 mg/m² i.v. twice daily)		x	x	x	x		
Cytarabine—200 mg/m²/24 hr		x	x	x	x		
(100 mg/m² i.v. twice daily)		x	x	x	x		
Cyclophosphamide—35 mg/kg/24 hr (short infusion over 60 min)		x	x	x	x		
Mesna—50 mg/kg/24 hr		x	x	x	x		
ABMT infusion (at least 48 hr after etoposide)							x

Abbreviation: ABMT, autologous bone marrow transplantation.

Table 4. Summary of Involved-field XRT, BEAC, and ABMT Protocol

Week	Monday	Tuesday	Wednesday	Thursday	Friday
1	XRT[a]	XRT	XRT	XRT	XRT
	XRT	XRT	XRT	XRT	XRT
2	XRT	XRT	XRT	XRT	XRT
	XRT	XRT	XRT	XRT	XRT
3	Carmustine[b]	Etoposide	Etoposide	Etoposide	Etoposide
		Cytarabine	Cytarabine	Cytarabine	Cytarabine
		CY	CY	CY	CY
4	ABMT				

Abbreviations: XRT, involved-field x-ray therapy; BEAC, carmustine, etoposide, cytarabine, cyclophosphamide; ABMT, autologous bone marrow transplantation; CY, cyclophosphamide.

Note: No therapy will be administered on Saturdays or Sundays.

[a] *Day 20 after the second course of dexamethasone, cytarabine, and cisplatin.*

[b] *Day 34 after the second course of dexamethasone, cytarabine, and cisplatin.*

ABMT (Table 4). The other will receive DHAP chemotherapy and involved-field XRT. This group will receive four additional rounds of DHAP therapy given every 28 days. Involved-field XRT will be administered day 28 post-DHAP (n = 6).

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International Randomized Trial in Non-Hodgkin's Lymphoma in Relapse

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For a decade, high-dose chemotherapy with autologous bone marrow transplantation (ABMT) has been used as salvage therapy in non-Hodgkin's lymphoma at relapse to increase the response and survival rates using the dose-effect relationship. In the same way, conventional regimens have been administered and a good response rate observed with acceptable toxicity. The results of high-dose therapy with ABMT (30% of patients surviving free of disease at 2 years) seem better than those obtained with conventional therapy (5-10% surviving free of disease at 2 years). However, it is obvious that the ABMT group is carefully selected and exclusive and not comparable with groups that include all patients who relapse, the typical organization of groups in conventional therapy studies. Although these two therapeutic strategies are equally used, their efficacies and toxicities have never been compared properly in a randomized clinical trial. Now this comparison is slated to be the objective of a phase III international randomized trial. In such a trial, several steps must be carefully considered by methodologists and statisticians; selection of patients, method of randomization, and method of evaluations can cause biases and invalidate results.

THEORETICAL OBJECTIVES AND JUSTIFICATIONS

The main objective of this trial might be described as determining if a high-dose chemotherapy regimen with ABMT can increase the survival rate for patients with relapsed non-Hodgkin's lymphoma over that produced by conventional chemotherapy. Other goals would be to compare toxicities, response rate, and progression-free survival rate in ABMT and conventional groups. However, the crude survival rate is not a valid criterion because other therapeutics will be used after these therapies fail. So progression-free survival should be taken as the main measure. To be valid, the study has to be based on a solid understanding of the two therapeutic strategies.

Background of ABMT and Conventional Therapy

Few phase II trials of high-dose chemotherapy with ABMT have been performed (1,2), but three conclusions may be drawn from these trials or retrospective studies. First, results obtained with high-dose chemotherapy and ABMT vary according to prior response to chemotherapy (complete response or not). Second, patients who relapse after achieving a complete response may be divided into two different groups: those whose disease remains responsive though in relapse (sensitive relapse) and those whose disease is resistant to therapy (resistant relapse). The former have a better prognosis than the latter, even if high-dose chemotherapy is administered later. Third, in a group of patients treated with high-dose chemotherapy and ABMT after conventional therapy, the disease-free survival rate is 30% at 2 years.

On the other hand, good results can be obtained with conventional therapy in terms of response and survival rates. In The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston's MIME (mitoguazone, ifosfamide, methotrexate, etoposide) study (3), a 67% response rate was obtained in a 52-patient group. With the DHAP protocol (dexamethasone, cytarabine, cisplatin), similar results were obtained in 17 patients also at M. D. Anderson Hospital. Findings about conventional therapy can be summarized as follows: 1) a high response rate to a multidrug regimen can be obtained; 2) evidence of prior complete response to chemotherapy is as important in conventional therapy as it is in ABMT; and 3) the disease-free survival rate is 5-10% at 2 years.

Justification of a Randomized Trial

After several years of experience in high-dose chemotherapy with ABMT in non-Hodgkin's lymphoma relapses, it is necessary to reevaluate this treatment. Based upon the results of open cohort studies, ABMT appears to have a higher response rate than does conventional therapy. However, ABMT is an expensive treatment and sometimes induces severe toxicity (the toxic death rate is estimated at 15%). Historical controls are not valid because of

disparity in selection criteria, statistical methods, and treatment modalities. Only a randomized clinical trial can properly address the question.

METHODOLOGICAL CHOICES

Patient Selection

The theoretical target population is the group of patients with high-grade or intermediate non-Hodgkin's lymphoma who are in relapse. To take into account ethical and methodological considerations, investigators must restrict admission to a responding patient group. Only those in sensitive relapse after prior complete response will be included. In addition, only patients who can receive either treatment without major risks (having no cardiac, pulmonary, renal, or hepatic dysfunction and being below 60 years of age) will be treated. No heavily pretreated patients (none with more than a second relapse or previous doxorubicin chemotherapy) should be allowed to participate. These criteria will produce a large subset of the theoretical target population. However, restrictions about chemosensitivity exclude patients, approximately 50%, who could be treated with ABMT and not with conventional chemotherapy (i.e., those in resistant relapse).

Only patients who respond to salvage conventional therapy can be included and randomized. The selection procedure of patients must be divided into two phases, the preinclusion phase (two courses of conventional chemotherapy) and the inclusion phase. If there is a complete response (CR) or partial response (PR) after two courses of chemotherapy and if all selection criteria are met, the patient is included and his therapy selected by randomization. This selection plan includes only responders in the trial, and all included patients are to be evaluated in their group.

Study Design (Fig 1)

Fundamental principles of controlled clinical trials are now well established (4). Ideally, all patients eligible for the study should be assigned in a random fashion to one of the study groups, and treatment should be administered without delay. Thereafter, follow-up procedures and assessment of study end points should be performed identically for all treatment groups. Double-blind procedures are recommended for use, whenever possible, to prevent follow-up and evaluation biases. For statistical purposes, after randomization no patient can be withdrawn from his original treatment group. Procedures should be designed to accommodate both methodological considerations and practical constraints.

Randomization

Treatment must be administered as close as possible to randomization (a delay between the second DHAP course and the next will theoretically be 28 days). However, it is necessary to inform the patient about the next treatment

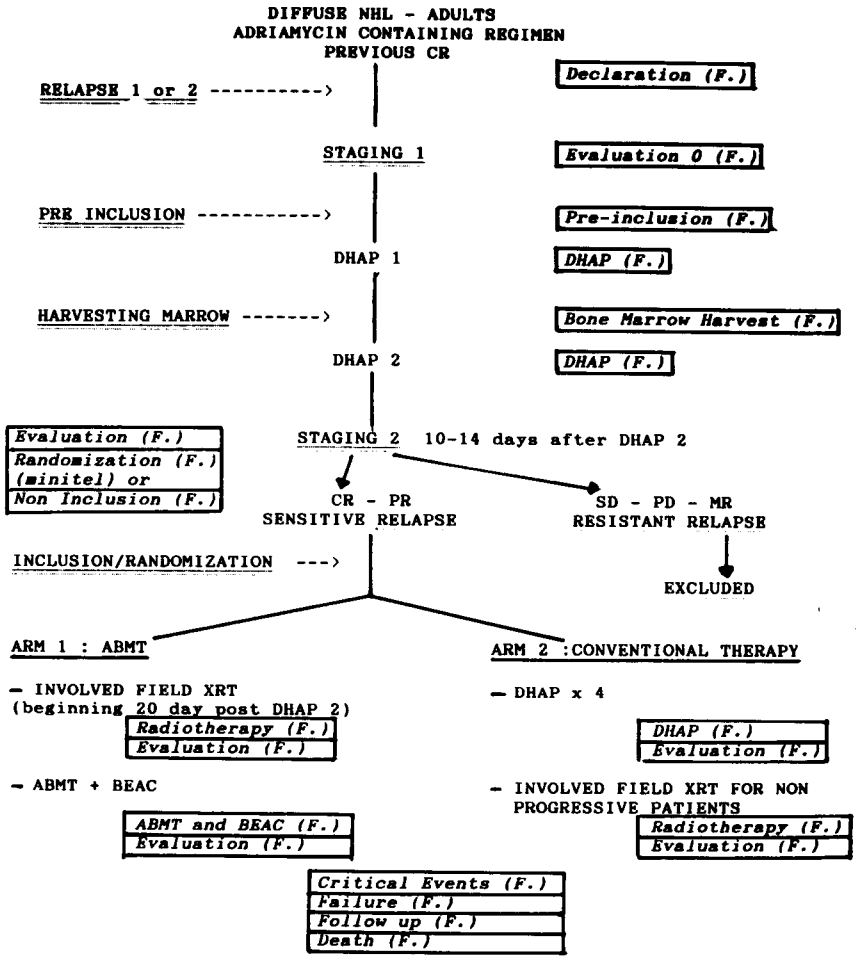

Figure 1. *Study plan.*

and to plan the bone marrow unit activity. So randomization will take place after the second evaluation, 10-14 days after the second course of chemotherapy. In spite of a 2-week delay between randomization and treatment administration, all randomized patients will be analyzed.

Since treatments can obviously not be given in a double-blind fashion, it is very important that no bias be introduced in treatment allocation. A centralized randomization procedure will ensure that 1) assessment of eligibility cannot be influenced by the possible knowledge of the next allocated treatment; 2) the allocation of all randomized patients is declared to the coordinating center; and 3) in addition, a final check of eligibility criteria is performed before randomization. After treatment allocation, patients should be scheduled for the allocated therapy. If for any reason the planned therapy

cannot be administered, the patient will still be followed up as scheduled until the end of the study.

All investigators will be able to connect their own personal computers to the coordinating center's computer by data process networks and TRANSPAC (in France). This computer provides a 24-hour service for allocation, interactive checking of all eligibility criteria, and confirmation of this allocation.

Follow-up

Because of the different treatment procedures in the two groups, follow-up of patients will vary between them during the treatment phase. However, assessment of the main end points will be done monthly in both groups and at the same fixed times.

ASSESSMENT OF END POINTS

The main end point is a binary criterion based on the evidence of an event (failure) or its absence (success). This event must be a hard end point, defined as evidence of a relapse (if CR was achieved); evidence of progression (if patient achieved only PR); appearance of a new tumor in a new locale; or death, whatever the cause, if observed before relapse or progression. Secondary end points concern death or toxicity. Records of deaths are necessary for computing the survival rate in the different treatment groups. A death could be early, related to toxicity, accidental, or disease related. Toxicity may be monitored on three levels: toxic deaths, major toxicities, and minor toxicities. Regular evaluations must be scheduled in the protocol before and after each treatment, at preinclusion, after two DHAP courses, after irradiation, and after chemotherapy. Relapse, failure, or death require specific evaluations.

SAMPLE SIZE AND STATISTICAL CONSIDERATIONS

Sample size computations for this trial must be based on the following two assumptions: First, the 2-year success rate in the conventional arm is estimated at 15% (i.e., event-free rate) (see above). In the ABMT arm, the success rate is expected to be 35% (improvement, 20%). Statistical errors are α = .05 and β =.20 (power = .80). Comparisons will be performed with two-sided tests. Thus, the number of patients under these specifications will be 71 patients per arm, that is to say, 142 patients to be randomized. The response rate to conventional therapy after two courses is estimated at approximately 50%. Therefore, 300 patients have to be screened in the preinclusion phase. Finally, based on an inclusion rate of 45 patients per year, the time necessary to complete trial recruitment will be 3 years.

ANALYSIS AND STOPPING RULES

Analysis will be performed based on the intention-to-treat principle: each randomized patient will be taken into account in the comparison of the two treatments. Every patient whose disease progressed, whose therapy failed, who died, or who was lost to follow-up after randomization will be considered as a failure of therapy. Final analysis will be performed 2 years after the last patient is included, probably 5 years after activation of the trial. Two interim analyses are scheduled 3 and 4 years after activation with $\alpha = .001$; however, final analysis will be performed with $\alpha = .05$.

Rules for stopping the trial have been decided about toxicity, response rate, and recruitment. The final decision definitely to stop the trial will be taken by the steering committee and the policy board (see "Organization" section) if abnormal events are observed or results reached.

QUALITY CONTROL

Emphasis will be put first on preventing errors and protocol violations. Using a manual of operation, conducting training sessions, and having direct contact between investigators and coordinating officers can increase quality control. Detecting and correcting errors will be ongoing throughout the study.

All data will be checked for validity and coherence using computerized procedures. Principles respected in this quality control include the following: 1) forms will collect data about one event at one moment in one place; 2) corrections will be the responsibility of investigators; 3) missing documents (forms or critical events reports) will be detected and claimed.

Inclusion and exclusion criteria will be checked a posteriori, though an interactive control is performed at time of randomization; however, any ineligible patient cannot be excluded from the study. Patient follow-up is monitored by the coordinating center computer. Any patient lost to follow-up more than 1 month after the scheduled time of the visit is considered provisionally lost to follow-up and an inquiry is made. Adherence to quality control is estimated; indices will measure regularly the distance between protocol requirements and reality (e.g., recruitment of patients, number of ineligible patients).

Events directly related to the evaluation of the main objective deserve attention. Documentation about these events with all relevant available material will be forwarded to the coordinating center. This documentation will then be reviewed by a committee (the critical events committee) for validation and classification. Critical events are defined as follows: evidence of a major toxicity, patient lost to follow-up or leaving treatment, or death.

ORGANIZATION

The participating investigators and centers in this international ABMT trial will collaborate through a study organization designed to maintain continuity of operation in the study and to facilitate effective communication and cooperation among the various functional units (Fig 2). Each clinical center with responsibility for recruiting, examining, evaluating, and treating patients will be known as a participating unit in this trial. Additional clinical centers may be recruited if patient recruitment does not proceed as expected. Responsible for the data collection, the data processing system, management of the study, randomization, and analysis is the coordinating center.

The writing committee initially develops the study protocol and dictates its direction until the establishment of the policy board. The study chairman, designated by the writing committee, has the major responsibility for the scientific direction of the study. In addition to the writing committee and the policy board, the administrative units of the study include a steering committee, a pathology committee, and the coordinating center.

The policy board is made up of permanent members who are senior scientists and who are not associated with the operation of the trial and not associated with its sponsor. The policy board has final control and full power for all study policy matters. Any decisions made by the policy board will be presented to the steering committee for review and discussion. Decisions made by the policy board will be final.

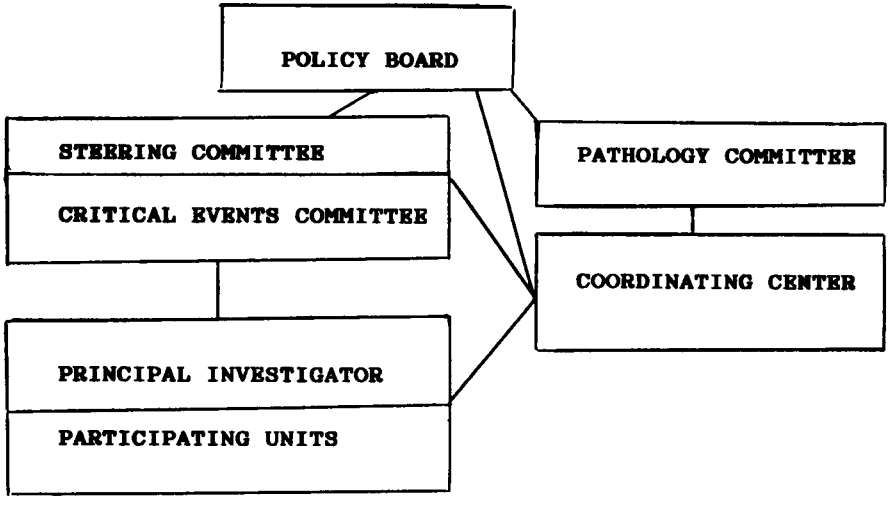

Figure 2. *Organization chart of the international study of relapsed non-Hodgkin's lymphoma.*

The steering committee will provide technical guidance for the study at the operational level. Permanent members of this committee include the study chairman, representatives of coordinating center, and representatives of the main groups of investigators. It is the critical events committee for this trial. The pathology committee is responsible for slide review. Each new case will be reviewed by the committee and a patient's acceptability determined before randomization. The duties of the last-named administrative unit, the coordinating center, are described above.

CONCLUSION

This trial, the first to compare ABMT with conventional therapy in a randomized study, may produce results of prime importance for determining ABMT's use in non-Hodgkin's lymphoma relapses. Technical problems in undertaking such a trial might make it as difficult to replicate as it is desirable.

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Lymphoma

T. Philip and G. Spitzer, Chairmen

DR. D. HEARD: The first question is how are you going to analyze those patients who have been randomized to chemotherapy and who may run off to another center to have bone marrow transplantation? The center is not cooperating in the international trial. Are they going to be excluded or are they going to be analyzed in the chemotherapy arm?

DR. F. CHAUVIN: No patient will be excluded from this study in the final analysis.

DR. HEARD: Once they have been randomized?

DR. CHAUVIN: Yes, and if the patients sign an informed consent in which they will agree to be randomized in the chemotherapy arm once they have been randomized, they will not go for autologous bone marrow transplantation (ABMT) before relapse.

DR. HEARD: Well, I mean they might sign the consent form, but they may change their minds later on and go to a different center. It brings up an issue that happens all the time—when someone gets treated on the opposite arm from which they were originally randomized.

DR. G. SPITZER: Well if it happens, it is going to be a very, very rare event.

DR. CHAUVIN: The point I want to emphasize is that we are really convinced, among the participants, that this randomized study should be

done because we don't know the results. Some people presume that bone marrow transplantation is better. We think they are wrong, and we have to prove this point now.

DR. SPITZER: Thierry (Dr. T. Philip) is going to update us on the timetable of this study.

DR. T. PHILIP: Yes, at the meeting yesterday it was decided among the participants that the study is still open to every group that wants to join. In this case, please contact me as soon as possible. Second, the study will begin as of January 1, 1987. The first 20 patients will not be randomized and will all receive the involved-field radiotherapy and the BEAC regimen with ABMT to confirm that the toxic death rate does not exceed 10-15%. It seems to be the case in the first 16 patients, as Dr. S. Jagannath told you, 1 in 16. However, we want to confirm this fact before starting the randomized study. The steering committee will meet when the data from the first 10 have been received, after which we will decide to open the randomized study.

IID. Purging and Detection

Chemoimmunoseparation of T Lymphoma Cells From Human Bone Marrow

Robert Bast, Jr., Bruce Montgomery, Azeem Haleem, Joanne Kurtzberg, Ann Rhinehardt-Clark, Sundaram Ramakrishnan, Greg Olsen, Carol Smith, David Leslie, William Peters, and Lou Houston

One requirement for effective autologous bone marrow transplantation (ABMT) in leukemia and lymphoma is the complete and selective elimination of malignant cells from remission bone marrow. In early studies we optimized removal of nonlymphocytic leukemic cells from rat bone marrow using polyclonal antibodies and complement (1). Availability of monoclonal antibodies reactive with determinants expressed by leukemias and lymphomas in the B-cell lineage facilitated attempts to eliminate tumor cells that tests showed positive for the common acute lymphoblastic leukemic antigen (CALLA) from human bone marrow (2). Limiting dilution techniques were utilized to estimate clonogenic Burkitt's lymphoma cells that remained following treatment with monoclonal antibodies and rabbit complement (3). The use of multiple monoclonal antibodies proved superior to treatment with single antibodies (3). Using optimal combinations of two monoclonals, about 3.5 logs of malignant clonogenic cells could be removed without affecting growth of granulocyte-macrophage colony-forming units (CFUs-GM), erythroid burst-forming units

(BFUs-E), or bipotent granuloerythropoietic precursors (CFUs-GEMM) (3-5). Antibodies reactive with cluster of differentiation 10 (CD 10), CD 9, and CD 20 proved particularly effective. Interestingly, the same monoclonal antibodies conjugated with ricin A chain were remarkably ineffective in eliminating malignant clonogenic Burkitt's cells in the absence of complement (6). Ricin A-chain conjugates with anti-Ia and anti-U monoclonal reagents could, however, eliminate up to 4 logs of malignant clonogenic tumor cells.

In an attempt to improve elimination of malignant B cells, we treated human bone marrow with a combination of monoclonal antibody, complement, and the cyclophosphamide derivative mafosfamide (Asta Z 7557) (7). Using this combination of chemoseparation and immunoseparation, we eliminated approximately 4 logs of malignant cells while studying the effect of combined chemoimmunoseparation on normal marrow precursors. Mafosfamide eliminated most CFUs-GM when marrow was cultured immediately after treatment (7). Incubation of mafosfamide-treated marrow in long-term culture permitted growth of new CFUs-GM, which was consistent with the possibility that more primitive progenitors had been spared (7).

During the last 2 years we have asked whether a combination of chemoseparation and immunoseparation would prove more effective than either single modality for selectively eliminating T lymphoma cells from human bone marrow. For chemoseparation we have used a combination of deoxycoformycin and deoxyadenosine. The former is an adenosine deaminase (ADA) inhibitor that has been shown to be selectively toxic for both normal and malignant T cells. Incubation with the ADA substrate deoxyadenosine potentiates deoxycoformycin toxicity. For immunoseparation we have utilized the 3A1 IgG2a murine monoclonal antibody that recognizes a CD 7 p40 cell-surface antigen expressed by nearly all T-cell leukemias and lymphomas. CD 7 is also expressed on a bipotent lymphohematopoietic-myelohematopoietic stem cell that can be isolated from human thymus, fetal liver, and bone marrow (8), but it is not expressed on the most primitive pluripotent stem cells. The 3A1 monoclonal antibody can lyse CD 7-positive cells in the presence of rabbit complement.

A limiting dilution assay has been used to count clonogenic units before and after chemoseparation and immunoseparation from human bone marrow (2; A. Haleem *et al.*, unpublished data). Bone marrow mononuclear cells have been separated over Ficoll gradients, irradiated with 50 Gy, and mixed in a 20-fold excess with 1×10^6 HSB-2 T lymphoma cells. Cell mixtures were then incubated with deoxycoformycin for 1 hour to inhibit ADA and with a combination of deoxycoformycin and deoxyadenosine for 4 additional hours. When immunoseparation was attempted, mixtures were incubated with 3A1 plus rabbit complement three times for 30 minutes. Cells were plated in limiting dilution assays. Plates were scored for clonogenic growth after 2 weeks' incubation. Scores were converted to clonogenic units using a Spearman-Karber estimator. In some experiments, T leukemia cells were cryopreserved

from the peripheral blood of patients; thawed; treated with deoxycoformycin plus deoxyadenosine, 3A1 plus complement, or both combinations; and counted in a clonogenic assay in semisolid medium.

With the clonogenic assay, about 6 logs of HSB-2 T cells can be measured (A. Haleem *et al.*, unpublished data). Three treatments with 3A1 antibody and complement eliminated more than 2 logs of malignant cells. Use of deoxycoformycin and deoxyadenosine for 5 hours eliminated almost 3 logs of malignant cells. Combined treatment with 3A1, complement, deoxycoformycin and deoxyadenosine eliminated more than 4 logs. Under these same conditions, treatment with 3A1 and complement with or without deoxycoformycin and deoxyadenosine failed to affect growth of CFUs-GM. When deoxycoformycin, deoxyadenosine, 3A1, and complement were used alone or in combination against five T leukemia cell lines positive for CD 7, additive toxicity was obtained against colony-forming units. With two other cell lines that did not strongly express CD 7, additive effects were not obtained. Using a combination of deoxycoformycin, deoxyadenosine, 3A1, and complement, all clonogenic units could be removed from samples of leukemic cells directly cryopreserved from six patients with T-cell malignancies.

In subsequent studies (R. B. Montgomery *et al.*, unpublished data), we have asked whether immunoseparation might be improved through the use of an immunotoxin conjugate containing 3A1 and pokeweed antiviral protein (3A1-PAP). When 3A1-PAP was used in concentrations ranging from 0.1-10 μg/ml, approximately 2 logs of malignant cells could be eliminated at the highest immunotoxin concentration. Addition of 100 μM chloroquine to the incubation mixtures eliminated 1 additional log of malignant cells. A combination of 3A1-PAP chloroquine with deoxycoformycin and deoxyadenosine proved more effective than either immunoseparation or chemoseparation alone, permitting the elimination of more than 4 logs of malignant cells. Increasing concentrations of 3A1-PAP proved toxic for human CFUs-GM. Similarly, deoxyadenosine concentrations in excess of 1 mM proved inhibitory for CFUs-GM. Using a concentration of 3A1-PAP, deoxycoformycin, and deoxyadenosine that proved optimal for eliminating malignant cells, we eliminated at least 50% of CFUs-GM.

In summary, a combination of chemoseparation using deoxycoformycin and deoxyadenosine with immunoseparation using either 3A1 and complement or 3A1-PAP and chloroquine was more effective than either single modality for eliminating malignant clonogenic T cells from human bone marrow. Chemoimmunoseparation with deoxycoformycin, deoxyadenosine, 3A1, and complement eliminated 4 logs of HSB-2 T lymphoma cells under conditions that did not affect CFU-GM, BFU-E, or CFU-GEMM growth. Chemoimmunoseparation with deoxycoformycin, deoxyadenosine, 3A1-PAP, and chloroquine could also eliminate at least 4 logs of clonogenic HSB-2 T lymphoma cells, but antitumor activity had to be weighed against inhibition of CFU-GM growth. Phase 1 studies with deoxycoformycin, deoxyadenosine, 3A1, and complement are anticipated in the near future to cleanse marrows from patients with T-cell malignancies.

ACKNOWLEDGMENTS

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Using a Liquid Cell Culture Assay to Measure In Vitro Elimination of Burkitt's Cells From Bone Marrow

I. Philip, M. C. Favrot, V. Combaret, J. C. Laurent, B. Kremens, and T. Philip

We have developed a liquid cell culture assay that enables the growth of Burkitt's lymphoma (BL) cells, whose culture is either Epstein-Barr virus (EBV) positive or negative, from cytologically normal bone marrow. As previously reported, we currently use such an assay in our patients' follow-up to detect minimal bone marrow involvement (1). This assay has permitted us to show that 40% of patients in partial remission or in relapse have bone marrow involvement, even if their bone marrow is cytologically normal (2).

We describe here how we have modified this assay to refine and quantify the purging procedure. In artificial models when irradiated allogeneic bone marrow is contaminated with BL cell lines, this liquid culture assay enables the growth of as few as one BL cell in 4×10^6 normal bone marrow cells in a wide range of BL cell lines. This culture assay also is useful in newly established EBV-negative or EBV-positive lines from our Caucasian patients who are candidates for autologous bone marrow transplantation (ABMT) (3). More recently, the assay has been used to quantify the elimination of fresh Burkitt's cells from our patients' autologous bone marrow and to evaluate the quality of therapeutic purging procedures, either in a preclinical assay or in the clinical procedure itself, and we will specially emphasize this last point here.

MATERIALS AND METHODS

Normal bone marrow obtained from healthy donors or from patients with Burkitt's lymphoma was collected on preservative-free heparin, diluted in phosphate-buffered saline (PBS) without calcium and magnesium (Gibco), and separated on a Ficoll gradient (Lymphoprep [1.077 density], Niegaard, Norway).

Four BL cell lines—IARC BL_{17}, IARC BL_{93}, IARC BL_2, and IARC BL_{99}—have recently been established from our patients' tumor cells, and their characteristics have been previously described (1,4,5). Fresh Burkitt's cells were obtained from pathological bone marrow, the effusions, or the initial tumors.

The assay was carried out under sterile conditions. In the artificial model, allogeneic normal mononuclear bone marrow cells were irradiated (50 Gy) and contaminated with BL cells from various cell lines. In the autologous model, patients' mononuclear bone marrow cells were contaminated with BL cells from their own tumors. The BL cells were added in five tenfold serial dilutions to samples of 4×10^6 BM cells: the initial and final BL cell concentrations in triplicate samples being 10^{-2} (4×10^4 total BL cells) and 10^{-6} (4 total BL cells). The last triplicate cultures contained a single BL cell. The triplicate samples from each dilution were poured into 5 ml of RPMI medium supplemented with 20% heat-inactivated irradiated fetal bovine serum, glutamine, and antibiotics in Corning 25100 plastic culture flasks (vertical position with 10 cm^2 MRC_5 feeder layer of embryonic irradiated fibroblasts) (Merieux, Lyons, France) and incubated at 37° C in 5% CO_2 in air. The cultures were checked twice weekly—we observed them with an inverted microscope and changed half of the medium. On the tenth day, samples were taken and the percentage of growing BL cells was evaluated in each aliquot by cytology on a cytocentrifuged smear. When no BL cell was seen on the smears, the culture was kept for 2 more weeks and checked again each week until the demise of the cells. Selected cultures with growing BL cells were also propagated for 3 weeks in order to show that the features of growing BL cells (Epstein-Barr virus-associated nuclear antigen reactivity, immunologic markers and cytogenetics) remained identical to that of the initial cell line used for the contamination (1-3).

Requirements for removing BL cells from excess bone marrow with two immunologic methods, the rabbit complement lysis procedure and the immunomagnetic purging procedure, have been extensively described elsewhere (6-8; see also Combaret *et al.* "Eliminating Burkitt's Cells From Excess Bone Marrow With an Immunomagnetic Purging Procedure," in this volume).

RESULTS

Four representative lines recently established from our patients' tumors were chosen from a panel of 100 BL cell lines for their distinct characteristics, including lines that were EBV negative or positive that were from North African or Caucasian patients. Lower BL contaminations were studied with three to five

replicates in order to analyze the reproducibility of the assay. The estimated average number of BL cells was one per flask (4×10^6 bone marrow cells) at the lowest dilution. In 12 of 13 experiments, the estimated single cell seed was sufficient in one to five of the triplicate samples to yield detectable outgrowth of BL cells after 10 days of culture. The mean value of the three was used as final result (Table 1). In three experiments (Table 2) only one of three of the triplicate samples did grow, and these cultures were kept until day 21, at which time the growth of BL cells was detectable in three more flasks.

The ability of BL cells to grow in culture may vary from one line to another and from one experiment to another. Thus, as shown by the range in Table 1, the absolute number of detectable BL cells varied at day 10. Nevertheless, within the same experiment, the growth of BL cells was closely correlated to the original number of BL cells added at day 0. This correlation indicates that for

Table 1. The Outgrowth of BL Cells in Liquid Cultures

Cell Line (no. of experiments)	Characteristics	BL Cells (%) 1 in 10^6	BL Cells (%) 1 in 4×10^6
BL_{93} (n = 5)	(EBV−) Caucasian	41% (5–98%)	26% (1–80%)
BL_2 (n = 2)	(EBV−) Caucasian	53% (6–100%)	49% (2–96%)
BL_{17} (n = 2)	(EBV+) North African	38% (35–42%)	8% (0–16%)
BL_{99} (n = 4)	(EBV+) Caucasian	70% (5–100%)	57% (1–100%)

Abbreviations: BL, Burkitt's lymphoma; EBV, Epstein-Barr virus.

Note: The numbers show the percentage of BL cells detected in cultures at day 10 by the ratio of BL cells to normal cells at the start. The results are means of three to five parallel samples in each experiment. The values in parentheses show the range observed in the different experiments.

Table 2. Optimal Detection of BL Cells in Long-term Liquid Culture

Cell Line	BL Cells (%) Day 10	BL Cells (%) Day 21
BL_{93}	0, 0, 3	0, 60, 100
BL_{17}	0, 0, 46	0, 80, 100
BL_{99}	0, 0, 2	0, 100, 100

Abbreviation: BL, Burkitt's lymphoma.

Note: Results are expressed in the percentage of BL cells at days 10 and 21 of the culture in the triplicate flasks. The contamination was one BL cell in 4×10^6 normal ones at the start.

each set of experiments the percentage of BL cells at day 10 within each sample, set up with serial BL cell dilutions, provides a calibration curve (Fig 1). Next, the effects of purging have been assessed in bone marrow samples contaminated with 1% BL cells and treated either by a complement-mediated cytolysis or immunomagnetic depletion. They were then grown in the same conditions as the untreated samples. At day 10 of the culture, the percentage of growing BL cells in the treated samples were compared with that in the untreated samples, and the number of residual BL cells that escaped treatment in each sample estimated (for example, see Fig 1). The estimates ranged from 1 in 10^3 (4000 total BL cells) down to 1 in 4×10^6 (i.e., 1 BL cell). The efficacy of the purging procedure can thus be quantitated from 2 to 4.6 logs (decimal logarithms) removal, or "kill," representing the outgrowth of cells related to the initial number of BL cells in the unpurged samples.

During the experiments, two additional findings emerged. First, when the samples contained more than 1 BL cell in 10^4 and the kill was less than 2 logs, the final proportion of BL cells in cultures grown for 10 days reached 90-100%. Consequently, this assay is not primarily suitable for measuring purging methods of low efficiency without starting with more highly diluted BL cells. In contrast, the assay described here is capable of detecting very low proportions of residual BL cells after purging. Second, by keeping the samples for more than 3 weeks in culture, it was possible to confirm the total elimination of all growing BL cells by the purging method used (Table 2).

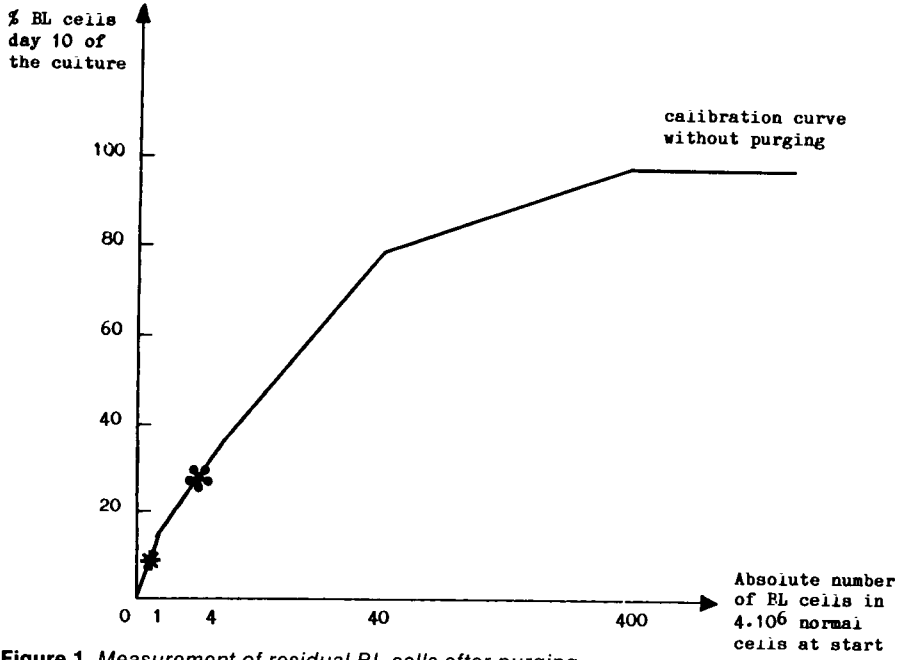

Figure 1. *Measurement of residual BL cells after purging.*

This liquid culture assay has already been used for seven patients to assay the efficiency of the rabbit complement lysis procedure. In four cases (Table 3), the culture showed that the autologous bone marrow was normal; this nonirradiated bone marrow was contaminated with decreasing concentrations of BL cells from the first site of the disease to provide a calibration curve. The assay was able to detect as few as one BL cell in 4×10^6 normal ones and to quantify a greater than 5-log depletion in patient 3.

Interestingly, in patient 1 the purging procedure was efficient when malignant cells were taken in relapse, but cells taken at the progressive phase of the disease were partially resistant to the same treatment. Burkitt's lymphoma cells taken at the progressive phase resulted in the BL_{93} cell line being shown to be partially resistant to the rabbit complement lysis procedure (see Favrot *et al.* "Comparative Efficiency of an Immunomagnetic Purging Procedure and a Rabbit Complement Lysis for Eliminating Burkitt's Lymphoma Cells From Bone Marrow," in this volume). As shown in Table 4, in four cases the bone marrow, even if cytologically normal, was shown to be pathological by the detection of a high percentage of BL cells at day 10 of the culture. The assay allows a qualitative evaluation of the purging methods. The purging procedure inhibited BL cell growth. In the first two cases, bone marrow had been harvested for ABMT, and the assay proved the quality of the therapeutic purging procedure. The purged autograft has been reinjected in patient 1. In patient 7, the cocktail of three monoclonal antibodies was shown to be more efficient than B_1 alone or Y29/55 combined with $RFAL_3$. Finally, and as shown in Table 4 for

Table 3. Quantitative Evaluation of the Elimination of Fresh BL Cells in Excess Autologous Bone Marrow by a Complement Lysis Procedure

	BL Cells (%)						
Patients	0	10^{-3}	10^{-4}	10^{-5}	10^{-6}	$1/4 \times 10^6$	$1/10^2$
1 (EBV−)[a]	0	100	80	20	1	ND	0
	0	60	20	10	ND	ND	20
2 (EBV+)	0	ND	10	8	7	ND	0
3 (EBV−)	0	ND	ND	35	10	3	0
4 (EBV+)	0	ND	ND	55	4	0	0

Abbreviations: BL, Burkitt's lymphoma; EBV, Epstein-Barr virus; ND, not determined.

Note: Numbers show the percentage of BL cells detected in culture at day 10 by the ratio of BL cells to normal cells at the start. All samples underwent rabbit complement lysis with one of two monoclonal antibody cocktails—$RFB_7 + SB_4$ or $B_1 + Y29/55 + RFAL_3$ (see Favrot et al. *"Comparative Efficiency of an Immunomagnetic Purging Procedure and a Rabbit Complement Lysis for Eliminating Burkitt's Lymphoma Cells From Bone Marrow," in this volume).*

[a]*For patient 1, the first experiment was made in first relapse; the second experiment was made in second relapse.*

Table 4. Qualitative Evaluation of Complement Lysis Purging on Pathological Bone Marrow

Patients	BL Cells (%)		
	Before Purging	After Purging	
1 (EBV−)	70	0	(Y29/55 + $RFAL_3$ + complement)
4 (EBV−)	60	0	(RFB_7 + Y29/55 + $RFAL_3$ + complement)
6 (EBV−)	63	0	(RFB_7 + SB_4 + complement)
		0	(B_1 + IMD)
7 (EBV+)	100	0	(B_1 + Y29/55 + $RFAL_3$ + complement)
		20	(B_1 + complement)
		10	(Y29/55 + $RFAL_3$ + complement)

Abbreviations: BL, Burkitt's lymphoma; EBV, Epstein-Barr virus; IMD, immunomagnetic depletion.

Note: Numbers show the percentage of BL cells detected in culture at day 10; negative cultures remained free of detectable BL cells at day 21. Bone marrow was pathological at the time of harvesting for autologous bone marrow transplantation. The assay demonstrated the efficiency of the therapeutic purging procedure.

patient 6, this assay allows us to compare the efficiency of two immunologic methods we recently defined.

DISCUSSION

Experimental models standardized for assessing purging procedures use malignant cells from various cell lines admixed with irradiated bone marrow cells, but very few models allow the evaluation of the quantitative or qualitative efficiency of the purging methods in preclinical or clinical assays. Serial dilution assays have been extensively used in an attempt to quantify purging procedures. According to published data (9-12), very few BL cell lines have been tested in such an assay because they have to be preselected for their high clonogenic efficiency to allow statistical evaluation of the remaining clonogenic units. None of them was newly established, however, and despite the selection of permanent lines, the clonogenicity would be between 15% and 90% and would be lower for EBV-negative cell lines (Table 5). Therefore, such an assay is unable to define the total disappearance of malignant cells from treated samples. Furthermore, it only detects cells with sufficient growth at day 14.

Our assay thus presents several advantages. Malignant cell lines can be established from more than 70% of our patients with EBV-positive and -negative BL (1). All of these newly established cell lines are able to grow in our assay, with a limit of detection of 10^{-6} cells and the growth of a single BL cell in 4×10^6 normal ones. The possibility of keeping these cultures for longer than 2 weeks enables us to confirm the BL cell growth when there are very few residual malignant cells and even when this growth is slow. Our assay can then be

Table 5. Summary of the Clonogenic Efficiency of BL Cell Lines in Serial Dilution Assay or Liquid Culture Assay

Cell Lines	Cloning Efficiency[a]	Author
Widespread		
BJAB (EBV−)	15%	de Fabritiis *et al.* (10)
JD-38 (EBV−)	26%	de Fabritiis *et al.* (10)
Manalwa (EBV+)	34%	de Fabritiis *et al.* (10)
Manalwa (EBV+)	50%	Bast *et al.* (9)
Status Undetermined		
BL cell line (?)	90%[b]	Uckun *et al.* (12)
Newly Established		
BL_{93}	> 90%[c]	Philip *et al.* (this chapter)
BL_2	> 90%	
BL_{99}	> 90%	
BL_{17}	> 90%	

Abbreviations: BL, Burkitt's lymphoma; EBV, Epstein-Barr virus.

[a]*Estimated on the basis of published results.*

[b]*Optimal growth in control samples at 1% BL cell concentration.*

[c]*Good outgrowth at a wide range of conditions (i.e., one residual cell among 4 × 10^6 normal bone marrow cells).*

exploited for quantifying the efficacy of purging against Burkitt's cells of various origins and different characteristics. Apparently the phenotypic features of these lines presumably reflect the heterogeneity of the disease in the individual patients (4,5). However, the liquid culture assay has the following limitations: this test indicates the range of residual malignant cells in the samples rather than their precise numbers, and though very sensitive in determining whether the cleaning of the bone marrow is complete (> 3 log kill), it is less sensitive in assessing the elimination under 2 logs.

More recently, we used the liquid culture to assay the efficiency of these purging procedures on fresh tumor samples, either invaded bone marrow or autologous nonirradiated bone marrow contaminated with fresh BL cells from the initial site of the disease. Preliminary results show that the level of sensitivity is close to that observed with the cell lines, and for four patients we were able to demonstrate the growth of malignant cells prior to the clinical purging procedure and its inhibition after the procedure. As shown for patient 7 (Table 4), this assay allows the selection of the relevant monoclonal antibodies in the complement lysis procedure or, as in patient 6, the comparison of the efficiency of immunomagnetic depletion and that of complement lysis.

Most purging procedures described have now been refined in terms of toxicity and efficiency and are capable of eliminating 3 logs of malignant cells. The next step is to transfer such procedures into clinics and to study the ability of such procedures to cleanse bone marrow with minimal involvement. As we

have already stressed elsewhere in this book (see Favrot *et al.* "Comparative Efficiency of an Immunomagnetic Purging Procedure and a Rabbit Complement Lysis for Eliminating Burkitt's Lymphoma Cells From Bone Marrow," and Combaret *et al.* "Eliminating Burkitt's Cells From Excess Bone Marrow With an Immunomagnetic Purging Procedure"), clinical pilot studies that permit an accurate quantification of residual malignant cells before and after the procedure for each individual patient are needed before extensive multicenter studies may be started. Our assay is promising in this respect.

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Comparative Efficiency of an Immunomagnetic Purging Procedure and a Rabbit Complement Lysis for Eliminating Burkitt's Lymphoma Cells From Bone Marrow

M. C. Favrot, I. Philip, P. Poncelet, V. Combaret, B. Kremens, G. Janossy, and T. Philip

Several monoclonal antibodies that react with B lymphocytes are now available for therapeutic use. We previously showed, according to the immunophenotype of 28 Burkitt's lymphoma (BL) cell lines, that three of them, B_1 and Y29/55—two pan B reagents—and AL_2 directed against CALLA (common acute lymphoblastoid leukemia antigen), were expressed on BL cell lines, especially in Caucasian ones (1). We used this cocktail of monoclonal antibodies to target BL cells, either in a purging procedure using rabbit complement or in an immunomagnetic depletion technique. A description of how to set up optimal working conditions in the two techniques has been previously published (2,3; see also Combaret *et al.* "Eliminating Burkitt's Cells From Excess Bone Marrow With an Immunomagnetic Purging Procedure," in this volume). The aim of this study was, first, to quantify the efficiency of two new IgM monoclonal antibodies, RFB_7 (CD 20-like) and SB_4 (CD 19), in the rabbit complement lysis procedure and, second, to compare in the same experiments the efficiency of both procedures in optimal conditions. Such experiments were performed on three cell lines freshly established from our patients' tumor cells, all three patients

being candidates for an autograft, and using a liquid cell culture assay to quantify residual malignant cells (4; see also I. Philip *et al.* "Using a Liquid Cell Culture Assay to Measure In Vitro Elimination of Burkitt's Cells From Bone Marrow," in this volume).

MATERIALS AND METHODS

Normal bone marrow samples were obtained from healthy donors on preservative-free heparin, separated on a Ficoll gradient to obtain the mononuclear cell fraction, and irradiated at 50 Gy. Samples were contaminated with 1% BL cells from three different cell lines: IARC BL_{99}, IARC BL_{93}, and IARC BL_2. The characteristics of these lines as well as the conditions of culture and viability have been previously described (1,4).

RFB_7 is a CD 20-like monoclonal antibody and SB_4 is a CD 19 monoclonal antibody. These two IgM monoclonal antibodies are directly coupled to the magnetic particles, as described below, or used at 10 μg/ml (final dilution) in the complement lysis procedure. Y29/55 (mouse IgG2 subclass) (Hoffmann-La Roche) is used at 20 μg/ml final dilution. B_1 (mouse IgG2 subclass) (Coultronics) is used at 20 μg/ml final dilution, and J_5 (mouse IgG2 subclass) (Coultronics) is also used at 20 μg/ml final dilution.

Monodispersable 4.5-μm polystyrene microspheres (ME 450) containing magnetite were used (Dynabeads 14002, Dynal, Norway). Affinity-purified rabbit antimouse IgG (Biosys, B 12013/6091) are coupled by physical adsorption on beads by a 4-hour incubation at 4°C in 1 volume of IgG in phosphate-buffered saline (PBS) (2 mg/ml), 3 volumes of particles (100 mg/ml in H_2O), and 1 volume of 0.5-M Na_2HPO_4 buffer (pH 7.5). Beads were then washed with saline (0.9% NaCl) containing 1% human serum.

When IgM monoclonal antibodies were used, they were directly coupled to beads by an 18-hour adsorption: 1 volume of beads (30 mg/ml) in H_2O and 1 volume IgM monoclonal antibody in 0.2 M phosphate buffer (150 μg protein/ml). Beads were then washed and kept as described above.

Samples were resuspended at 20×10^6 cells/ml, incubated half an hour at 4°C with monoclonal antibodies at appropriate dilution, and washed twice before the incubation with beads in PBS medium without albumin. Mononuclear bone marrow cell samples (10×10^6 cells/ml) were then incubated with 2 mg of beads/ml for 30 minutes at 4°C under continuous slow rotation and passed through the magnetic system. The number of beads needed depends on the total number of cells that are to be treated, either normal or malignant, 2 mg of beads being sufficient to clean samples containing up to 10% malignant cells. The second step of the procedure involving the incubation with the beads and the magnetic separation is repeated twice. Cells are then resuspended in appropriate medium for the freezing procedure.

All experiments were performed at 24°C. Baby rabbit complement was made in collaboration with Institut Pasteur (Lyons, France) (2). Samples (2 ×

10^7 cells/ml in Hanks' medium) were incubated 20 minutes with monoclonal antibodies and then twice with complement, with one wash between the two complement treatments. Complement was used at one-third final dilution. DNase I (sterile nonpyrogenic pancreatic DNase from Sigma, St. Louis, MO) (5 U/ml) was added to each complement treatment. All media were buffered with HEPES (Tebu).

Residual malignant cells were quantified by a liquid cell culture assay (4; see also the above-named chapter by I. Philip *et al.* in this volume). After treatment, samples initially contaminated with 1% BL cells are grown in a liquid culture system, and the percentage of growing BL cells at day 10 compared to a calibration curve enables us to quantify the number of residual BL cells after treatment. If no BL cells are detectable at day 10, samples are kept for 2 more weeks in culture before we conclude that all BL cell growth has been inhibited, a finding usually correlated to the complete elimination of these cells in this assay.

Mean results are then expressed in decimal logarithms of the initial ratio of malignant and normal cells divided by the final ratio after the purging procedure. In individual experiments, results are expressed as percentages of growing BL cells in the culture at day 10.

RESULTS AND DISCUSSION

Results shown in Table 1 prompt three comments. First, whatever monoclonal antibody is used, the BL_{93} cell line is partially resistant to the complement. Second, contrary to general opinion, SB_4 monoclonal antibody alone is as efficient as the two cocktails of monoclonal antibodies. RFB_7 shows a

Table 1. Efficiency of the Complement Lysis Procedure

Cell Lines (no. of experiments)	Mean Cell Kill (log)					
	RFB_7	SB_4	$RFAL_3$	Y29/55	RFB_7 + SB_4	RFB_7 + SB_4 + Y29/55
IARC BL_{99} (n = 8) EBV (+) (8,22) Caucasian	5	>5	4.5	3	>5	5
IARC BL_{93} (n = 5) EBV (-) (8,14) Caucasian	2.5	1.5	2	2	3	2.5
IARC BL_2 (n = 5) EBV (-) (8,22) Caucasian	4	>5	5	5	>5	5

Abbreviation: EBV, Epstein-Barr virus.

very similar activity. Therefore, a single monoclonal antibody with high cytolytic activity is able to kill, in a heterogeneous malignant population, cells with the lowest density antigen. However, in such a procedure, the use of a cocktail could remain essential in therapeutic purging if the initial phenotype of the tumor is unknown; in addition, the antigen density on tumor cells in the bone marrow might be more heterogeneous than on a cell line.

Our results are rather different from those obtained and published on the African cell line Manalwa (5). They emphasize the point that a purging procedure must be tested in a model as close as possible to the clinical situation and, as described here, on cell lines obtained from European or American patients.

Finally, these results demonstrate the difficulty of passing from the experimental model to clinical practice. Indeed, when purging procedures were started on our patients the only antibody available was Y29/55, and the BL cell elimination ranged from 2 to 5 logs. We then used a cocktail consisting of Y29/55, AL_3, and RFB_7, and more recently we were able to use for 10 patients a cocktail of RFB_7 and SB_4. With such a cocktail, we can obtain a full elimination of malignant cells in most cases, but it is likely that a few patients will have resistant malignant cells. We do not have criteria predictive of failure; therefore, if possible, a preclinical test will have to be performed for each patient before the therapeutic assay (see the above-named chapter by I. Philip *et al.* in this volume).

Results of using the immunomagnetic purging procedure are reported and discussed in the chapter by V. Combaret *et al.* in this volume. Work had shown that the monoclonal antibody B_1, used as single reagent in an indirect method, is the most effective reagent to be used in immunomagnetic depletion (IMD).

Experiments reported in Table 2, performed either with complement lysis or IMD, have been performed in parallel on the same samples and on the same day. They permit a comparison of the two methods. BL_{99} and BL_2 cell lines were

Table 2. Comparison of Two Immunologic Procedures

Cell Lines (no. of experiments)	Mean Cell Kill (log)	
	Complement-dependent Lysis Procedure $RFB_7 + SB_4$	Immunomagnetic Depletion B_1
BL_{99} (n = 8)	>5	4
BL_{93} (n = 5)	3	>5
BL_2 (n = 5)	>5	4

Note: The efficiency of the procedure has been evaluated by liquid cell culture assay in a short- and long-term culture.

very sensitive to the complement lysis; in contrast, the IMD procedure provided an optimal BL cell elimination on the BL_{93} cell line partially resistant to complement. The analysis of individual experiments, in which one or the other procedure failed, supplied complementary data, as shown in the three examples of Table 3. Indeed, we demonstrated a complementarity between the two immunologic procedures. It has been previously reported that chemical (mafosfamide) and immunologic purging methods have additive effects (6-10). Such a complementary effect could appear more surprising in the case of two immunologic methods. It is in fact quite logical if one remembers that the resistance of malignant cells to complement lysis (as shown for BL_{93}) is relatively independent of the monoclonal antibody used and that quality criteria required from monoclonal antibodies and the recognized antigen in the IMD are different from those required in the complement lysis procedure (see V. Combaret *et al.* in this volume).

CONCLUSION

Our work shows that one or the other procedure enables us to purge a bone marrow having minimal involvement (i.e., 1% or less), but a few unpredictable partial failures were observed with both methods. Considering such results, we are tempted to propose combining the purging methods, to allow the cleansing of bone marrow contaminated by more than 1% malignant cells and to avoid the few failures we observed. We will, however, strongly reject such an idea. Indeed, in the first hypothesis, if the marrow contains more than 1% malignant cells, it could probably be purged by a combination of these procedures, but the peripheral disease would not be eradicated by high-dose chemotherapy. In the second hypothesis, in order to avoid a few failures in some patients it is unacceptable to increase for all of them the toxicity and the cost of the purging by combining two procedures. We will, therefore, suggest a purging "a la carte" for which optimal monoclonal antibodies and consequently optimal methods will be selected for each patient in a preclinical assay (4; see also I. Philip *et al.* in this volume). Such an approach is certainly a very difficult one for laboratory investigators but could allow the evaluation of the purging method

Table 3. Complementary Effects of Two Immunologic Procedures

Cell Line	Mean Cell Kill (log)	
	Complement	Magnetic Depletion
BL_{99}	>5	2
BL_{93}	2	>5
BL_2	>5	3

Note: These three individual experiments (one on each line) are part of Table 2 results and are given as examples. When one of the two techniques fails, the other one is highly efficient.

for each patient in clinical pilot trials. This step is fundamental before any controlled or even randomized multicriteria studies can be proposed.

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Susceptibility of Lymphoid Malignant Cells to Ricin A-chain Immunotoxins

G. Laurent, J. M. Derocq, F. Roubinet, E. Kuhlein, M. Chiron, M. Attal, and F. K. Jansen

Immunotoxins may improve in the future the efficacy and specificity of the conventional treatment of neoplastic blood diseases. In autologous bone marrow transplantation (ABMT), immunotoxins may represent an interesting approach not only to eliminating residual malignant cells that contaminate bone marrow but also to treating leukemia and lymphoma patients in vivo in an additive conditioning therapy in association with conventional preparative regimens. However, it is now well established from in vitro studies that a given immunotoxin may induce different cytotoxic effects on cell lines expressing the same relevant antigen (1-3), suggesting that in clinical conditions, neoplastic cells' sensitivity to a given immunotoxin might vary from patient to patient. Unfortunately, the sensitivity of individual leukemia cells to immunotoxins has not been explored fully, mainly because of methodological obstacles such as their low level of protein synthesis and their low cloning efficiency in semisolid medium. Now, however, a study from a Minneapolis group points out the feasibility of a cloning assay to evaluate, under sophisticated conditions of culture, the sensitivity of fresh ALL cells to immunotoxins (4).

In order to test rapidly the sensitivity of leukemia cells to an immunotoxin in routine clinical conditions, we designed a method based on a dye exclusion

assay (DEA) that represents a simple and reliable procedure that can give after standardization a semiquantitative indication of the sensitivity of bulk tumor cells (3). The present study aimed to evaluate in vitro sensitivity of various neoplastic blood cells expressing the T65 antigen to the antigen-binding fragment (Fab) of T101 ricin A-chain immunotoxin (Fab T101-RTA) and to explore possible correlations between efficacy and mean density of T65 molecules on target cells. This study was performed with and without NH_4Cl because NH_4Cl (10 mM) has been found to be a safe and potent enhancer agent suitable for ex vivo bone marrow treatment with ricin A-chain immunotoxins (5,6) but inappropriate for in vivo use because of its general toxicity at concentrations needed to obtain an optimal potentiating effect.

MATERIALS AND METHODS

A CEM III subclone was derived from CEM wild-type cell line by sorting and selected on the basis of T1 antigen expression (30,000 T1 molecules/cell) (3). Heparinized blood samples obtained from leukemia patients were separated on a Ficoll-Hypaque gradient. Mononuclear cells were then washed and resuspended in macroplate 2-ml wells at a final concentration of 10^6 cells/ml in RPMI and 10% fetal bovine serum.

One million cells were incubated with Fab T101-RTA at a final concentration of 10^{-8} M expressed in A-chain content with and without NH_4Cl (10 mM) in a total volume of 1 ml. Cells were incubated at 37° C in 5% CO_2 in air with gentle agitation during 24 hours. Controls consisted of treatment with RPMI alone, ricin A-chain alone, and NH_4Cl alone. All experiments were performed in quadruplicate.

After incubation with immunotoxin, treated cells were washed and then a DEA was performed as previously reported (3). Fluorescein diacetate (FDA) staining propidium iodide (PI)-negative living cells were enumerated at different times after immunotoxin treatment—24, 48, and 72 hours—in order to improve the sensitivity of the test by counting cells after a long period of time following the end of immunotoxin treatment. A 48-hour delay was found to be a good compromise for serving the interests of the sensitivity of the assay and viability of control cells. Sensitivity to immunotoxin was determined by the ratio between the number of viable cells after immunotoxin treatment and the number of viable cells in control samples, and it was expressed in a percentage.

A cloning assay was performed on CEM cells as previously described (2) and used to standardize the DEA by comparing the percentage of CEM living cells evaluated with DEA 48 hours after treatment and the level of cytoreduction as assessed by cloning assay at various doses of Fab T101-RTA (3). T65 antigen mean density was measured by cytofluorometric quantification according to the technique described by Poncelet and Carayon (7).

RESULTS

In the presence of NH_4Cl (10 mM), Fab T101-RTA showed three distinct ranges of cell killing efficacy on CEM III cells as evaluated by DEA (Table 1). First, the high cell killing efficacy for immunotoxin concentrations ranging from 10^{-8} to 10^{-10} M left from 5.1% to 19.7% residual viable cells. For these concentrations, the cloning assay showed a cytoreduction of 6 logs. Second, the medium-range cell killing efficacy for a concentration of 10^{-11} M left from 63% to 77% residual cells. For this concentration, the cloning assay showed a cytoreduction of 2.8 logs. Third, poor or no cell killing efficacy existed for immunotoxin concentrations of 10^{-12} M, which left percentages 90.3% and higher. No significant cytoreduction was observed by the cloning assay at these concentrations.

In the absence of NH_4Cl, Fab T101-RTA could induce at 10^{-8} a cytoreduction of 2.9 logs. At this concentration, DEA could detect 44-57% (mean, 48%) residual viable CEM III cells. Examined together, these results showed that DEA could identify three distinct levels of sensitivity to Fab T101-RTA: 1) a high level of sensitivity with percentages lower than 16%, 2) an intermediate range of sensitivity with percentages varying from 40% to 80%, and 3) a low level of sensitivity with percentages higher than 90%.

Malignant T cells displayed very distinct sensitivities to Fab T101-RTA, depending on the presence or absence of NH_4Cl. In the presence of NH_4Cl (10 mM) T cells of both chronic lymphocytic leukemia (CLL) and acute lymphocytic leukemia (ALL) showed extremely high susceptibility to Fab T101-RTA equal to or greater than that observed for CEM III cells (see Table 2). Without NH_4Cl, malignant T cells displayed a wide range of susceptibility

Table 1. Dose Effect of Fab T101-RTA on CEM III Cells With and Without NH_4Cl as Assessed by Cloning Assay and DEA

	Concentration of Fab T101-RTA (M of A-chain)				
Measure	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Log kill with NH_4Cl (cloning assay)	6.0	6.0	6.0	2.8	0.5
R[a] with NH_4Cl (DEA) (%)	5.1–13.8 (m =10.2)	5.0–16.3 (m = 10.8)	11.7–19.7 (m = 14.5)	62.9–76.8 (m = 69.4)	90.3–104.5 (m = 98.1)
Log kill without NH_4Cl (cloning assay)	2.9	1.8	0.5	0	0
R[a] without NH_4Cl (DEA) (%)	44.1–57 (m = 48)	81.9–97.4 (m = 88.1)	92.0–116.6 (m = 103.4)	NT	NT

Abbreviations: DEA, dye exclusion assay; NT, not tested.

[a]*R = percentage of residual viable CEM III cells 48 hours after treatment.*

Table 2. Sensitivity of Fresh Leukemia T Cells to Fab T101-RTA With and Without NH_4Cl as Assessed by FDA-PI Dye Exclusion Test

Patient	R[a] with Fab T101-RTA Without NH_4Cl	R[a] with Fab T101-RTA With NH_4Cl (10^{-2} M)	Mean Density of T65 Antigen
1 (T-CLL)	14.2	3.6	123,000
2 (T-CLL)	37.5	8.6	13,000
3 (T-ALL)	46.0	0	23,000
4 (T-CLL)	56.0	21.3	65,000
5 (T-ALL)	67.0	3.9	10,000
6 (T-CLL)	101.0	3.4	25,500
CEM III	48.1 ± 6 (log kill = 2.9)	10.2 ± 5.7 (log kill = 6)	30,000

Abbreviations: FDA-PI, fluorescein diacetate-propidium iodide; T-CLL, T-cell chronic lymphocytic leukemia; T-ALL, T-cell acute lymphocytic leukemia.

[a]R = percentage of residual living cells 48 hours after treatment (24-hour incubation) with immunotoxin (10^{-8} M).

with percentages varying from 14.2% (much lower than that observed with CEM cells) to 101.0%. No correlation with mean density of T65 antigen (range, 10,000-123,000 T1 molecules/cell) could be established.

Malignant B-CLL cells showed generally poor, although variable, susceptibility to Fab T101-RTA (equal to or lower than that of CEM cells) with or without NH_4Cl as shown in Table 3. No correlation with mean density of T65 antigen (range, 2500-35,000 T1 molecules/cell) could be established.

DISCUSSION

This study confirmed the clinical feasibility and the value of the DEA evaluation system as a simple semiroutine procedure to evaluate, after standardization, the susceptibility of tumor cells to immunotoxins. The DEA allowed three distinct ranges of sensitivity with an excellent correlation with a cloning assay as shown on CEM cells.

In a series of 13 patients (6 with T-cell malignancies and 7 with B-CLL), DEA showed a wide range of in vitro sensitivity to Fab T101-RTA. These results confirmed the heterogeneity of the response of malignant cells to immunotoxins as it has been previously reported in other systems (1-4). These results suggest that predictive determination of the sensitivity of malignant cells to immunotoxins is clearly needed in order to select patients who might benefit from treatment with them. However, despite large variations of cell-killing efficacy, it has to be pointed out that in the presence of NH_4Cl as an enhancing agent, most malignant T cells showed a very high

Table 3. Sensitivity of Fresh B-CLL Cells to Fab T101-RTA With and Without NH_4Cl as Assessed by FDA-PI Dye Exclusion Test

Patient	R[a] with Fab T101-RTA Without NH_4Cl	R[a] with Fab T101-RTA With NH_4Cl (10^{-2} M)	Mean Density of T65 Antigen
1	36.7	27.9	2,500
2	43.5	15.0	35,000
3	46.0	34.6	20,000
4	53.9	37.0	10,000
5	78.0	39.6	5,500
6	93.2	74.8	10,000
7	100.0	86.2	6,000
CEM III	48.1 ± 6 (log kill = 2.9)	10.2 ± 5.7 (log kill = 6)	30,000

Abbreviations: B-CLL, B-cell chronic lymphocytic leukemia; FDA-PI, fluorescein diacetate-propidium iodide.

[a]R = percentage of residual living cells 48 hours after treatment (24-hour incubation) with immunotoxin (10^{-8} M).

sensitivity to Fab T101-RTA (equal or higher than CEM cells) and B-CLL cells displayed a lower sensitivity. Furthermore, without NH_4Cl, high or medium range cytoreduction (higher than or equal to 3 logs) could be expected in most T-cell malignancies, suggesting the high potential of Fab T101-RTA for in vivo use, even if fully resistant T101-positive malignant cells were found.

No correlation could be established between mean density of T65 antigen and Fab T101-RTA cell-killing efficacy within a range of 2500-123,000 molecules/cell. Those findings are not in line with those recently reported from studies on continuous cell lines in which the influence of mean density of target antigen on immunotoxin cell-killing efficacy was clearly demonstrated (3). These conflicting results may demonstrate that other factors can influence individual susceptibility of target cells to relevant immunotoxins.

Finally, despite its relatively low sensitivity compared with the cloning assay, DEA allowed the detection of distinct susceptibilities of fresh malignant blood cells to Fab T101-RTA. However, especially in malignant T cells, Fab T101-RTA could induce in most cases a significant cell-killing efficacy greatly improved by the use of NH_4Cl as an enhancing agent.

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Lymphoma: Purging and Detecting

K. Anderson and G. Phillips, Chairmen

DR. C. GORIN: Dr. Barlogie, did you try to purge marrow with J5 or with any other anticommon acute lymphoblastic leukemic antigen monoclonal antibody?

DR. B. BARLOGIE: No, we didn't.

DR. A. GEE: I was interested in asking Bob Bast whether addition of a second monoclonal antibody rather than a chemotherapeutic agent increased the cell kill or whether your cells that were left behind were, for example, low density and sensitive in that way.

DR. R. "BOB" BAST: The problem didn't seem to be killing the lymphoma cells so much, at least with the immunotoxin, as it was killing the normal marrow precursors. We could eliminate every last lymphoma cell that we could measure, provided that you can go high enough on the dosage. It may be with the second antibody that we might get additive effects against the lymphoma and not additive effects against the CFUs-GM. We have looked for bystander effects with a B-lymphoma line, mixed with the T lymphoma and the immunotoxin. And we don't see bystander killing. So, presumably, there is something going on that is a bit more specific in terms of the marrow toxicity.

DR. C. READING: I just want to ask you if you used immunotoxins and then froze the marrow and thawed it again, would the bystander effects be much worse? Have you investigated that?

DR. BAST: We have not. Before this goes to clinical trial, which we anticipate doing in the not too distant future, that is one of the essential controls. We are aware of that problem, but we have not investigated it. We have been looking for the optimal dose of immunotoxin which we have only recently discovered.

DR. G. SPITZER: Bob, I wanted to ask you if you noticed any patient variability in the sensitivity to chemoimmunopurging.

DR. BAST: Within the limits of the data of the six patients that I showed you, we did not see dramatic differences in that we could eliminate all of the colonies that we could measure with the combination. However, this is a suboptimal system. A subset of these, two leukemic patients, are responsive to IL-2 or conditioned medium, and we will probably be able to improve that system by a log in the near future. I think that we may be able to find more subtle differences in the susceptibility of different patient cells. In our experience to date, virtually every leukemia and lymphoma that we have measured at Duke University has been 3A1 positive in contrast to the data with some of the cell lines that I presented. So there is some hope that this will be a relatively universal reagent, although there may be occasional patients in which a second immunotoxin might be useful because of the heterogeneity issue rather than the saturation.

DR. M. FAVROT: I would like to ask you two more questions. When you are working with fresh tumor samples, do you use the limiting dilution assay to detect residual tumor cells? What is your limit of detection compared with immunofluorescence?

DR. BAST: I suspect, Marie (Dr. Favrot), that you could be much more sensitive with a morphologic technique or with an immunofluorescence technique. Whether it is more relevant, I think, is the difficult issue. Needless to say, I think that from your work, as from ours, we are interested in getting rid of the clonogenic cells; however, cases in which we are growing only 20 or 50 colonies per 10^5 cells, the sensitivity of that technique is so low that I am sure that tumor cell detection can be done better with immunofluorescence.

III. Neuroblastoma

Autologous Bone Marrow Transplantation for Poor-Prognosis Neuroblastoma

Robert C. Seeger, C. Patrick Reynolds, Thomas J. Moss, Carl Lenarsky, Stephen A. Feig, Michael Selch, John Ugelstad, and John Wells

Neuroblastoma, a neoplasm of the sympathetic nervous system, is the most common extracranial tumor of childhood (1). Approximately 60% of all patients have only a 10% probability of long-term, disease-free survival if given conventional therapy that includes chemotherapy, local irradiation, and surgery (2-5). Recent pilot studies of intensive chemotherapy and total body irradiation (TBI) followed by allogeneic bone marrow transplantation (BMT) or autologous bone marrow transplantation (ABMT) have produced encouraging results (6,7). In this report, we update our original study in which 20 patients with advanced neuroblastoma underwent intensive four-drug chemotherapy, TBI, and ABMT or allogeneic BMT; in addition, we provide data from our current study regarding the clinical use of sedimentation, filtration, and magnetic immunobeads for the ex vivo removal of neuroblastoma cells from autologous marrow.

TOXICITY AND EFFICACY OF INTENSIVE CHEMORADIOTHERAPY AND BMT

In our initial study (patient entry from January 1983 to October 1985), we investigated toxicity and efficacy of intensive chemoradiotherapy (teniposide [VM-26], doxorubicin, L-phenylalanine mustard, cisplatin, and TBI; VAMP-TBI) followed by allogeneic or ABMT (Table 1). Twenty patients, all of whom were diagnosed after 1 year of age and who had stage IV (n = 18) or stage III (n = 2) disease, received VAMP-TBI and bone marrow. Thirteen patients were given bone marrow transplants before they developed progressive disease (3 received allogeneic marrow from a human leukocyte antigen [HLA]-compatible sibling and 10 received autologous marrow); 7 received transplantations after they developed progressive disease (5 allogeneic BMT and 2 ABMT).

Eight patients received bone marrow from HLA-mixed leukocyte culture (MLC) matched, nonidentical siblings (median of 3×10^8 and range of $2\text{-}4 \times 10^8$ nucleated marrow cells/kg). These patients were given methotrexate after BMT for prophylaxis of graft-versus-host disease (10 mg/m^2 on days 1, 3, 6, 11, and then weekly to 100 days).

Twelve patients received cryopreserved autologous marrow. Autologous marrow was used to restore hematopoiesis only if tumor cells were not detectable in an aliquot of the cryopreserved specimen by immunoperoxidase staining with anti-cell-surface monoclonal antibodies (mixture of antibodies 390, 459, HSAN 1.2, and 126-4) and anti-neuron-specific enolase serum; analysis of 3×10^5 bone marrow mononuclear cells gives a 95% probability of detecting one neuroblastoma cell among 10^5 normal cells (8,9). Marrow was prepared for cryopreservation by equilibrium density centrifugation over Ficoll-Hypaque (patients 1-7) (10); by sedimentation and filtration (patient 8); or by

Table 1. Pretransplant Intensive Chemoradiotherapy Regimen (VAMP-TBI)[a]

Day	Treatment
-9	Cisplatin, 90 mg/m^2 i.v.
-8	No therapy
-7	VM-26, 150 mg/m^2 i.v.; doxorubicin, 45 mg/m^2 i.v.
-6	Melphalan, 140 mg/m^2 i.v.
-5	Melphalan, 70 mg/m^2 i.v.
-4	VM-26, 150 mg/m^2 i.v.
-3	TBI, 3.33 Gy, 0.08-0.1 Gy/min
-2	TBI, 3.33 Gy, 0.08-0.1 Gy/min
-1	TBI, 3.33 Gy, 0.08-0.1 Gy/min

[a] *For patients <2 yrs old or weighing <12 kg, the doses of cisplatin, VM-26, melphalan, and doxorubicin are calculated according to weight, assuming 1 m^2 = 26 kg (e.g., cisplatin, 3.5 mg/kg; doxorubicin, 1.7 mg/kg; melphalan, 5.4 and 2.7 mg/kg; VM-26, 5.8 mg/kg). Fractionated TBI is administered with a 6-MeV linear accelerator.*

Abbreviations: VM-26, teniposide; TBI, total body irradiation.

sedimentation, filtration, incubation with monoclonal antibodies, and then goat antimouse immunoglobulin-coated magnetic beads (marrows from patients 9-11 were treated with antibodies 390, 459, HSAN 1.2, BA-1, and RB21-7; marrow from patient 12 was treated with antibodies 459, BA-1, and 126-4) (11,12). Recipients of autologous marrow were given a median of 7×10^7 nucleated marrow cells per kilogram (range, $2\text{-}54 \times 10^7$ cells/kg).

Severe oral mucositis and enteritis were observed in all patients after treatment with VAMP-TBI. Total parenteral nutrition via central venous catheter was necessary after BMT for all patients because of mucositis, enteritis, and anorexia; for those surviving the first month after transplantation, the median time until parenteral nutrition was discontinued was 2 months. Skin desquamation was significant in the allogeneic BMT group but not in the ABMT group.

Four deaths occurred during the first month after transplantation among the eight patients undergoing allogeneic BMT. The causes of these early deaths, which all occurred prior to documented engraftment, were renal failure, hepatic veno-occlusive disease, disseminated aspergillosis, disseminated candidiasis. Graft-versus-host disease was not observed in any of the allogeneic marrow recipients.

Among the 12 patients receiving autologous marrow, there were no deaths in the first posttransplantation month. One patient, who failed to recover platelets even though megakaryocytes were present in the marrow, died of a cerebral hemorrhage 3 months after BMT (patient 9); and one whose marrow engrafted died 7 months posttransplantation of bacterial sepsis due to suspected child abuse (patient 8).

Early death as well as morbid toxicity appeared to be greatest in the allogeneic group. Analysis of differences between the two groups suggested that methotrexate given for prophylaxis of graft-versus-host disease was the most probable cause of the added toxicity. Methotrexate toxicity would be increased if renal clearance was impaired secondary to chemotherapy (e.g., cisplatin) and radiation. Thus, it is advisable to assess renal function before administering methotrexate, to monitor clearance and adjust dosage during treatment, and to rescue with Leucovorin as necessary.

Engraftment was defined as follows: 1) absolute neutrophil count greater than 500/mm^3 for 3 consecutive days; 2) platelet count greater than 30,000/mm^3 without transfusion; and 3) hemoglobin count greater than 8 g/dl sustained without transfusions. Engraftment occurred in all 4 patients receiving allogeneic marrow who survived more than 1 month and in 11 of 12 receiving autologous marrow.

Outcome for the 13 patients who received bone marrow before they developed progressive disease was encouraging (Fig 1). Seven are tumor-free survivors for 3+ to 40+ months after BMT; four patients relapsed (all from the ABMT group); one died secondary to toxicity (allogeneic BMT group); and one died of sepsis secondary to suspected child abuse (ABMT group). The estimated disease-free survival rate is 45%, and the actual survival rate is 56% at

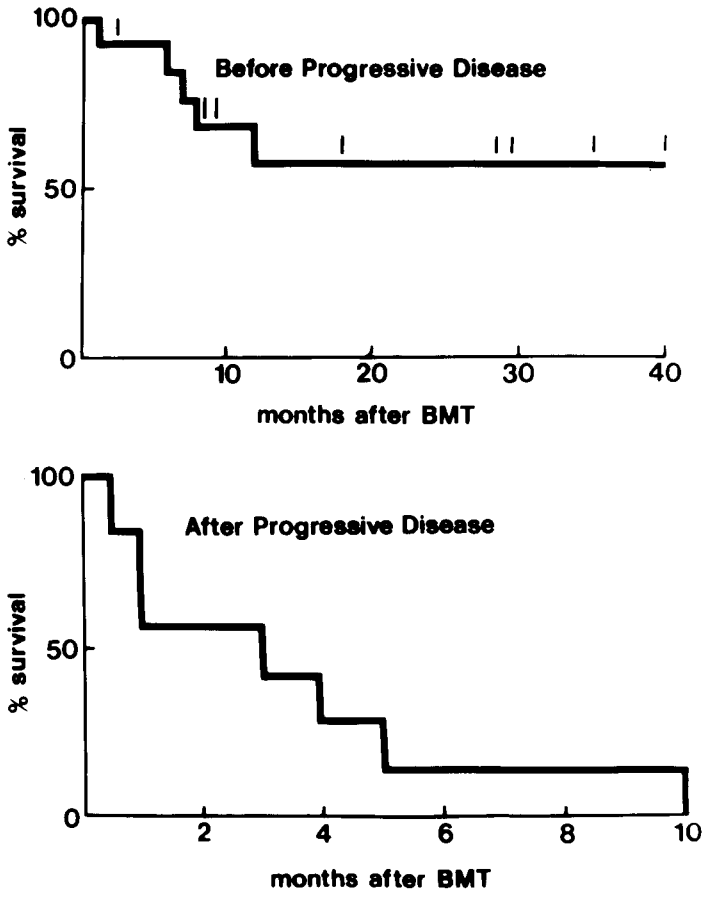

Figure 1. *Survival rates of patients who undergo BMT before or after they develop progressive disease. Initially, all patients received conventional chemotherapy (various regimens); the median times from diagnosis to BMT for groups whose transplantation occurred before and after progressive disease were 6.8 and 11 months, respectively. Tumor status at the time of BMT was as follows: among those who received BMT before progressive disease, five had a complete remission and eight had a partial remission; among those transplanted afterward, one had a complete remission, five had stable disease, and one had progressive disease. Eight patients survive who received BMT before developing progressive disease, and seven are tumor free (two recipients of allogeneic BMT and five of ABMT).*

40+ months. In contrast, all seven patients who received transplants after developing progressive disease had died 10 months after BMT; three deaths were toxicity related, one death occurred from brain hemorrhage, and three deaths followed relapses (Fig 1). These data suggest that treatment with VAMP-TBI followed by BMT improves outcome if carried out before progressive tumor growth occurs.

EX VIVO REMOVAL OF TUMOR CELLS FROM AUTOLOGOUS MARROW WITH SEDIMENTATION, FILTRATION, AND MAGNETIC IMMUNOBEADS

In our current study (CCG-321P3, which opened for patient entry October 1985), we are determining the toxicity and efficacy of aggressive induction chemotherapy followed by surgery and local irradiation (as indicated) and then by VAMP-TBI and BMT. A major objective is to perform BMT by approximately 20 weeks after diagnosis before progressive tumor growth occurs. For those requiring ABMT, we are determining if neuroblastoma cells can be removed ex vivo without impairing the ability of the marrow to restore hematopoiesis. Ex vivo purging will be discussed, since the study is too new to assess long-term efficacy.

Ex vivo purging of autologous marrow employs sedimentation, filtration, and monoclonal antibody-coated magnetic beads (Fig 2). To plan the purging procedure, the numbers of normal and tumor cells in posterior iliac crest marrow are determined 3 days before the large-scale harvest. Sufficient marrow then is obtained from the large-scale harvest to provide approximately 10^8 marrow cells per kg after ex vivo purging. Whole marrow is mixed 1:1 with 3% hetastarch and allowed to sediment, after which supernatant cells are filtered through nylon wool, washed, and mixed with magnetic beads that have been coated with a mixture of monoclonal antibodies via goat antimouse immunoglobulin. Attaching the monoclonal antibodies to the beads, which is a

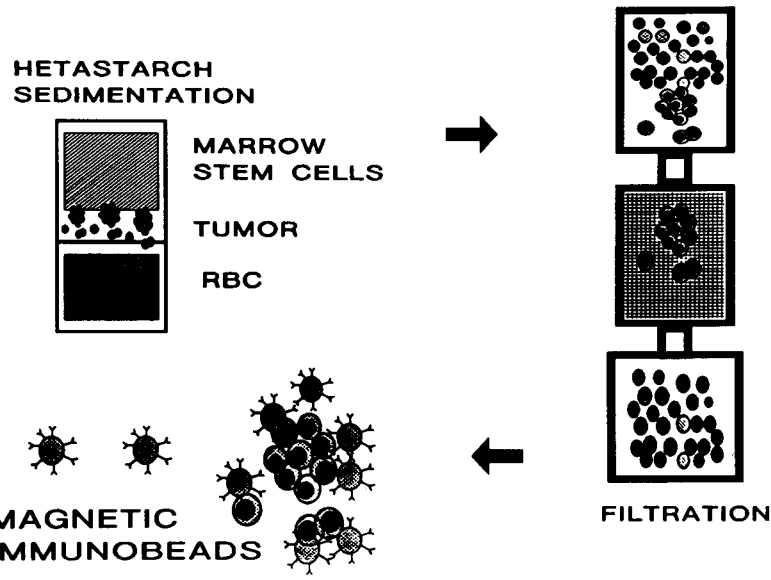

Figure 2. *Procedure for removing tumor cells from autologous marrow by sequential sedimentation, filtration, and monoclonal antibody-magnetic bead treatment.*

modification of our previous method of first binding them to cells and then to beads (11,12), increases the speed of purging and decreases nonspecific cell loss because cell-washing steps are not required. Following one-half hour of rotation with immunobeads, tumor cells attached to the beads are removed with samarium-cobalt magnets; the immunobead depletion step is repeated if the preharvest marrow contained more than one tumor cell per 10^3 normal cells. Total cell recovery is approximately 66% after the first two steps and 50% after each magnetic immunobead step; thus, 35-40% of the initial cells are recovered after one full sequence. An aliquot of marrow (10^8 cells/kg) that is treated only by sedimentation and filtration is cryopreserved as a backup in case antibody-treated marrow does not engraft.

This procedure has removed immunohistologically detectable tumor from 17 of 17 marrows that have been treated. Approximately 50% had no detectable tumor cells after the filtration step; generally, these were the ones in which the original tumor burden was 0.5-2 tumor cells per 10^5 normal cells. However, magnetic immunobeads were necessary for purging more heavily contaminated marrows. One cycle of treatment with immunobeads was adequate when tumor-cell concentration was less than one per 10^4 normal cells, but two cycles were necessary when it was more than one per 10^3 normal cells. One marrow containing 2% and another with 1% tumor cells were successfully purged with this procedure. Data from the treatment of four representative marrows are shown in Figure 3.

Engraftment of purged marrows is evaluable for 11 patients. Marrows from the first three patients enrolled in this study were purged with a new combination of monoclonal antibodies that was highly effective for removing tumor cells (antibodies 459, BA-1, and 126-4); however, two of these marrows engrafted slowly (82 and 74 days) and one did not engraft. Either antibody 126-4 (anti-G_{D2}) or the particular combination of antibodies was the most likely cause of this complication, because poor engraftment was not a problem previously when antibodies 390, 459, BA-1, HSAN 1.2, and RB21-7 were used. Subsequently, marrows were treated with beads coated with antibodies 390, 459, BA-1, and HSAN 1.2; using a bead to total cell ratio of 1:1 and two cycles of treatment, these immunobeads can clear at least as much as 2% tumor from marrow. Engraftment has occurred in all eight patients given marrow treated with these immunobeads.

SUMMARY

Our initial study suggests that intensive chemoradiotherapy (e.g., VAMP-TBI), if administered relatively soon after diagnosis and before progressive disease develops, may improve the long-term survival rate of patients with advanced neuroblastoma. Our current study of newly diagnosed patients is testing aggressive induction chemotherapy, ex vivo purging of autologous marrow, and VAMP-TBI followed by BMT. Our objectives are to get 90% of newly

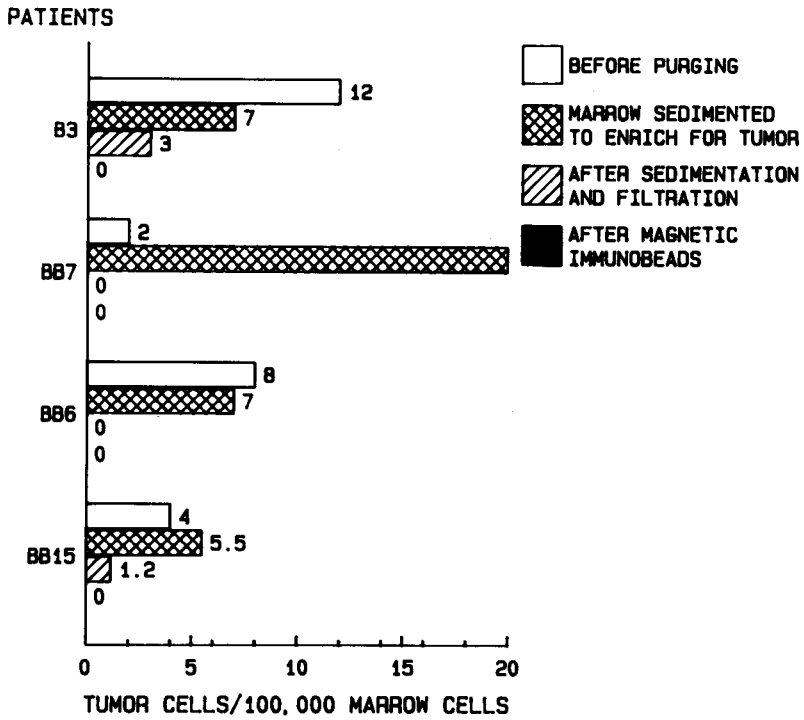

Figure 3. *Removal of neuroblastoma cells from autologous marrow by sequential sedimentation, filtration, and magnetic immunobead treatment. Marrow from four patients (B3, BB7, BB6, and BB15) was treated with one cycle of immunobeads coated with goat antimouse immunoglobulin and monoclonal antibodies 390, 459, HSAN 1.2, and BA-1. Tumor cells were identified in marrow by immunoperoxidase staining with anti-cell-surface monoclonal antibodies (mixture of antibodies 390, 459, HSAN 1.2, and 126-4) and with anti-neuron-specific enolase serum.*

diagnosed patients into the BMT phase by 20 weeks after diagnosis without progressive disease and to emerge from the BMT phase with a 90% survival rate. Because all patients are not expected to remain tumor-free following BMT, efforts are being made to identify prognostic factors for this very aggressive therapeutic approach; additional or different therapy will need to be developed for those who are likely to develop progressive disease after BMT. Collectively, these new strategies may further increase the percentage of patients who survive tumor free.

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Repeated High-Dose Chemotherapy Followed by Autologous Bone Marrow Transplantation as Consolidation Therapy in Metastatic Neuroblastoma

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The use of high-dose chemotherapy in the treatment of metastatic neuroblastoma in children older than 1 year of age at diagnosis has been studied extensively. This new approach has been developed because of the poor results obtained with conventional therapy (1). In a previous study (2) using high-dose melphalan as the conditioning regimen, our results appeared more promising when this therapy was used as consolidation therapy rather than when it was used in patients with measurable disease. Other investigators have found similar results (3) and the results of a randomized trial using high-dose melphalan as the consolidation therapy are under evaluation in the European Neuroblastoma Study Group. We therefore designed a new study with two courses of combined high-dose chemotherapy as the consolidation therapy. We report here the results obtained in 33 patients with metastatic neuroblastoma who were more than 1 year old at diagnosis.

PATIENTS AND METHODS

Since January 1, 1982 in our institution, according to Evans' classification (4), 62 children who were over 1 year of age with stage IV disease and who received diagnoses before January 6, 1985 completed conventional primary therapy before entering the high-dose consolidation chemotherapy. As a result of this therapy, a complete disappearance of bone marrow involvement was not obtained in 29 patients. These patients were excluded from further consolidation therapy. The 33 patients in whom normalization of bone marrow was obtained entered this study of consolidation therapy. Their median age at diagnosis was 41 months (range, 11-160 months) and the male:female ratio was 20:13. The primary tumor was abdominal in 27 cases, thoracic in two, thoracoabdominal in three, and of unknown origin in one case. Nineteen patients received 6-10 courses of a single combination of drugs as conventional primary chemotherapy (Table 1). In 14 patients, metastases did not disappear using the first-line conventional chemotherapy. Because some metastatic signs were still present, the patients received two (13 patients) or three (1 patient) combinations of drugs. Overall, patients received three to eight different drugs with a median of five drugs. Surgical excision was attempted in all patients with a detectable primary. Radiation therapy of the residual primary was performed in two patients prior to the consolidation therapy. As a result of these treatments, 23 patients entered

Table 1. Conventional Chemotherapy[a]

Regimen	Day 1	Day 2	Day 3	Day 4	Day 5
CADO					
Cyclophosphamide ($300\ mg/m^2$)	x	x	x	x	x
Adriamycin ($60\ mg/m^2$)					x
Oncovin (vincristine) ($1.5\ mg/m^2$)	x				x
OPEC					
Oncovin (vincristine) ($1.5\ mg/m^2$)	x				
Cyclophosphamide ($600\ mg/m^2$)					
Cisplatin ($60\ mg/m^2$)		x			
VM-26 (teniposide) ($150\ mg/m^2$)				x	

[a] *Six to eight courses were administered every 3 weeks.*

complete remission (CR) with no detectable disease, and 10 patients presented complete disappearance of all metastatic signs and a small macroscopic residual primary tumor (< 5% of initial size). Their response to primary therapy was termed good partial remission (GPR) (5).

High-Dose Chemotherapy

The chemotherapy regimen consisted of a combination of BCNU (carmustine), VM-26 (teniposide), and melphalan. On day 7, carmustine (300 mg/m^2) was infused intravenously over 60 minutes. On days 7, 6, 5, and 4, teniposide (250 mg/m^2/day) was infused intravenously over 60 minutes. On day 3 melphalan (180 mg/m^2) was administered as an intravenous bolus through a central venous line. During the five days of chemotherapy, hydration (31 cc/m^2/day) was carried out with 5% dextrose. Thawed bone marrow was infused on day 0.

Patients were to receive two courses of this regimen 3-4 months apart. Each course was followed by autologous bone marrow transplantation (ABMT). The second course was performed if the patient remained in remission after the first and if the toxicity of the first course had been acceptable.

No patient received post-ABMT chemotherapy before relapse occurred.

Bone Marrow Procedures

At the time of bone marrow harvest, all patients were in CR or GPR. The technique of bone marrow harvesting has been described elsewhere (6). Samples of bone marrow harvest were shown cytologically to be free of residual involvement. The cells were fractionated into three parts, each of 2×10^8 cells/kg. Two parts were purged by Asta Z (100 mmol/ml) as described by Beaujean *et al.* (7). The technique allowed two purged bone marrow grafts from a single collection. The unpurged sample was kept frozen for use in case of prolonged aplasia. The technique of marrow thawing and infusion is described by Beaujean *et al.* (8).

Supportive Care

All patients were treated under simple reverse isolation barrier conditions. Prophylactic oral nonabsorbable antibiotics were not used. Febrile episodes were promptly treated intravenously with broad-spectrum antibiotics.

Evaluation of Toxicity

The intensity of gut toxicity was established as described elsewhere (9,10). The criteria for the evaluation of infectious complications were the duration of fever over 38° C and the occurrence of sepsis and/or documented infections.

RESULTS

Thirty-three patients received the first course of high-dose chemotherapy followed by ABMT. Of these, 18 received the second course 3-4 months later. The 15 remaining patients received only one course of the high-dose chemotherapy. The reasons for exclusion from the second course were: early deaths, two patients; relapse occurring before the second course, seven patients; life-threatening toxicity during the first course excluding the patient from the second course, five patients; and one other patient is presently scheduled for a second course.

Survival

At present, 16 patients are alive in continuous CR, with a median follow-up postdiagnosis of 28 months (range, 8-50 months). Ten patients received two courses of high-dose chemotherapy and six received one course. The median follow-up since the last ABMT is 15 months for the twice-grafted patients (range, 2-38 months) and 19 months for the six remaining patients (range, 2-30 months). Fourteen patients relapsed, seven before the second course of high-dose chemotherapy and seven after. One of these relapses was exclusively local, at the primary site. The remainder were distant, involving the bone and/or bone marrow. Four complication-related deaths occurred, two following the first course of high-dose chemotherapy and two after the second course. The probability of the overall disease-free survival rate at 2 years postdiagnosis is 50.5%. Taking into account the entire population of 62 patients with stage IV neuroblastoma referred to our institution during the same period, the overall disease-free survival rate at 2 years postdiagnosis was 27.4%; the survival rate of patients who did not respond to primary therapy and were therefore not grafted was very poor (7.7%). These results are summarized in Figure 1.

Various factors with a possible influence on the results were studied. The factors were gender, age, extent of disease at diagnosis, status before high-dose chemotherapy, and response to primary therapy aimed at CR. No prognostic factors could be found related to gender, age, extent of disease, or status of the disease prior to high-dose chemotherapy in these patients with stage IV disease who were more than 1 year old at diagnosis. The quality of the response to primary chemotherapy might be of prognostic value. Of the 33 patients who received grafts, 24 received three to seven courses of CADO (cyclophosphamide, Adriamycin [doxorubicin], Oncovin [vincristine]) as the first-line conventional chemotherapy. With the use of this single combination and surgery at the primary site, 13 patients entered CR or GPR (group I). Eleven failed to respond to this first-line chemotherapy, and the use of a second-line conventional chemotherapy (combination of cisplatin + VP-16-213 [etoposide]) (11) was necessary for them to enter CR or GPR (group II). Patients from group I responded well to conventional chemotherapy, whereas

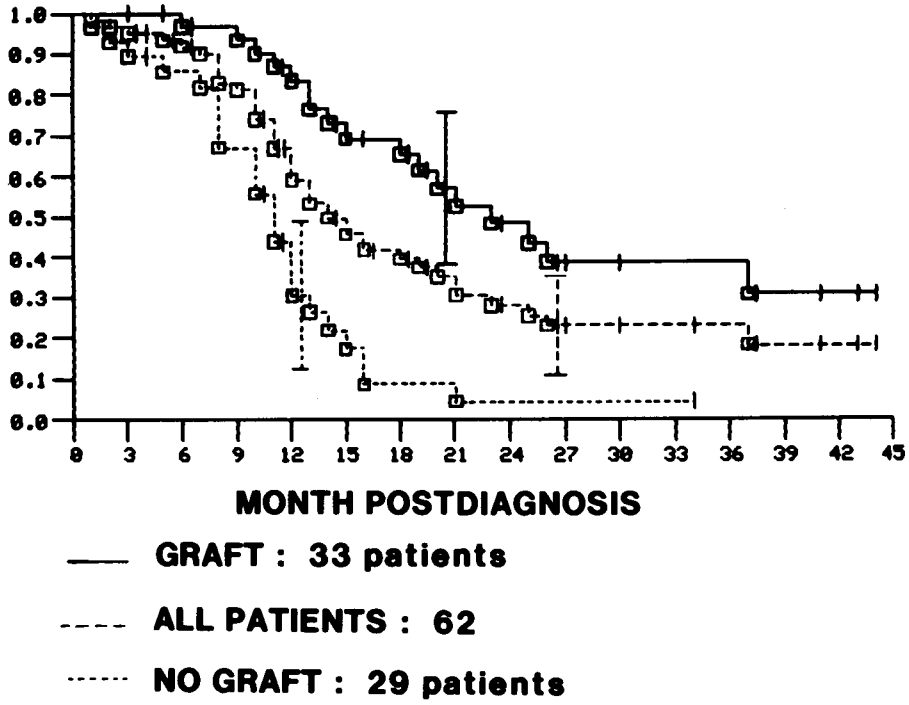

Figure 1. *Disease-free survival stage IV neuroblastomas.*

group II patients responded poorly. The time elapsing between graft and relapse would appear to be shorter in group II than in group I, but this difference is not statistically significant (Fig 2).

Toxicity

No difference was observed in the complications following the first or second course of high-dose chemotherapy. All the complications occurring in the 51 courses in 33 patients are therefore grouped.

All patients experienced profound myelosuppression; the median duration of granulocytopenia less than $0.5 \times 10^9/l$ was 26 days (range, 2-55 days). Leukocytopenia less than $1 \times 10^9/l$ had median durations of 25 (range, 9-52 days) and 37 (range, 5-140 days) days, respectively. No difference in the duration of these parameters was observed between the first and second courses of high-dose chemotherapy and ABMT.

Gut toxicity was often severe. Moderate or severe vomiting was observed in 15 of 51 courses (29%). Diarrhea was absent or of mild intensity in 26 courses and of moderate or severe intensity in 25 (49%). Mucositis occurred frequently and was of moderate or severe intensity in 34 courses (67%). Liver toxicity was rare: in four courses, a transient elevation of SGOT to three or four times the

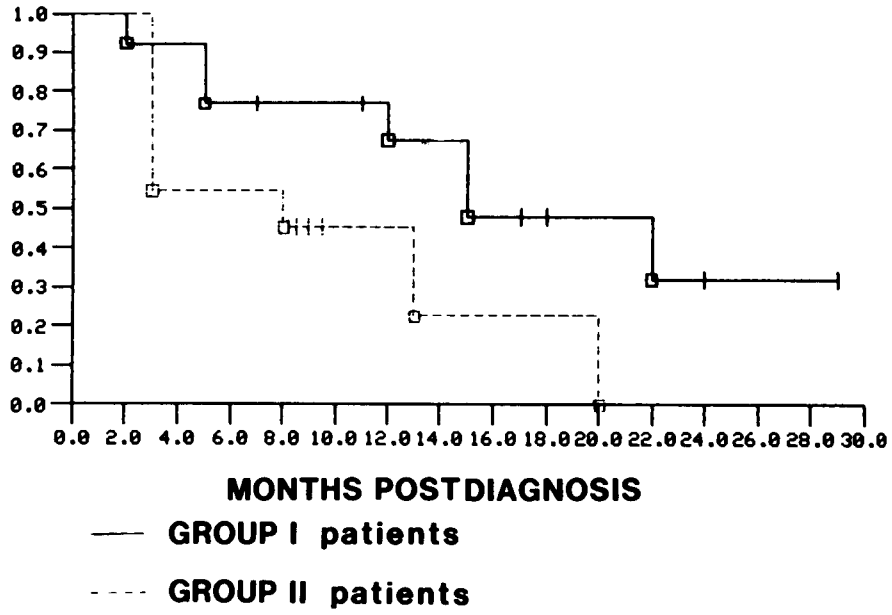

Figure 2. *Disease-free survival according to response to primary therapy.*

normal value was observed. Two episodes of veno-occlusive disease were observed and rapidly resolved under symptomatic therapy.

On day 6 post-ABMT, one patient had generalized seizures with a sudden elevation of glycemia from 4.6 mmol/l to 120 mmol/l during the same day. No direct explanation for this hyperglycemia was found. In spite of progressive improvement of this condition, he remained in a deep coma and died on day 11 post-ABMT. Post mortem histological examinations were not performed.

No cardiac, renal, or cerebral toxicity was found to be directly attributable to this high-dose chemotherapy.

Infectious Complications

All patients experienced fever for a median duration of 10 days (range, 1-49 days). Following the first ABMT, 12 out of 33 patients (36%) had bacteremia. Of the 18 patients who received a second ABMT, 4 (22%) had bacteremia. One patient died of aspergillosis; the other patients recovered under appropriate antibiotherapy.

Nine cases of interstitial pneumonitis were observed: three were related to cytomegalovirus (CMV) infection and two were lethal; two were related to *Pneumocystis carinii* and resolved under cotrimazole therapy; two occurred during a septicemia (one bacterial and one candidial) and resolved under antibiotherapy; one was of unknown origin and improved spontaneously; and one was related to aspergillosis and was lethal.

Viral Infections

Thirteen viral infections (four herpes simplex, six herpes zoster, two varicella, and one CMV) in 8 of 33 patients were observed after the first ABMT. After the second ABMT, seven viral infections, one herpes simplex, two herpes zoster, two CMV, one hepatitis B virus, and one human T-cell leukemia virus (HTLV) infection responsible for an acquired immunodeficiency syndrome (AIDS), which proved lethal 23 months after the second ABMT were observed in 5 of 18 patients.

Complication-related Deaths

Four complication-related deaths were observed; two occurred after the first ABMT and were related to a CMV infection in one case and to acute metabolic disorders in the other. Two occurred after the second ABMT and were related to diffuse aspergillosis and AIDS.

DISCUSSION

The use of high-dose chemo-radiotherapy as consolidation therapy in stage IV neuroblastoma is being investigated by several teams. The majority of the protocols published consist of chemotherapy plus total body irradiation (TBI) (12,13). The use of TBI appears logical according to the results published concerning studies both in vivo and in vitro (14,15). However, the role of radiotherapy in the treatment of stage IV neuroblastoma remains controversial (16,17). Taking into account these results and the delayed complications of TBI, especially in children (18), we chose to study a polychemotherapy regimen. It was based on a possible dose-effect relationship as already demonstrated in other diseases (10) and especially in a situation of minimal residual disease (19).

The combination chosen was based on phase II studies previously performed on neuroblastoma, and especially the wide experience acquired with high-dose melphalan (2,3). The use of two courses of high-dose chemotherapy followed by ABMT was based on the same concept of dose effect on minimal residual disease. Using this approach, encouraging results have already been published on other malignancies (20).

In the present study, the disease-free survival of the grafted patients would appear to be significantly higher than that of patients treated with conventional therapy (1,21). The results are similar to those published concerning patients in CR or GPR in smaller series (12,13).

Most of the relapses occurred a few months after the first consolidation course. The bone marrow was the principal site of relapse. In this study, all patients received bone marrow treated in vitro. It is impossible to know whether these bone marrow relapses were related to a failure of the conditioning regimen to eradicate the residual disease or to a failure of the purging technique to eliminate the residual cells in the graft, or both. The

higher relapse rate in poor responders to primary conventional chemotherapy might point to a failure of the high-dose chemotherapy to eradicate the residual disease in the patients. In another study, using another conditioning regimen, the relapse rates after autologous or allogeneic bone marrow transplantation are similar. For these relapsing cases, the failure of the conditioning regimen is obvious (22).

The myelotoxicity of this regimen was high and the duration of the aplastic phase was longer than that observed with nonpurged ABMT (7). Nevertheless, it is noteworthy that all patients recovered from their aplasia. Gut toxicity was frequently severe. The role played by melphalan in this adverse effect has already been demonstrated (2,3,23,24). The high rate of viral infections occurring during the first 6 months post-ABMT is probably related to lengthy immunosuppression. A long-term longitudinal study of the immunologic status of these patients is in progress.

The patients remained isolated for a median duration of 42 days (range, 27-88 days). Despite the supportive care techniques, 4 of 33 (12%) complication-related deaths were observed. Recent progress in the management of these patients, such as the use of HTLV- and CMV-negative blood products and the infusion of high-titer anti-CMV immunoglobulins, should decrease this incidence in the future.

CONCLUSION

With the present follow-up, the disease-free survival of the patients treated with this protocol is encouraging and would appear to be different from that observed with conventional therapy. Intensive, supportive care rendered this high-dose therapy tolerable. The use of this consolidation therapy deserves further investigation and a longer follow-up is necessary to determine whether some of these patients are really cured. New combinations of high-dose chemotherapy are now under investigation in order to improve these results, especially in those with poor responses to conventional chemotherapy (25).

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High-Dose Therapy and Unpurged Bone Marrow Rescue for Neuroblastoma With Poor Prognosis

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The prognosis for neuroblastoma has remained extremely poor despite modern multidisciplinary approaches (1-3). However, it has been shown that ablative therapy (AT) with or without total body irradiation (TBI) followed by allogeneic or autologous bone marrow rescue is capable of inducing long-term remissions in children with end-stage neuroblastoma (4-11). In October 1984 we began a study in which patients having neuroblastoma with poor prognosis were treated with intensive induction chemotherapy consolidated by AT and autologous bone marrow transplantation (ABMT) with unpurged marrow. The purpose of this chapter is to report the efficacy and toxicity of AT with ABMT.

MATERIALS AND METHODS

Eligibility requirements were that patients have resistant or relapsed neuroblastoma that had failed to respond to other treatment regimens (group A) or have previously untreated disseminated neuroblastoma (group B). From

October 1984 to September 1986 34 children, from 7 months to 7 years of age, were enrolled in the study. Twenty patients were in group A (7 with resistant disease and 13 in relapse) and 14 in group B.

Eligible patients received treatment including one cycle of peptichemio (450 mg/m^2) (12) and two cycles of the OC-HDP regimen: vincristine (1.5 mg/m^2), cyclophosphamide (600 mg/m^2), and cisplatin (200 mg/m^2) (13) (Fig 1). Patients with documented disappearance of metastatic lesions who continued to present with operable residual primary masses underwent surgery. Subsequently patients received one or two courses of Adriamycin (doxorubicin) (45 mg/m^2) and teniposide (375 mg/m^2). Response to induction therapy was assessed by change in size of tumors, urinary catecholamine excretion, and bone marrow infiltration. Complete response (CR) was defined as the disappearance of all measurable tumor. Partial response (PR) was defined as a more than 50% reduction of each measurable lesion without appearance of new lesions.

Bone marrow was harvested after two evaluations performed at 4-week intervals produced negative findings. Each evaluation included aspirates obtained from four different sites as well as trephine biopsy specimens from two sites. Aspirates were studied by traditional cytomorphology and immunofluorescence utilizing monoclonal antibody UJ13A (14). Marrow was considered to be free from neuroblastoma cells when results of cytomorphology and trephine biopsy were negative (< 2% of isolated immunofluorescent cells). After

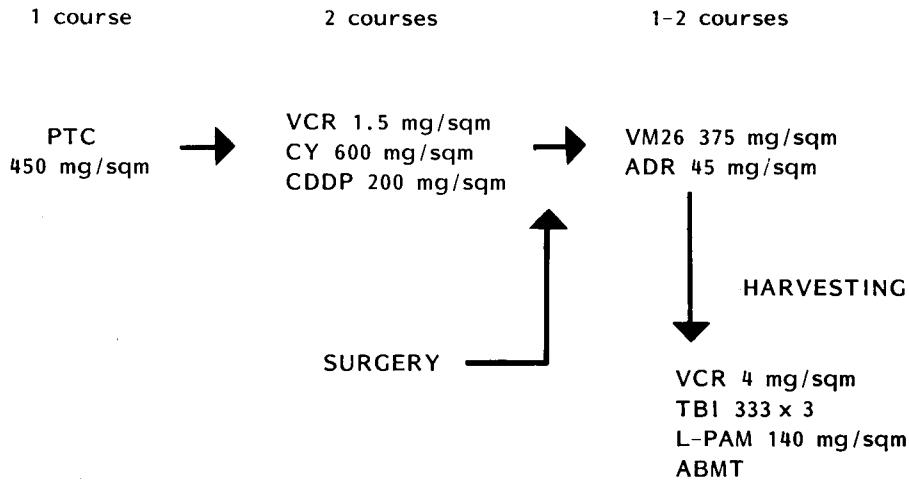

Figure 1. *Patients received peptichemio (PTC), vincristine (VCR), cyclophosphamide (CY), and cisplatin (CDDP) in the first three courses. Afterward they received one or two courses of teniposide (VM26) and Adriamycin (doxorubicin) (ADR). Following bone marrow harvest, patients would undergo ablative therapy including vincristine, total body irradiation (TBI), and melphalan (L-PAM) with autologous bone marrow transplantation (ABMT) rescue.*

collection, bone marrow was filtered and processed with the IBM COBE 2991 blood cell processor and cryopreserved as described by Herve *et al.* (15).

Ablative therapy consisted of vincristine, melphalan, and fractionated TBI (Fig 1). TBI was delivered with a cobalt 60 source (Theraton 780). The source-to-skin distance was 3 m; dose rate was 3.5 cGy/minute in midplane of the pelvis. Bone marrow was reinfused on day 7, 24 hours after melphalan administration. No maintenance chemotherapy was administered after ABMT.

Patients were kept in reverse isolation with one parent constantly present. They did not receive antibiotic or immunoglobulin prophylaxis, nor did they undergo gut decontamination. All children received cooked foods only. Total parenteral nutrition was administered when oral caloric intake was less than 50% of the daily requirement. Broad-spectrum antibiotics (amikacin and ceftazidime) were given when a temperature rose above 38°C. Filtered red blood cells were transfused in order to maintain a hemoglobin above 10 g/dl. Platelets were administered to maintain counts above 10,000/mm^3. All blood products were irradiated.

RESULTS

Ten of the 20 patients in group A achieved CR after induction therapy, and one child had a PR. Nine of these patients, including the one who achieved PR, underwent consolidation therapy with AT and ABMT. In two cases parents refused to have their child undergo the procedure. Of the remaining nine patients, six had rapid disease progression and were excluded from the study, and three had persistent primary lesions or increasing urinary catecholamine levels with disease-free bone marrow. These three were treated with AT and ABMT.

Twelve patients underwent transplantation in this group. Three of four patients who underwent ABMT with evidence of disease had brief remissions of 2-3 months; one child has had slow disease progression. Eight patients who received AT and ABMT in CR were evaluable for duration of response. Four patients continued to be in CR at 7 (two patients), 13, and 16 months post-ABMT. Four relapsed after 4, 7, 8, and 17 months. The last patient is in subsequent CR after surgical removal of an isolated lesion that appeared in relapse (Table 1).

Two of the fourteen patients in group B developed progressive disease and were excluded. Eleven of fourteen achieved CR, and one achieved PR. Parents of two patients in CR refused ABMT, but the remaining ten patients underwent consolidation therapy. Nine out of ten patients having ABMT were evaluable for duration of response. One child died in CR on day 3 because of multiple organ failure. Six of the nine patients undergoing ABMT in CR are alive with no evidence of disease at 4, 5 (two patients), 8, 11, and 15 months. Two patients relapsed: one at 4 months with disease in the abdominal lymph nodes and one at 9 months with disease in the bone marrow. The last patient, treated in PR, had

Table 1. Characteristics and Clinical Course of Patients Who Underwent Ablative Therapy and Autologous Bone Marrow Transplantation

Patient	Interval DX/AT (mo)	Status Before AT	Status After AT	Relapse		Status/ Survival (mo)
				Site	Time (mo)	
Group A						
1	63	CR	CR	Primary	17	ACR[a]/25+
2	11	↑VMA	CR	BM + primary	2	DWD/6
3	9	CR	CR	BM + bone	8	DWD/9
4	19	LR	PR	Primary	2	DWD/5
5	28	CR	CR	Primary	2	DWD/9
6	8	↑VMA	CR	Primary	3	DWD/4
7	17	CR	CR	—	—	ACR/16+
8	12	CR	CR	—	—	ACR/13+
9	31	PR	PD	Primary	—	AWD/12+
10	9	CR	CR	LN	4	DWD/6
11	15	CR	CR	—	—	ACR/7+
12	19	CR	CR	—	—	ACR/7+
Group B						
13	6	CR	CR	—	—	ACR/15+
14	5	CR	CR	BM	9	AWD/14+
15	6	CR	CR	LN	4	DWD/8
16	5	CR	CR	—	—	ACR/11+
17	6	PR	CR	BM	4	AWD/8+
18	4	CR	CR	—	—	ACR/8+
19	6	CR	NE	NE	—	TD in CR
20	6	CR	CR	—	—	ACR/5+
21	4	CR	CR	—	—	ACR/5+
22	4	CR	CR	—	—	ACR/4+

Abbreviations: AT, ablative therapy; CR, complete remission; ACR, alive in complete remission; VMA, vanillylmandelic acid; BM, bone marrow; DWD, dead with disease; LR, local relapse; PR, partial remission; PD, progressive disease; AWD, alive with disease; LN, lymph nodes; NE, not evaluable; TD, toxic death.

[a] *In subsequent CR after radical surgery.*

stable disease for 4 months but then had a relapse with disease in the bone marrow (Table 1).

Patients were reinfused with 1.2-20 × 10^7/kg viable bone marrow mononuclear cells (median, 6.7 × 10^7/kg). The nadir of peripheral blood cell counts (neutrophils < 0.1 × 10^9/l; platelets < 10 × 10^9/l) was observed between days 1 and 4 after AT and ABMT. Peripheral blood cell recovery is illustrated in Figure 2.

Linear regression analysis was done to determine the correlation between the number of reinfused mononuclear cells and parameters of hematopoietic recovery. The latter was expressed in terms of time necessary to reach stable

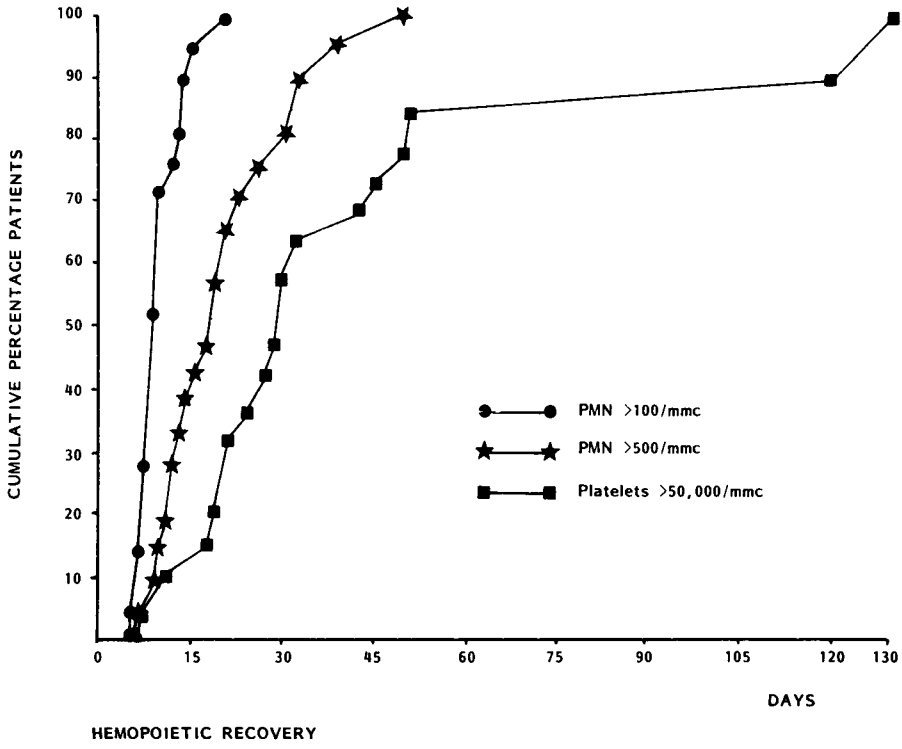

Figure 2. *Peripheral blood cell recovery (PMN, polymorphonuclear neutrophil leukocytes).*

blood cell counts (neutrophils > 0.1 × 10^9/l and > 0.5 × 10^9/l; platelets > 50 × 10^9/l). A significant correlation was found between the number of reinfused mononuclear cells and recovery of neutrophils > 0.1 × 10^9/l (P = .013) and > 0.5 × 10^9/l (P = .002). There was no correlation for platelet recovery (P = .1).

One patient died 3 days after ABMT because of multiple organ failure. Toxicity essentially involved the gastrointestinal tract, and severe mucositis was observed in all patients. Seventeen patients had moderate or severe diarrhea, but nausea and vomiting were absent or of mild intensity. Sixteen patients experienced paresthesias because of vincristine (seven mild cases, seven moderate, and two severe). All patients had fever during the aplastic phase for a median duration of 11 days (range, 6-38 days). Infections were microbiologically documented in 11 patients; 6 had septicemia.

DISCUSSION

High-dose peptichemio followed by the OC-HDP regimen has been shown to be an effective induction regimen in patients with neuroblastoma of poor

prognosis: 11 of 20 (55%) patients with resistant disease or in relapse and 12 of 14 (86%) patients who had newly diagnosed disseminated neuroblastoma achieved a major response. The consolidation regimen utilized in this study was first proposed by Philip *et al.* (16) for patients with disseminated neuroblastoma. Fractionated TBI was given with two chemotherapeutic agents known to be effective in neuroblastoma: the dose-effect relationship for melphalan in neuroblastoma and other solid tumors has been documented (5), and the efficacy of high-dose vincristine in continuous infusion has been demonstrated in solid tumors resistant to first-line treatment (17). The role of TBI in treating neuroblastoma is not yet clear; however, this pediatric tumor, which is often disseminated, is known to be radiosensitive (18).

In our group of patients the response obtained in four of five children with measurable disease shows the additional therapeutic benefit of AT and ABMT. However, the brief duration of remission confirms that AT and ABMT cannot be considered the solution for patients who do not completely respond to conventional therapy. This group is the best patient population for the use and study of new therapeutic strategies.

The brief follow-up does not allow us to draw any conclusions about the long-term efficacy of our program, but the fact that 11 of 20 patients are in CR with a median follow-up of 7 months from ABMT indicates that this therapeutic approach may improve prognosis. Ten out of 20 evaluable patients relapsed: six in extramedullary sites, three in bone marrow, and one in both types of sites. The role of minimal residual disease in reinfused marrow in inducing relapse is unclear. Some groups have included purging in their study of ABMT (19,20). In this study utilizing unpurged marrow, the bone marrow relapse rate is low.

Adequate supportive therapy and careful evaluation of patients during treatment has permitted us to perform AT and ABMT in normal reverse isolation without major complications. Despite significant infectious morbidity and severe gastrointestinal toxicity, the mortality rate is less than 5%.

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ENSG 1—Randomized Study of High-Dose Melphalan in Neuroblastoma

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The outcome for children with advanced neuroblastoma has improved with the introduction of intensive chemotherapy, but, despite high initial remission rates, prolonged survival remains limited to around 10% of patients with stage 4 disease who are older than 1 year at diagnosis. After almost any of the current regimens, about 70% of children achieve partial or complete responses following chemotherapy and surgery. In 1983 the European Neuroblastoma Study Group (ENSG) set out to answer the question of whether intensive consolidation chemotherapy would produce in such patients better results than no further treatment, in terms of either disease-free progression or survival. Phase 2 studies with high-dose melphalan followed by autologous bone marrow transplantation (ABMT) had shown activity in patients with relapsed or chemoresistant disease; therefore this was a logical agent to evaluate. Because of the morbidity, prolonged hospitalization, and emotional trauma of isolation nursing inevitable with high-dose chemotherapy and ABMT procedures, we believed it essential to evaluate this regimen with a prospective, randomized study rather than just a historical comparison. The latter would have been particularly difficult due to the recent introduction of more intensive conventional dose-induction regimens.

The ENSG comprises several European pediatric oncology centers, each of which treats between 5 and 20 new cases of neuroblastoma per annum. The outline of ENSG 1 is shown in Figure 1. In summary, all newly presenting patients with Evans' stage 3 or 4 disease aged over 6 months at diagnosis were to receive standard induction chemotherapy with OPEC (Fig 2). If, after surgery and 6 to 10 courses of chemotherapy, the patient had achieved either complete remission (CR) or "good partial remission" (GPR = > 50% reduction at primary site and catecholamines; normal marrow; improved bone scan), he or she was to be randomized to either no further treatment or high-dose melphalan (180 mg/m^2) with nonpurged ABMT (Fig 3). Informed consent was obtained from parents for the entry of patients into the study, and the protocol was approved by the respective ethical committees of each participating center.

RESULTS

Between January 1982 and March 1985, 140 patients were registered in the study. Ninety-five of these patients (68%) achieved CR or GPR after chemotherapy and surgery. Of these eligible patients only 65 were randomized, for either medical reasons, physician preference, parental reluctance, or death in remission. Thirty-two received high-dose melphalan and 33 no further treatment. The toxicity of high-dose melphalan was generally as predicted, including inevitable diarrhea and oral mucosal ulceration. There were two treatment-related deaths, both from staphylo-

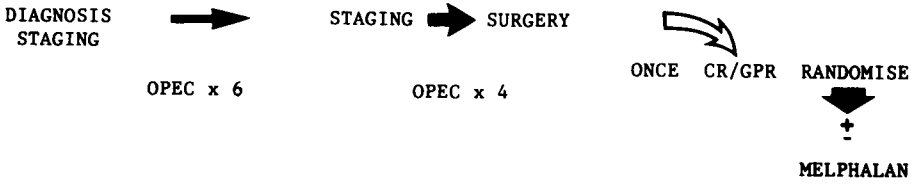

Figure 1. *Outline of the ENSG 1 study.*

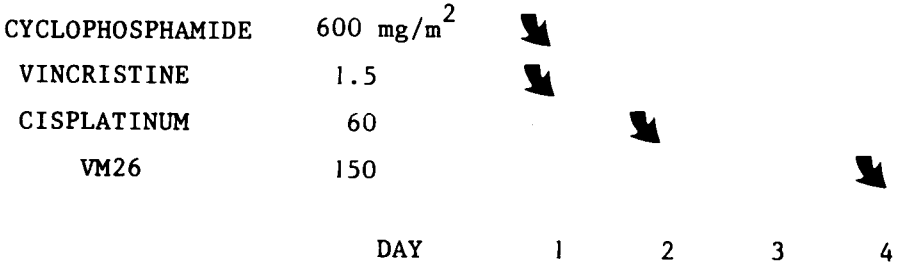

Figure 2. *OPEC induction chemotherapy.*

BONE MARROW HARVEST UNDER GENERAL ANAESTHESIA

MONONUCLEAR CELL YIELD RANGE 2.3-7.0 x 10^8/kg

MEDIAN 5.0

MARROW STORED AT $4^{o}c$

MELPHALAN 180 mg/m^2 IV BOLUS

HYPERHYDRATION FOR 24 HOURS

MARROW REINFUSED 24 HOURS AFTER MELPHALAN GIVEN OVER 4 HOURS

NO ATTEMPT MADE TO PURGE MARROW

Figure 3. *High-dose melphalan and autologous bone marrow transplantation procedure.*

coccal septicemia. In most patients marrow reconstitution was prompt; the median duration of a neutrophil count lower than 1000/mm³ was 14 days (range, 10-26 days) and of a platelet count less than 50,000/mm³ was 29 days (range, 19-70 days).

For the purposes of this analysis, only those 84 patients with stage 4 disease who were over 1 year old at diagnosis are considered. This is the patient group that is currently and universally regarded to have a very poor prognosis. The outlook of those between 6 months and 1 year or those with stage 3 disease is more varied and generally more hopeful.

Of the 24 patients over 1 year old with stage 4 disease who were randomized to melphalan, 2 relapsed before consolidation treatment. One died of toxicity, 9 have relapsed, and 11 remain disease progression free at a median follow-up of 32 months post-ABMT. Of the 26 who received no further treatment, 18 have relapsed and 9 remain progression free (Fig 4). The actuarial curve is shown in Figure 5. Overall median progression-free survival time for the melphalan group was 23 months postrandomization compared with 6 months for the no-treatment arm (log rank test $P < .02$).

DISCUSSION

This study demonstrated that high-dose melphalan prolonged significantly both the median duration of disease progression-free remission and the time to death in children with advanced neuroblastoma. This conclusion is the same whether the small numbers of patients with stage 3 disease or between 6 months and 1 year of age are included or excluded. An important consideration in the data interpretation is the number of patients who were

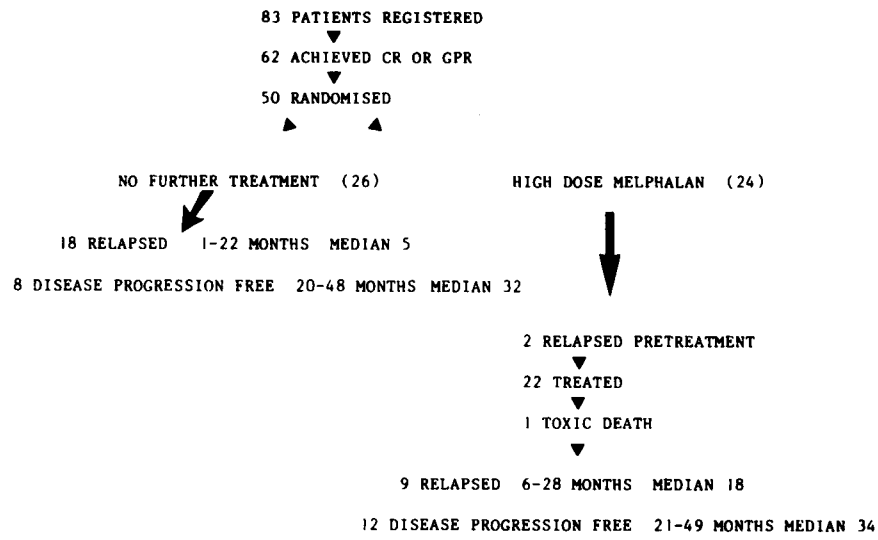

Figure 4. *Summary of outcome in the two randomized groups.*

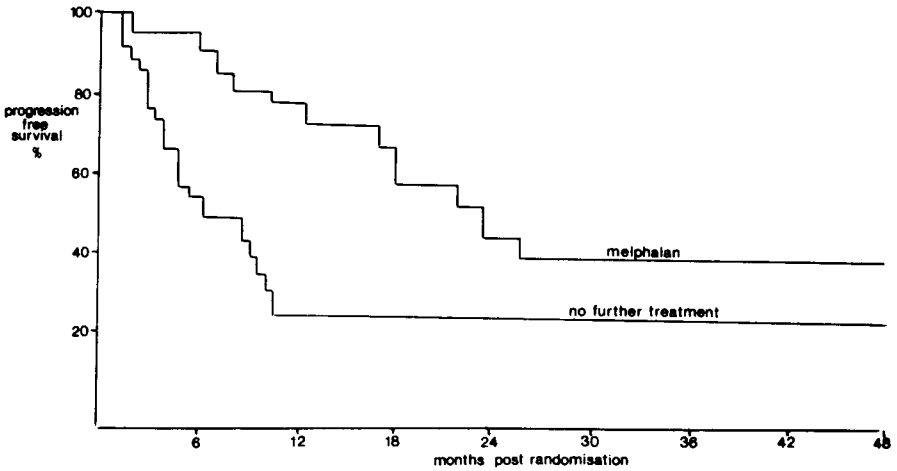

Figure 5. *Actuarial disease progression-free survival for the two randomized groups.*

eligible for randomization (i.e., achieved CR or GPR after induction chemotherapy and surgery), but were not entered into the study. This is an almost inevitable consequence in a study in which informed consent was sought prior to entry, particularly where the difference between the two treatment arms was so marked (i.e., megatherapy or no further treatment). It is of note, however, that the patients not entered were distributed equally between those

who chose (or the physician involved chose) melphalan or no treatment. Although these exclusions necessitated prolonging the trial to achieve a statistically significant end point, there is no reason to believe that they in any way imbalanced the patient distribution in the randomized group. Even if physicians had elected to remove all those believed to be at an especially high risk and treat them with ABMT or vice versa for those at a lower risk, all those remaining would have been equally distributed between the two treatments. Review of those patients not entered does not, however, indicate any such physician bias at any of the participating centers.

We conclude that high-dose melphalan is an active agent in neuroblastoma and had a significant advantageous effect when given as consolidation in chemosensitive patients. It is impossible to say whether continuing conventional-dose chemotherapy beyond 6 to 10 courses would have had a comparable effect, but there is little evidence to suggest that therapy prolonged beyond 12 months has any influence on disease progression. The current study has provided a rational basis for including high-dose melphalan in multiagent procedures for patients with neuroblastoma. Melphalan alone probably does not increase the overall cure rate, and the curves of the two study groups appear to be converging. The addition of other modalities such as total body irradiation or other high-dose chemotherapy agents (VM-26 [teniposide], cisplatin, Adriamycin, vincristine) are under investigation in several centers. Moreover, an important problem that remains is the quality of remission at the time of high-dose consolidation. Currently, less than one third of patients are in CR, and new treatment approaches are urgently required. The use of more aggressive initial therapy such as very high dose cisplatin or high-dose ifosfamide is under evaluation, and targeting techniques such as radiolabeled monoclonal antibodies may have a future role to play.

Bone Marrow Transplantation in an Unselected Group of 65 Patients With Stage IV Neuroblastoma

T. Philip, J. M. Zucker, J. L. Bernard, P. Biron, B. Kremens, E. Quintana, J. C. Gentet, P. Bordigoni, D. Frappaz, G. Souillet, I. Philip, F. Chauvin, and M. Favrot

Neuroblastoma is the commonest malignancy disease of childhood before the age of 5 years (1). Despite considerable progress in pediatric oncology, neuroblastoma has remained a fatal disease for 90% of patients with stage IV disease and for at least 70% of children who have the disease after age 1. Several reports using high-dose melphalan followed by autologous bone marrow transplantation (ABMT) have shown promising response rates in phase II studies (2-4), even in some chemoresistant patients, and, in some cases, long survival (5,6). The Philadelphia group (7,8) achieved a 36% survival rate at 12 months in a group of patients in relapse selected as responders to irradiation rescue protocols. These investigators were the first to use fractionated total body irradiation (TBI) associated with a melphalan-containing, high-dose chemotherapy regimen, and the preliminary results in the relapsed patients were better than those reported for patients receiving high-dose melphalan alone or in combination with other drugs (2,4,9). Moreover, in vitro studies using cell lines have produced further support for the use of TBI in this disease (10). Several groups have reported encouraging

preliminary results from using intensive therapy and ABMT as early consolidation for stage IV neuroblastoma in children over 1 year of age who are either in partial remission (PR) or complete remission (CR) (6-9). A randomized study of the use of high-dose melphalan alone with unpurged marrow as consolidation of first CR is now in progress under the responsibility of the European Neuroblastoma Study Group (ENSG) (11,12, and T. Philip *et al.* "A Single Institution's Experience of Autologous Bone Marrow Transplantation for Neuroblastoma," this volume).

Extensive bone marrow staging procedures have shown clearly that at least 25% of patients in so-called CR still have some malignant cells in their bone marrow (9,13). These clinical observations, linked with those of the various animal models in leukemia, which showed that no more than one or two reinjected cells might explain failure after ABMT (14,15), were the background for setting up ex vivo procedures to try to purge the remaining malignant cells prior to ABMT in neuroblastoma. An in vitro purging procedure with Asta Z, the stable active metabolite of cyclophosphamide (4-OH-cyclophosphamide or mafosfamide) is widely used despite little clear evidence of its efficacy in vitro (6). The 6-OH-dopamine purging procedure has been shown to be clinically feasible (9,16), but in vitro data have shown that this technique alone will not produce more than a 1-log reduction of tumor cell load (16). For immunological purging procedures, complement lysis techniques were unsuitable because of the absence of cytotoxicity with use of the majority of the currently available monoclonal antibodies (2). The development of an immunomagnetic technique suitable for ex vivo treatment provided an alternative method of utilizing monoclonal antibodies (17-21; see also Combaret *et al.* "Eliminating Burkitt's Cells From Excess Bone Marrow With an Immunomagnetic Purging Procedure" and Favrot *et al.* "Comparative Efficiency of an Immunomagnetic Purging Procedure and a Rabbit Complement Lysis for Eliminating Burkitt's Cells From Bone Marrow," both in this volume).

In this report we analyze our experience since 1983 of the use of intensive therapy as consolidation for an unselected group of patients older than 1 year who have stage IV neuroblastoma.

PATIENTS AND METHODS

Patients

All patients with stage IV neuroblastoma according to Evans *et al.* (22) and more than 1 year of age entered the pilot protocol. The first 37 patients received induction regimens including cisplatin, epidophyllotoxin, VM-26 (teniposide) alternating with cyclophosphamide, Adriamycin (doxorubicin), and vincristine (PE/CADO). Surgery was performed after a maximum tumor regression (i.e., four courses of chemotherapy in most cases). After surgery four additional courses of conventional therapy were given, except for four

patients (quick responders) who received fewer than eight courses, and three others (who had continuous incomplete responses after eight courses) who received more. Intensive therapy and fractionated TBI was thus performed within 12 months of diagnosis in all patients. Median time from diagnosis to consolidation for the whole group was 8 months.

The next 28 patients received induction regimens of high-dose VP-16-213 (etoposide) and cisplatin alone or in combination with ifosfamide and actinomycin. As soon as possible after surgery, bone marrow transplantation (BMT) was attempted with the same conditioning regimen as shown in Figure 1. For this group, median time from diagnosis to consolidation was 4 months.

Among the 65 patients eligible for consolidation during this period (January 1983 to November 1986) 6 are still on the protocol preceding surgery and only 59 are evaluable. Among these 59 patients, 23 were girls, and their median age was 3 years, 8 months. Bone marrow invasion was documented in all cases except one (a child who had bone lesions only), and 42 patients had bony involvement (two or more lesions). A retroperitoneal primary tumor was present in 53 cases including three initial thoracic and one jaw tumor, and two tumors of unknown origin.

At the time of intensive therapy consolidation, which always followed surgery, and 2 months later, patients were defined as in CR, very good partial remission (VGPR), or PR according to the following criteria:

—CR represents complete surgical excision, normal catecholamines, normal marrow, as determined by a minimum of four assays of aspirates and four biopsies (under general anesthesia), and a normal bone scan (or biopsy-proved negative residual lesion).

—VGPR represents a more than 90% removal of the lesion at surgery,

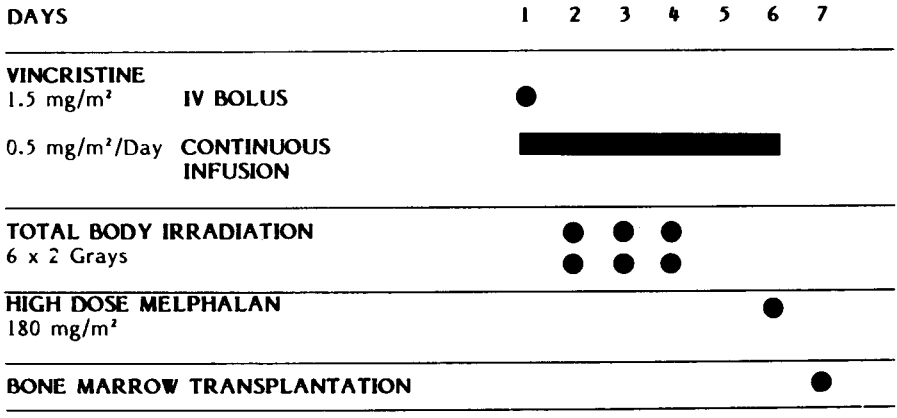

Figure 1. *Intensive therapy regimen used by our group since January 1982.*

normal catecholamines, normal marrow by the same criteria as CR, and improved bone scan but without biopsy examination.

—PR represents a 50% or greater improvement in at least two of the criteria (initial tumor, catecholamines, marrow, bones) with no progression at any sites.

Parents were fully informed of the protocol background and risks, and they gave formal consent. The protocol was reviewed and accepted by the Comite d'Ethique des Hospices Civils de Lyons et de l'Universite Claude Bernard. The cooperative group France Autogreffe de Moelle Osseuse also reviewed and accepted the protocol.

Intensive Therapy Regimen for Consolidation

The intensive therapy conditioning regimen used for all patients in this study is shown in Figure 1. The rationale for each drug was reported previously (9). For the fractionated TBI delivered over 3 days as six fractions of 2 Gy, lung protection was given at 10 Gy.

Bone Marrow Transplantation

Forty-nine of the 65 patients were given consolidation treatment with intensive therapy and fractionated TBI, followed by BMT. Forty-six patients received purged autologous marrow because only three had a histocompatible allogeneic donor that allowed allogeneic marrow transplantation resulting in 46 autologous and three histocompatible allogeneic transplantations.

Autologous Bone Marrow Transplantation

All bone marrows were harvested and frozen under conditions previously reported in detail (9,18). Bone marrow was harvested immediately or after surgery, following four to seven courses of chemotherapy (median, 5 months postdiagnosis). One patient's marrow was purged at Centre Leon Berard, Lyons, using the 6-OH-dopamine technique. Two patients' marrow was purged with 50 mg/ml of mafosfamide at Centre de Transfusion, Besancon, using the procedure previously described (23,24). In the 44 other cases, the original or a modified Kemshead immunomagnetic procedure was used (18,20). Monoclonal antibody against neuroblastoma (11,18) and the sheep anti-mouse immunoglobulin were provided by J. Kemshead (ICRF, London) and the beads kindly by J. Ugelstad (Oslo, Norway).

Allogeneic BMT

In three patients, bone marrow was harvested and purged with a cocktail of T monoclonal antibody as described previously (9).

Analysis of Results

Because 63% of the patients who received grafts were not in CR at the time of transplantation, despite a high response rate to the intensive regimen,

it was impossible to use disease-free survival curves. We therefore constructed curves using the Kaplan-Meier method only for probability of nonprogression. Nonprogression or progression of the disease was defined either clinically or with bone marrow, bone scan, or biological evaluation. Patients who died of complications were considered in the curves as progressive at the time of death.

RESULTS

For the 59 patients who completed induction therapy, the results can be summarized as follows:

Ten (17%) were not grafted because of bone marrow progression and lack of allogeneic donors (three), death at induction (four, two of whom were in CR) or during surgery (one), and parents' refusal (two). All these patients are dead or have relapsed.

Forty-nine patients underwent consolidation treatment with vincristine, melphalan, and fractionated TBI, with the following results:

Thirty-one patients were in PR at time of BMT. Four died of toxic reactions before day 90 post-ABMT, one of veno-occlusive disease (VOD), two of pneumonitis, and one of gastric bleeding. One patient had a late-occurring toxic reaction on day 413, with fatal bleeding related to persistent thrombocytopenia. Three patients had progressive disease and died 147, 190, or 192 days post-ABMT. Nine relapsed between 3 and 12 months post-BMT and died 172, 179, 231, 323, 358, 388, 389, 538, or 631 days post-BMT. Fourteen patients are alive and progression free 29-1118 days post-BMT (median, 461 days). However, the probability of nonprogression at 20 months postgraft is 34%, as shown in Figure 2.

Seven patients received grafts while in VGPR. Three died of toxicity 28, 36, or 62 days postgraft (two of VOD and one of cytomegalovirus). One relapsed 17 months postgraft and is alive with disease more than 845 days post-BMT; and three patients are alive and nonprogressing 383+, 474+, and 800+ days post-BMT.

Eleven patients received grafts during CR. One died of toxicity on day 42 of encephalitis and one on day 52 of VOD; one Algerian child was lost to follow-up on day 50 when he had VOD, and he is considered as dead in our data. One patient relapsed at 5 months postgraft and died 278+ days postgraft. One relapsed at 7 months and died in relapse on day 236, and one relapsed at 11 months and died with disease 452+ days postgraft. Four patients are still alive and progression-free 90+, 145+, 202+, and 950+ days post-BMT. One patient was lost to follow-up.

As shown in Figure 2, the probability of nonprogression at 20 months postgraft of the 18 patients grafted during VGPR or CR is 37%.

The projected progression-free survival of the whole group of 49 patients at 35 months postgraft is 33% (Fig 2). If the 10 patients excluded from

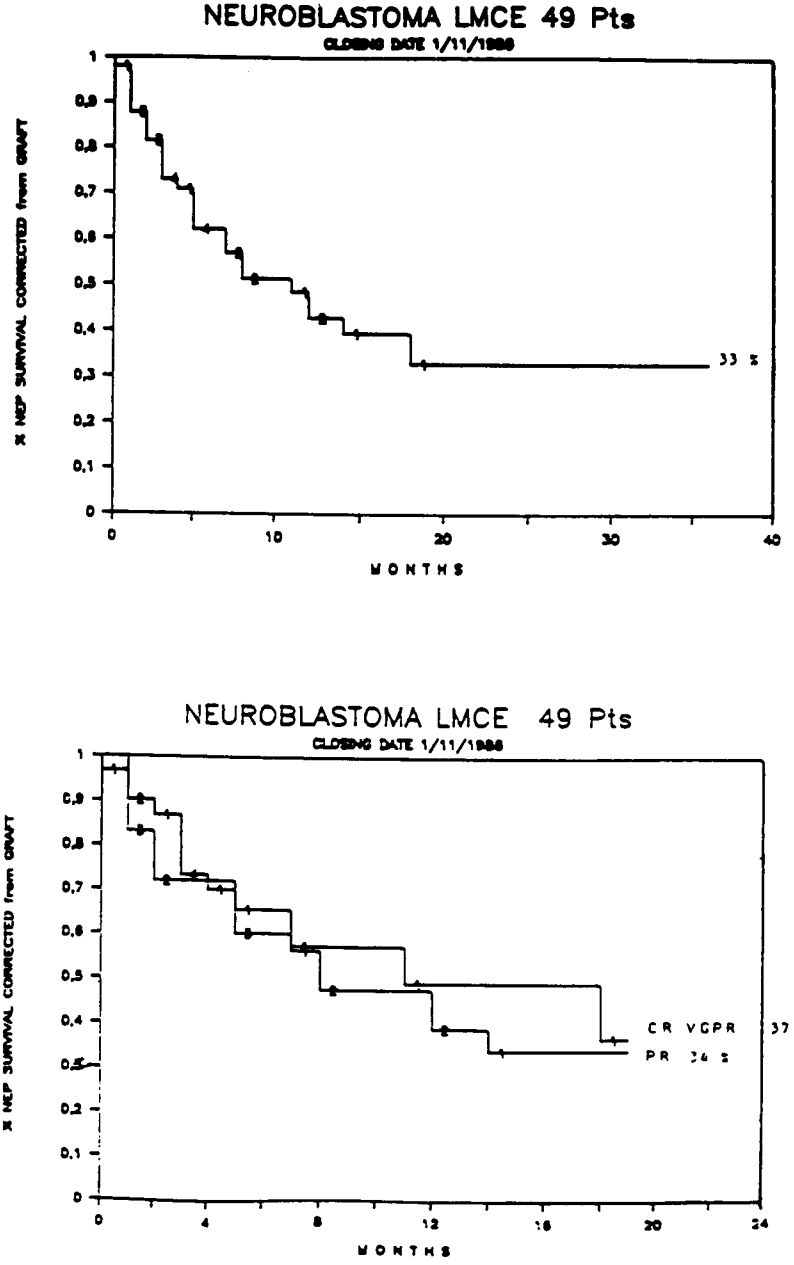

Figure 2. *Probability of nonprogression (Kaplan-Meier method) of the 49 BMT patients according to status: PR or CR/VGPR* (bottom), *whole group* (top). *Toxic death was taken into account as death.*

grafting and the 6 patients still in induction treatment are included as shown in Figure 3, the projected overall progression-free survival at 27 months postdiagnosis is 24%, compared with 1% for the same cooperative group's previous experience.

Among the 44 patients purged with the Kemshead technique, the median time to reach a WBC count of 1000 post-ABMT was 23 days. The median time to reach a count of 500 polynuclear cells was 24 days; 200 polynuclear cells, 19 days; and 50,000 platelets, 39 days. The capability of immunomagnetically purged autologous bone marrow graft to repopulate marrow after TBI was thus demonstrated. Five graft failures were, however, observed in this group and are reported in detail elsewhere (Favrot *et al.* [25], and the above-named chapter in this volume).

The toxicity of this particular association of continuous infusion of vincristine, high-dose melphalan, and TBI has been reported in detail (9). In this group of patients, the following features were of note:

In 50% of the patients, continuous infusion of vincristine produced some toxicity (e.g., leg pain lasting up to 3 days, maxillary pain, and mild ileus [25 patients]). Oral mannitol (100 cc/kg/day) as laxative was generally sufficient to control these problems in 20% of these patients. One patient had a small bowel obstruction on day 26, but the role of vincristine in this is not certain because intensive therapy was given only 1 month following this patient's abdominal surgery. Other toxic reations were related specifically to the

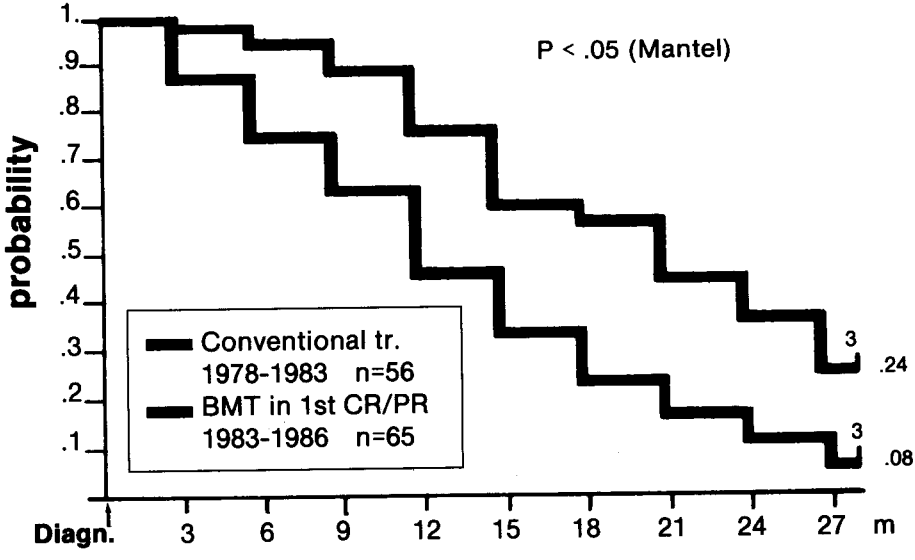

Figure 3. *Survival of stage IV patients 1 year old and older in two groups of patients treated during two consecutive periods in the same cooperative group (no selection and no exclusion during the period considered).*

protocol. Parotitis was observed in 10 patients despite fractionated TBI and lasted a maximum of 2 days. Diarrhea occurred in 25 patients but was never severe. Severe mucositis was observed in 18. Three herpes viral infections were seen, all in patients with severe mucositis. Nine cases of sepsis were recorded among the 49 patients who underwent BMT.

Ten therapy-related deaths were observed among the 49 patients: four of VOD on days 11, 28, 36, and 52 post-BMT, one of encephalitis on day 42 post-BMT, three of viral pneumonitis on days 29, 62, and 82 post-BMT, one of fatal gastric bleeding on day 39 post-BMT, and one of late toxicity of thrombocytopenia on day 413.

The toxic death rate was 20%, and 13 patients (26.5%) relapsed. Three patients' conditions progressed despite therapy, the first site of progression being the bone marrow or bones, except in the case of one patient in whom an unusual pulmonary relapse presented as pneumonitis. All relapses occurred between 4 and 17 months after diagnosis.

DISCUSSION

Although new, more intensive induction regimens have undoubtedly improved the CR rate in neuroblastoma (12), the long-term survival of stage IV disease among children over 1 year old has not improved significantly. Elective consolidation with intensive therapy and BMT is, therefore, now widely considered in stage IV patients. The important, soon to be completed ENSG 1 study is the first to consider the role of intensive therapy on the basis of a randomized study. The current problem is not only whether to use intensive therapy, but also at what time, with what type of combination chemotherapy, whether to use TBI, whether two procedures are better than one, and finally whether there is any advantage in attempting to purge the marrow (2,6,15). The 24% overall survival rate at 27 months postdiagnosis for the group of 65 unselected patients with stage IV neuroblastoma and more than 1 year old at diagnosis is encouraging compared to our previous experience (Fig 3). Despite numerous reports of ABMT in neuroblastoma (2-7), this is the first time a nonselected group of patients presenting during a specific period were treated in this way. We believe that only with such a group of patients can we clearly define the future role of intensive therapy and bone marrow transplantation in neuroblastoma. Despite our induction regimen, which compares favorably with those of all other published reports (12), strict criteria of defining response showed that only 36% of the patients were in CR or VGPR at time of BMT. Thus, almost two thirds of stage IV patients never reached more than a PR at the end of induction. We note that, despite strong commitment by every member of the group to use BMT as consolidation for all patients, 17% of the patients did not receive grafts. This included three patients for whom it was not reasonable to harvest a progressing marrow, and others either because of parents' refusal or the patient's early death.

As is shown in Figure 2, the probability of nonprogression at 20 months postgraft is not better for patients grafted in CR or VGPR (37% versus 34%). At this time, with a median follow-up of 18 months postdiagnosis, results for the PR group are better than those of our early reports (19). But this is an interim analysis with a rather large interval of confidence, and more follow-up is needed for this particular conclusion. The difference between the two groups (CR-VGPR and PR) has not been significant since the study began. In the current study, a clear improvement is shown at 2 years postgraft (24% progression-free survival compared with 1% for the historical control), but no conclusions can be reached at this time regarding long-term survival. Patients in CR or VGPR at the time of BMT would obviously be a group for whom long-term survival might be expected. The ENSG study will provide an answer to how effective high-dose melphalan alone is as late consolidation in patients in CR or "good PR," and it will also show survival data for patients achieving CR who were not grafted. The inclusion of TBI in many intensive therapy regimens is a result of the Philadelphia experience, at a time when alternative high-dose chemotherapy regimens had not been introduced into pediatric practice (7,8). Recent studies such as those of Hartmann *et al.* (26) suggest that it may be possible to achieve comparable results without TBI, using two courses of intensive chemotherapy for the CR-VGPR group. The result of this experience is clearly different, however, for the subgroup of 12 patients who are comparable to our PR group (no progression-free survivors at 12 months for the Hartmann *et al.* group and 40% progression-free survivors in this group of 31 PR patients (6, see also Hartmann *et al.* "Repeated High-Dose Chemotherapy Followed by Purged Autologous Bone Marrow Transplantation as Consolidation Therapy in Metastatic Neuroblastoma, in this volume). The long-term toxicity of two courses of high-dose alkylating agent is as yet unknown (26,27). The short- and long-term complications of TBI are clearer from experience in children with leukemia (2), and its omission, if it were possible, would be welcome. The young age of many of the patients with neuroblastoma adds to this concern. Nevertheless, the reduction in pulmonary and possibly CNS toxicity with fractionated TBI, our preliminary results from the group of PR patients, and the experimental background that favors this therapeutic modality inclines us toward continuing this procedure. In the present study, the treatment-related morbidity was not markedly different from our early experience with intensive therapy for lymphoma, in which we used a protocol without TBI but in which the incidence of VOD was of concern (23).

The issue of ex vivo purging is also important. In the present study, the use of all techniques described was highly experimental in nature—these are essentially phase I studies. We demonstrated that they do not harm, and graft take is not inhibited. The occurrence of early marrow relapse, despite purging, and the even more striking occurrence in one patient of what seemed to be tumor embolization, might be seen as indicating that purging

was not fully effective (19,25). Conclusions should be drawn from clinical observations with caution, however, as marrow relapse may be the first relapse site in patients simply because intensive therapy has failed to eradicate the source. Moreover, after ABMT the deranged immune system may alter the pattern of metastatic disease in a misleading fashion. A major problem with neuroblastoma is the difficulty of accurately assessing purging efficiency in vitro. Although the use of monoclonal cocktails and, more recently, Hoechst dye techniques (21,25,28) has improved this, it may be necessary to develop highly sensitive culture systems that will detect very low levels of marrow infiltration, as in the case of Burkitt's lymphoma (15). Only then can methods be refined and their results assessed in individual patients.

It seems probable that the future lies in a combination of purging techniques rather than any single system. For example, new cytolytic monoclonal antibody may have a role to play in other ex vivo chemotherapy methods (2,15). Only if the effectiveness of purging systems can be demonstrated convincingly in the marrow of individual patients, in vitro, will a randomized study of purged versus nonpurged marrow be of value (15). The fact that patients with neuroblastoma relapse after allogeneic transplants is evidence that current intensive therapy regimens leave much to be desired, irrespective of the contribution of reinfusion of tumor cells. It seems appropriate, therefore, that while purging systems are improved, allogeneic grafts are used when possible and that at the same time every effort is made to improve not only the intensive chemotherapy but also the induction regimens that precede it (28).

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APPENDIX

The investigators who included patients in this study were: Drs. Brunat-Mentigny, T. Philip, Pinkerton, Dutou, Biron, Chauvin, I. Philip, Favrot, Souillet, Paris, and Philippe, Lyons; Drs. Zucker, Quintana, Vilcoq, Lopez, and Urresola, Paris; Drs. Bernard, Gentet, and Raybaud, Marseilles; Drs. Olive and Bordigoni, Nancy; Drs. Plouvier and Herve, Besancon; Drs. Robert, Carton, and Roche, Toulouse; and Dr. Lutz, Strasbourg.

A Single Institution's Experience of Autologous Bone Marrow Transplantation for Neuroblastoma

T. Philip, D. Frappaz, P. Biron, E. Bouffet, L. Dutou, I. Philip, R. Pinkerton, B. Kremens, M. C. Favrot, and M. Brunat-Mentigny

The experiences of single institutions concerning treatment of tumor are frequently reported because of the homogeneity of clinical decisions, care, and final evaluation. For a rare disease such as neuroblastoma, however, referral of patients previously treated elsewhere by various modalities may represent a bias of selection, and it may lead to false conclusions if these patients are mixed with those referred at diagnosis at the institution. We participate in the ongoing LMCE study of unselected stage IV patients older than 1 year of age (1,2) but are also a referring center for bone marrow transplantation in this disease. Our report here concerns 29 patients with poor-prognosis neuroblastoma (patients referred at relapse were excluded) who received grafts in Lyons since 1983 and the 4 patients with stage IV neuroblastoma referred to us at diagnosis who received no grafts. These 33 patients were divided as follows:

Group 1—Nineteen patients included in the LMCE study who received autologous bone marrow transplantation (ABMT).

Group 2—Four patients included in the LMCE study who did not receive ABMT (two suffered drug-related early deaths, one died during surgery, and one patient's parents refused ABMT).

Group 3—Ten patients referred from outside, including 3 patients who were either in complete remission (CR, i.e., complete surgical excision, normal catecholamines, normal marrow as tested by at least four aspirates and four biopsies under general anesthesia, and a normal bone scan or biopsied negative residual lesion) or very good partial remission (VGPR, i.e., more than 90% surgical removal, normal catecholamines, normal bone marrow by the same criteria as for CR, and improved bone scan but without evidence from biopsy). Seven patients were in partial remission (PR) (50% or greater improvement of at least two of the criteria concerning initial tumor, catecholamines, marrow, bones, with no progression at any site). The 10 patients had received various treatments and were therefore excluded from the LMCE study but were included in a protocol of single (5 patients) or double (5 patients) massive therapy with ABMT.

Our goal was to learn whether inclusion of these different groups in the final evaluation of the unselected patients would change our conclusions concerning neuroblastomas with poor prognosis.

PATIENTS AND METHODS

In *group 1*, all patients were classified as having stage IV neuroblastoma according to Evans' criteria. The group included 11 male and 8 female patients with a median age at diagnosis of 3.15 years (range, 1.03-7 years). The primary tumor in all patients was retroperitoneal but included one jaw, one thoracic, and one tumor of unknown origin. The patients initially had bone and bone marrow involvement, except one who had bone marrow invasion only. Induction treatment included OPEC/CADO (five patients), NB 84 (four patients), NB 85 (five patients), NB 86 (three patients) (1), ENSG (European Neuroblastoma Study Group) B (two patients). Induction duration was a median of 7.9 months (range, 4-11 months).

Surgical removal could be assessed in all but two patients (those with tumors of jaw and tumors of unknown origin); there were five cases of complete removal, nine of microscopic residue, two of macroscopic residue, and one tumor was unresectable.

All patients received similar massive therapy including vincristine given as a bolus injection on day 1 followed by a 24-hour infusion from days 1 to 5 (total 4 mg/m^2). High-dose melphalan (180 mg/m^2) was given on day 5. Fractionated total body irradiation was delivered over 3 days in 6 fractions of 2 Gy, with lung protection at 10 Gy.

All bone marrow grafts had been purged by an immunomagnetic

procedure described elsewhere (3; see also Combaret *et al.* "Eliminating Burkitt's Cells From Excess Bone Marrow With an Immunomagnetic Purging Procedure," this volume), except for two patients who received a T-cell depleted allograft and one whose marrow had been treated by 6-OH dopamine.

At the time of intensive consolidation, which was always performed after surgery, and 2 months thereafter, patients were defined as in CR, VGPR, or PR according to criteria outlined above.

All in *group 2* had stage IV disease. Age at diagnosis was 2.7 years (range, 1-4.6 years), and the gender ratio was 2:2. The patients' primary tumors were located in the retroperitoneum. The induction regimen consisted of PE/CADO (three patients) or ENSG III C (one patient). Before the graft could be done, all patients died between 4 and 7 months after treatment had begun.

Group 3 patients were at stage IV, except for two at stage III who had had grossly incomplete surgery, and one who had a localized esthesioneuroblastoma (second cancer). There were seven males and three females, the age at diagnosis being 5.41 years (range, 0.10-18 years). All patients' primary tumors were located in the retroperitoneal region except for the temporal esthesioneuroblastoma. Most stage IV patients had bone and bone marrow metastasis, but two patients had only bone, and one had only bone marrow invasion. Induction treatment was heterogeneous because most patients had been referred from elsewhere. Induction duration was 10.4 months (5-15 months). Surgical removal of tumor could be assessed in seven patients: three had complete removal, one had microscopic residue, one had macroscopic residue, and two tumors were unresectable.

Massive therapy consisted of one or two ABMT: five patients received only one graft. Two had the same conditioning as in group 1, one of them receiving a local boost of 20 Gy to the primary tumor. One patient underwent similar conditioning, omitting total body irradiation because he had had previous local irradiation for the esthesioneuroblastoma. One patient received VM-26 (teniposide), carboplatinum, and high-dose melphalan (see below for dosages) because of young age at diagnosis. One patient received carmustine (BCNU), teniposide, and cisplatin (see below) and a local 24-Gy boost because of young age at diagnosis. Five patients received one or the first of two successive high-dose chemotherapy protocols followed by ABMT. The first conditioning regimen included BCNU (300 mg/m^2 in i.v. bolus on day 10), teniposide (250 mg/m^2 in i.v. bolus on days 2-5), and cisplatin (40 mg/m^2 in i.v. bolus on days 2-6) with marrow infusion on day 7. The second graft followed the same schedule as described for group 1.

Three patients have completed the two courses, and two patients have received only one graft as of now.

All patients received immunomagnetically purged marrow once or twice. Only the patient with an esthesioneuroblastoma received unpurged marrow.

RESULTS

Among group 1 patients, two died of toxicity, including one of septic shock on day 34 in CR and one of graft failure and veno-occlusive disease on day 52. Nine patients relapsed: one did not respond to massive therapy and the others relapsed from 1 to 12 months post-ABMT. Eight of these patients have died of progressive disease 28, 192, 224, 226, 238, 382, 382, and 542 days post-ABMT; one is alive in second CR 471 days after the graft.

Eight patients are alive and progression-free 50, 66, 120, 333, 350, 408, 993, and 1607 days post-ABMT.

Among the 7 patients grafted during CR or VGPR, 3 are alive and progression-free, compared with 5 of 12 patients grafted during PR.

The estimated percentage of survival rate at 35 months is 28% (Fig 1).

All group 2 patients died before they could receive grafts. Thus 17% of the patients referred to our institution from elsewhere were not grafted, which is similar to the percentage of unselected patients recruited into the LMCE study (17%). If groups 1 and 2 are added, the estimated percentage of patients who survived at 35 months is 23%, which is not significantly different from the estimated 28% survival rate of group 1 patients.

Among group 3 patients, no death occurred as a result of toxicity. All patients are surviving and with nonprogressive disease. For those who received a single graft protocol, follow-up has been 60, 60, 90, 515, and 1125 days postgraft. For those who are included in the two successive massive

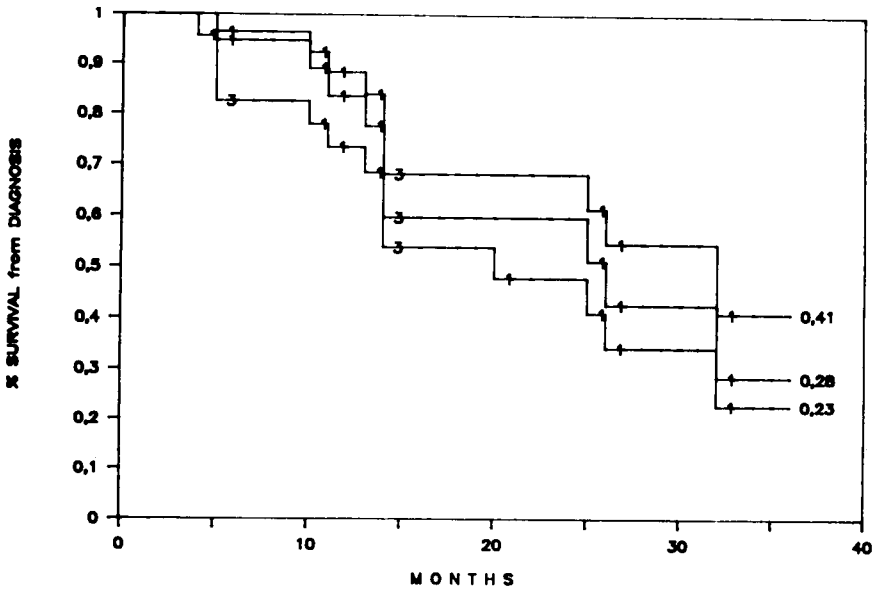

Figure 1. *Experience of Centre Leon Berard with treating 33 patients for neuroblastoma.*

therapies, follow-up after the first graft has been 60, 60, 120, 150, and 395 days. Status at first graft was CR-VGPR for four patients and PR for six patients. If groups 1 and 3 are combined, estimated survival at 35 months is 41% (Fig 1).

CONCLUSIONS

1. The overall results of ABMT in referring centers tend to be biased by selective referral of patients with good prognosis from outside.

2. To obtain unbiased results that are comparable to one another, studies must either include outcome in a complete regional recruitment of patients or indicate status at diagnosis, previous therapy, and outcome for each patient entered.

ACKNOWLEDGMENT

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Transplantation for Neuroblastoma Using Immunomagnetically Purged Autologous Bone Marrow

Adrian P. Gee, John Graham-Pole, Carlos Lee, Karen Bruce, Terry Pick, Walter Harvey, Diana Worthington-White, Marilyn Hintz, William Janssen, and Samuel Gross

Although using autologous bone marrow avoids limitations associated with allogeneic transplantation, namely, nonavailability of donors and reactions between the graft and host, its use is restricted in neuroblastoma by the high incidence of marrow infiltration by malignant neuroblasts. If this infiltration could be overcome, then all these patients could potentially benefit from autologous bone marrow infusions for hematologic support.

In vitro immunomagnetic purging (1,2) appears to be one of the safest and most effective methods of selectively eliminating malignant cells from bone marrow. In this procedure, target neuroblasts are identified using monoclonal antibodies. They are linked to magnetic microspheres, and then the bead-coated cells are removed by passing the bone marrow through a magnetic field. Unlike complement-mediated purging, this method does not depend on using antibodies of a particular isotype or subclass, is unaffected by anticomplementary factors associated with normal bone marrow cells (3), and can be used to eliminate cells expressing low levels of target antigen (4). Here we report preliminary results of a pilot study using immunomagnetically purged bone

marrow and autologous transplantation in the treatment of 30 patients with disseminated neuroblastoma, giving special attention to factors that influence marrow engraftment. These studies were carried out at five institutions—the University of Florida College of Medicine in Gainesville; Brooke Army Medical Center, Fort Sam Houston, TX; Cook Children's Medical Center, Fort Worth, TX; Montreal Children's Hospital; and the Cleveland Clinic, Cleveland, OH—on a Pediatric Oncology Group pilot protocol. Purging was performed at the University of Florida.

PATIENTS AND METHODS

Thirty patients 1-14 years old (median age, 4 years) with disseminated (stage D) neuroblastoma were enrolled in the study. All had been initially treated with combinations of cyclophosphamide, Adriamycin (doxorubicin), cisplatin, and teniposide or etoposide. Cyclophosphamide, vincristine, and etoposide were most often used for reinduction following relapse. Equal numbers underwent transplantation in initial remission (group 1) and in subsequent remission (group 2). All patients received high-dose melphalan (60 mg/m^2 i.v. daily for 3 days), total body irradiation (200 cGy twice daily for 3 days, with or without irradiation for local lesions of 150 cGy twice daily for 5 days), followed by autologous bone marrow infusions.

Bone marrow was harvested when restaging indicated that patients were in clinical remission (grossly normal aspirate and biopsy specimens of >75% cellularity). Bone marrow (10-15 ml/kg) was aspirated (with the patient under general anesthesia) from the posterior iliac crests bilaterally using syringes primed with 10% by volume of phenol red-free medium 199 containing preservative-free heparin at a concentration of 20 U/ml. The bone marrow was filtered through a 400-μm steel mesh screen followed by filtering through a 100-μm steel mesh screen and transferred to 600-ml blood bags. A minimum of 1×10^8 nucleated cells/kg body weight was collected. All bone marrow was immunomagnetically purged within 12 hours of collection and before cryopreservation in liquid nitrogen. Bone marrow from referring centers (for 16 patients) was sealed in blood transfer packs, wrapped loosely in insulating material, and placed on wet ice packs for transportation by air to the University of Florida for purging. Purged bone marrow was cryopreserved and returned in liquid nitrogen.

Six monoclonal antibodies (MAbs)—UJ13A, 223.8, 181.4, H11, Thy 1, and 127.11—were used. They have been demonstrated to bind selectively to neuroblastoma cells in bone marrow (1). All were purified on immobilized Protein A or by fast-performance liquid chromatography and titrated by indirect immunofluorescence on tissue culture neuroblastoma cell lines.

Paramagnetic microspheres, which are monodisperse polystyrene beads of 4.5-μm diameter, contain about 20% magnetite by weight. Before use they were dispersed in water, sonicated briefly, and sterilized by incubation in 70%

ethanol. After extensive washing in sterile phosphate-buffered saline (pH 7.7), they were resuspended in affinity-purified sheep antimouse immunoglobulin antibody at a concentration of 3.4 mg antibody/100 mg beads. The mixture was rotated slowly overnight at 4°C, and the beads washed four times immediately before use in phenol red-free medium 199 containing 10% plasma protein fraction (PPF).

Forty-two-milliliter aliquots of bone marrow were drawn into 60-ml syringes containing 8 ml Hespan (6% hetastarch) (American McGaw, Irvine, CA). Following mixing, the syringes were incubated vertically at ambient temperature for 30 minutes. The supernatant cells were then collected and pooled. The remaining red cell fraction was increased in volume to 42 ml with normal saline, an additional 8 ml Hespan added with mixing, and the sedimentation process repeated. The second supernatant was pooled with the first, and the nucleated cells were collected by centrifugation. The cells were washed three times, twice in saline with 10% PPF and once in 199 with 10% PPF, before the MAbs were added. A final packed cell volume of 20-25 ml was routinely collected by the addition of autologous erythrocytes from the sedimented fraction, which reduced nucleated cell losses during the procedure.

Sterile MAbs were added to the suspension of washed nucleated cells in a total volume of 199 and 10% PPF not exceeding 50 ml. The antibody concentration used was in excess of that required to saturate the binding sites on neuroblastoma cells at a 15% level of infiltration (i.e., a level in excess of that present in remission patients). The mixture was incubated with occasional mixing for 30 minutes on ice. It was then divided between two tubes and washed at least three times with 199 and 10% PPF. The final cell pellets were each resuspended in a volume of 40-45 ml, to which was added 5 ml 199 with 10% PPF, containing half the washed bead suspension. From 150 mg to 200 mg ($2.1\text{-}2.8 \times 10^9$) beads were added to the bone marrow. The bead and cell mixture was rotated slowly on ice for 30 minutes at 4°C and transferred through wide-bore tubing to a 300-ml blood bag, which was then connected to the separation apparatus.

Bone marrow from the cooled transfer pack was drawn into a single, disposable separation chamber at a rate of 1.5 ml/minute. This low-ceilinged, rectangular cross-section chamber is placed over a carrier containing an array of samarium cobalt magnets that produce a controllable magnetic field. From the chamber the bone marrow was drawn through a flow meter using a peristaltic pump and into a collection pack on ice. This apparatus represents a considerable modification of that originally described by Treleaven *et al.* (1).

Thirty-milliliter aliquots of bone marrow were mixed with an equal volume of chilled medium 199 containing 20% PPF and 20% dimethyl sulfoxide (Cryoserv, Research Industries, Salt Lake City, UT) in 120-ml cryopreservation bags (Stericon, Broadview, IL). The bags were rapidly transferred to aluminum freezing cannisters (Stericon), frozen in a programmable freezer to −90°C with eutectic point compensation, and transferred to liquid nitrogen. The cannisters

were stored in the liquid phase until immediately before use. For reinfusion the bone marrow was thawed rapidly in the cannisters in a 37°C bath at the bedside.

All patients were nursed in isolation, and strict precautions were followed to reduce contamination. Central venous catheters were placed to facilitate blood drawing, blood component and antibiotic administration, and total parenteral nutrition. All blood products were irradiated with 1,500 cGy to prevent graft-versus-host disease from transfused lymphocytes. Systemic antibiotics were given for fever that developed during the neutropenic phase after appropriate cultures were collected. Amphotericin was added if fever persisted for 7 days. Patients were discharged when they were clinically stable, afebrile, off systemic antibiotics, and taking adequate nutrition by mouth. This was most often between 4 and 6 weeks after the bone marrow transplantation (BMT). They were monitored closely in the outpatient clinic but received no further specific therapy.

RESULTS

We show in Table 1 the clinical results achieved in this pilot study and the current status of the patients. The data are divided according to whether the patients were treated in initial remission (group 1) or following disease progression (group 2). Sixteen patients (53.3%) are alive and disease free with a mean follow-up of 7.6 months (range, 2-16 months) post-BMT. Nine patients have relapsed a mean of 4.8 months (range, <1-14 months) post-BMT. Although it is too early to draw firm conclusions, there is a higher incidence of transplant-related fatalities and post-BMT relapses in the group 2 patients.

Transporting bone marrow between centers for purging proved entirely feasible, as indicated by the time to successful engraftment (2 consecutive days with a total WBC count over 1,000/μl and an absolute phagocyte count over 500/μl), which was 33 (±14 SD) days for University of Florida patients (n = 14) and 28 (±15 SD) days for patients receiving transported bone marrow.

Eight patients have been excluded from this analysis. Of these, three died

Table 1. Results of Immunomagnetic Purging and Autologous Bone Marrow Transplantation in 30 Patients With Stage D Neuroblastoma

Variable	First Remission (group 1, n = 15)		Postrelapse (group 2, n = 15)	
Days to engraftment (±SD)	33.4	(±18)	39.4	(±21)
Transplantation-related deaths (%)	1	(6.7%)	3	(20.0%)
Progression/relapses (%)	3	(20.0%)	6	(40.0%)
Months to relapse (range)	6.3	(4-10)	5.6	(1-14)
Disease-free patients (%)	10	(66.7%)	6	(40.0%)
Mean months of follow-up (range)	6.6	(4-15)	8.7	(2-16)

Note: patients in group 1 were treated in first remission. Those in group 2 had relapsed at least once but were in remission at the time of marrow harvesting and transplantation.

early from progressive disease prior to engraftment, one died at day 19 of infection, and two patients had marrow that failed to engraft and died of associated complications at days 53 and 88. Two patients are alive, but their marrow has not fully engrafted at the time of this report (days 60 and 69).

Having delayed engraftment or nonengraftment in patients receiving autologous bone marrow is of major concern. This could be owed to the effects of previous therapy on the harvested marrow, its subsequent manipulation during immunomagnetic purging, or both. Inasmuch as all patients had their marrow purged using the same general procedure, variation in the quality of the bone marrow harvested is a more likely explanation. We have therefore attempted to correlate engraftment data with previous therapy received by the patients, as shown in Table 2. The amount of prior chemotherapy has been expressed as the product of the number of individual drugs given and the number of doses administered.

Although each patient group is small, several trends are apparent from these data. There appears to be a direct correlation between the time to engraftment and the amount of previous chemotherapy. This trend is even clearer in the more heavily treated patients (those receiving >20 chemotherapy doses) who had, in addition, received prior abdominal or skeletal irradiation or both. However, radiotherapy, when considered as a separate factor, did not significantly affect the time to engraftment. More rapid engraftment in patients who had received less prior therapy may be owed in part to the increased numbers of nucleated cells that they received at transplantation. A similar trend is apparent when numbers of granulocyte-macrophage colony-forming units

Table 2. Relationship Between Rate of Engraftment and Prior Therapy in Patients Receiving Immunomagnetically Purged Autologous Bone Marrow

Prior Therapy	Nucleated Cells ($\times 10^8$/kg ± SD) Harvested	Reinfused	Days to Engraftment (range)
Chemotherapy doses[a]			
> 20 (n = 10)	3.27 ± 1.29	1.79 ± 0.77	42.2 ± 21.5 (12-88+)
≤ 20 (n = 15)	4.37 ± 2.51	1.95 ± 1.34	27.2 ± 13.2 (15-53+)
Radiotherapy[b]			
Yes (n = 10)	3.63 ± 1.64	1.54 ± 0.89	37.7 ± 23.8 (12-88+)
No (n = 16)	3.72 ± 2.03	2.09 ± 1.03	36.3 ± 18.1 (15-68+)
Combined chemotherapy and radiotherapy			
RT + >20 CT (n = 5)	3.82 ± 1.96	1.79 ± 0.81	46.4 ± 29.1 (12-88+)
RT + ≤ 20 CT	—	—	—
No RT + >20 CT (n = 16)	3.16 ± 1.03	1.68 ± 0.86	37.4 ± 18.8 (14-68+)
No RT + ≤20 CT (n = 5)	5.15 ± 3.09	2.60 ± 1.41	25.4 ± 12.0 (15-46)

Abbreviations: CT, chemotherapy; RT, radiation therapy.

[a]*Number of drugs multiplied by number of times administered.*

[b]*Abdominal and/or skeletal irradiation given.*

(CFUs-GM) are considered (data not shown). Of interest is the observation that marrow harvested from patients receiving previous radiotherapy often contained fewer CFUs-GM ($12.6 \pm 8.3 \times 10^4$/kg compared with $17.5 \pm 9.3 \times 10^4$/kg), although the total nucleated cell numbers were similar in harvests from both irradiated and nonirradiated patients (3.63×10^8/kg compared with 3.72×10^8/kg).

DISCUSSION

This pilot project shows the feasibility of hematologic support through autologous marrow infusions for children with disseminated neuroblastoma undergoing marrow-ablative therapy with curative intent. The purging technique that we have used compares favorably in both safety and efficacy with other in vitro physical separation and cytotoxic methods. No complement is needed, no toxin is introduced, and normal hematopoietic cells are unaffected. It is also adaptable for removing other malignant cells from marrow.

We encountered no difficulties with transporting marrow for in vitro treatment, and the viability of hematopoietic cells is such that marrow can be shipped throughout the North American continent. With careful coordination, this capability permits the development of multicenter clinical trials in which the purging procedure is carried out at a single institution.

Although we cannot prove that immunomagnetic purging eliminates the risk of reseeding the patient with malignant cells, extensive testing indicates that it consistently removes all neuroblastoma cells detectable by currently available assays. Of concern is the delayed engraftment and nonengraftment that we observed in four patients. We believe that these problems are owed primarily to the quality of the marrow harvested rather than the procedure used for purging. In particular, marrow collected from patients who suffered prior relapse and who were heavily pretreated, particularly with a combination of chemotherapy and skeletal and/or abdominal irradiation, may have limited hematopoietic potential, even when the nucleated cell count appears adequate. For this reason and because patients who have relapsed are probably more resistant to chemotherapy, we believe that it is essential to harvest bone marrow for purging when patients enter initial remission and their marrow is cytologically free of tumor. More extensive studies are needed to establish if ablative therapy is more effective when given at an early clinical stage before signs of disease progression.

We cannot draw firm conclusions yet about the efficacy of our protocol for treating patients with neuroblastoma at high risk of relapse. Others have achieved similarly promising results in single-arm studies of such therapy. The Pediatric Oncology Group is about to undertake a prospective multicenter study to compare autologous or allogeneic marrow transplantation with the best available conventional therapy in newly diagnosed children with disseminated neuroblastoma. This is the essential next step in establishing the value of such therapy in this refractory cancer.

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Digital Image Analysis System for Detection of Infrequent Cells in Bone Marrow

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One danger inherent in autologous bone marrow transplantation is the possibility of reinfusing malignant cells with the marrow. This has led to development of various methods for purging malignant cells from marrow harvested for transplantation (1-5). To assess the efficacy of such purging procedures requires methods that can detect very small numbers of malignant cells in bone marrow (6). Antibodies that bind to malignant cells but not to normal marrow cells have been used to detect small numbers of malignant cells in blood or marrow using flow cytometry or manual microscopy (Table 1). Some of these assays are capable of detecting as few as 1 malignant cell per 100,000 marrow cells. The sensitivity of such assays is limited by several factors, the most important being the number of cells analyzed for each specimen and the number of false-positive and false-negative signals generated by the assay.

We sought to develop an instrument that would improve sensitivity in detecting minimal disease in bone marrow. To avoid false-positive results the system should have a means of confirming positive signals as true tumor cells. Furthermore, to avoid false-negative results the system would need to avoid excluding clumps of cells (a problem with flow cytometry). Finally, the system would need to analyze a large enough number of cells to provide the desired sensitivity in a short enough period of time to be practical. We

Table 1. Immunoassays for Minimal Disease Detection in Human Bone Marrow

Target Cell	Tissue	Method	Sensitivity	Reference
Neuroblastoma	Marrow	ADCC[a]	1/40	7
B-cell lymphoma	Blood	Flow cytometry	1/1000	8
Leukemia	Blood	Manual microscopy	1/1000	9
Leukemia	Blood	Manual microscopy	1/10,000	10
Neuroblastoma	Blood or marrow	Flow cytometry	1/1000	11
Melanoma	Marrow	Flow cytometry	5/100	12
Leukemia	Blood	Flow cytometry	1/100,000	13
Neuroblastoma	Marrow	Manual microscopy	1/100,000	6,14,15
Neuroblastoma	Marrow	Manual microscopy	1/1000	16
Neuroblastoma	Marrow	Manual microscopy	1/10,000	17

[a]*ADCC, antibody-dependent cell-mediated cytotoxicity.*

describe here a system we have developed using a microcomputer-controlled scanning stage on a fluorescence microscope. Intensified video signals from the microscope are analyzed by the microcomputer to permit semiautomatic detection of fluorescent cells in bone marrow.

MATERIALS AND METHODS

The system is based on an IBM personal microcomputer (PC AT). For data storage the system uses a 10-megabyte Bernoulli cartridge disk drive (Iomega, Roy, UT) formatted with an interleaf factor of 2, which increases the speed of data storage. Software for the system was developed in Turbo PASCAL (Borland International, Scotts Valley, CA), using serial input-output procedures from the Turbo Asynch Tools Package (Blaise Computing, Berkeley, CA) to control the scanning-stage MDACE 1000 motorized stage controller (Ludl Electronics, Scarsdale, NY) via the serial interface. We have developed a complete set of software tools to control the scanning stage, scan various patterns on a slide, and record the locations of cells of interest (Lee, Ludd, Reynolds, unpublished data).

Detection capability is provided by a Dage MTI intensified silicon intensifier tube (ISIT 66) intensified video camera (Dage-MTI, Michigan City, IN) (18-20) on a Leitz Orthoplan Fluorescence Microscope (E. Leitz, Rockleigh, NJ). The ISIT camera provides an RS-330 video signal to a PC Vision real-time digitizer (Imaging Technology, Woburn, MA) located in the IBM PC AT. The PC vision board digitizes an image of 512 × 480 pixels with 256 gray levels at a rate of 30 frames per second (21). The aspect ratio of the 512 × 480 pixel image matches that of a standard video image.

Cell preparations consisted of Hoechst 33342 (H342) premarked leukemia cells (MOLT-3, from American Type Culture Collection, Rockville, MD) (22) seeded into human vertebral-body marrow as previously described (23). To provide optimal detection of the H342-marked cells, the microscope was equipped with a 100-watt mercury lamp and an ultraviolet excitation (350 nm)/blue emission (460 nm) "D" cube. Cytospins were prepared by using centrifugal cytology buckets (CCB) (International Equipment, Needham Heights, MA) to centrifuge marrow/leukemia mixtures onto poly-L-lysine-coated microscope slides at 200 × g for 10 minutes (24,25). Slides were prepared by covering one surface with greater than 100,000 molecular weight poly-L-lysine (Sigma Chemical, St. Louis, MO) (1 mg/ml in 0.1 M potassium-phosphate buffer, pH 7.0) and allowing the solution to dry (26). The CCBs were modified by placing a silicon rubber gasket between the slide and the cell chamber, defining a cell-preparation area of 13 mm × 15 mm. Marrow/leukemia mixtures were adjusted so that the correct number of cells for a well (usually 2 million) was suspended in 0.4 ml of medium (Iscove's Dulbecco's modified Eagle medium + 10% fetal bovine serum), which was then pipetted into the well. The pipette was rinsed and the rinse (0.1 ml)

added to the well. After centrifugation, medium was aspirated via the outlet port, and 70% methanol was added to the inlet port. After 10 minutes, the slide was carefully removed from the CCB and covered with a mounting medium (Aquamount, Lerner Lab., New Haven, CT) and a coverslip.

RESULTS

The configuration of the system is outlined in Figure 1. Under control of the microcomputer, the scanning stage moves the slide in increments of one field of vision (for a 25 X objective lens). In order to ensure that every area of the cytospin was digitized and analyzed by the microcomputer, the scan was programmed with overlaps in each field of vision. The computer digitizes each area to be analyzed into a 512 x 480 pixel image in the PC vision graphics memory. A threshold algorithm is applied to the image so that any pixels brighter than the defined threshold are set to 1 and any below the threshold are set to 0. This speeds analysis of the image for positive events. Next, the image is scanned for positive events; it was determined that scanning every eighth pixel increased scanning speed and provided enough overlap to avoid missing any cell. The scanning program is capable of digitizing and analyzing a field of vision for positive events in 0.61 seconds.

When a positive event is detected, the coordinates of the event on the slide are stored in a file on the cartridge disk drive. After scanning the entire cytopreparation, the computer then "plays back" each positive event under

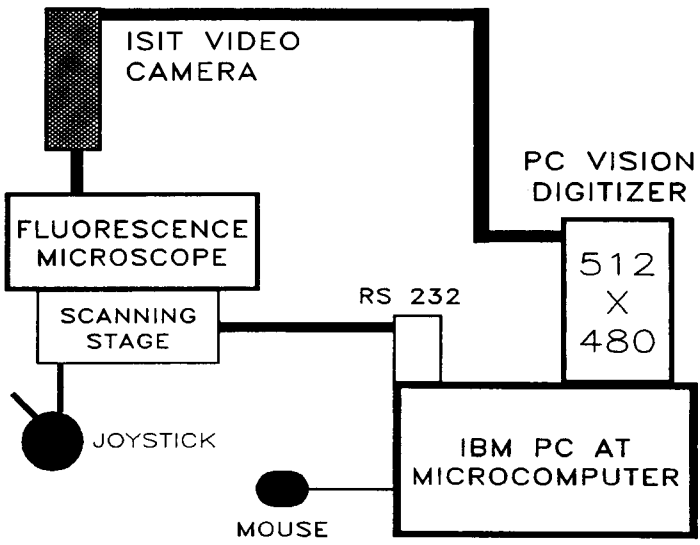

Figure 1. *Diagram of digital image analysis system for detecting minimal disease using fluorescent probes.*

control of the operator, who can observe the positive cell in the microscope to either confirm the event or exclude it as a false positive. When the operator enters his decision on the keyboard, the computer moves to the next positive event, allowing rapid user confirmation of the positive events. Computer-controlled scanning of a single cytopreparation requires 24 minutes, and semiautomatic playback for visual confirmation by the user averages 5 minutes.

In order to provide an efficient detection system, a method of placing a large number of cells on a slide for analysis was required. We found that if we used poly-L-lysine-coated slides, the CCBs could deposit efficiently up to 2 million cells per preparation area (the buckets provide three rectangular cytospins per slide, 13 mm × 15 mm each) (Fig 2). Careful observation of the supernatant from the CCBs aspirated after depositing cells failed to reveal any cells. As a further test, H342-marked MOLT-3 cells were seeded into marrow at a concentration of 10 cells per million, and then 0.5, 1, 2, and 3 million total cells per preparation were deposited using the CCBs. We found the deposition of the leukemia "tracer" cells to be linear until greater than 2 million cells were deposited. Thus, if one limits each preparation to 2 million cells or less, the number of cells loaded into the CCB is effectively the number of cells analyzed. Also, cytospins of 2 million cells or less provide a good distribution of cells on the slide for analysis, avoiding cell stacking.

To determine the reproducibility of the system in scanning slides, a cytopreparation of 1 million cells seeded with 100 fluorescent cells was scanned repeatedly. As shown in Table 2, the reproducibility of the system is

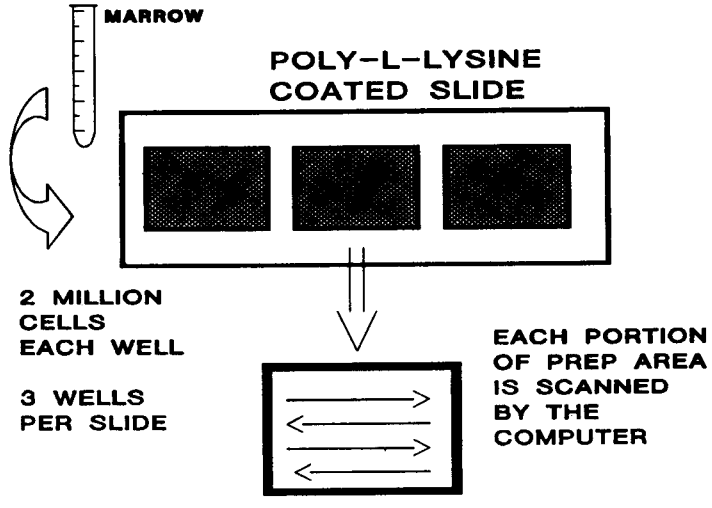

Figure 2. *Use of centrifugal cytology system to prepare marrow specimen for rare-event analysis.*

Table 2. Reproducibility of Detecting Cells With Digital Image Analysis System

Trial	Cells Detected
1	91
2	89
3	90
4	88

quite good; we attribute the decrease in detected cells in later scans to quenching of the H342 stain owing to repeated analysis.

To determine the sensitivity of the system, we seeded human bone marrow with H342-marked leukemia cells at concentrations of 50, 10, and 1 target cell per million marrow cells. Figure 3 shows the number of H342-stained leukemia cells detected in marrow at various seeding concentrations; each bar represents analysis of one cytopreparation (for one target cell per million, 2 million cells per preparation were scanned). Thus, detection of seeded cells with the system was reliable down to one target cell per million marrow cells.

DISCUSSION

Now that relatively specific antibodies for detecting malignant cells in bone marrow are becoming available, assay systems that maximize the ability of the antibodies to detect a given target cell are needed. Sensitive detection of infrequent leukemia or tumor cells in blood or marrow using flow cytometry has been reported (8,11-13). However, flow cytometry is less than ideal for detecting infrequent malignant cells, because target cells found in clumps (especially common in solid tumors) are excluded from analysis owing to the narrow aperture of the flow chamber. Slide-based detection assays (which do not exclude tumor clumps) of cells stained for tumor-associated antigens using immunofluorescence or immunoperoxidase have demonstrated sensitivities from 1/10,000 to 1/100,000, but require labor-intensive manual scanning of the slides (Table 1). To overcome this limitation of slide-based assays, we have developed a semiautomated system for scanning slide preparations for infrequent cells. To use the system efficiently, we employ a modified centrifugal cytology method that allows highly efficient depositing of 2 million cells into a preparation area ideally suited for "rare event" scanning.

The ability of the system to "replay" positive events for user confirmation can be exploited in a variety of ways. In our model-system studies, we used the replay to exclude false fluorescent signals based on the morphology of the H342-stained cells. In using the system with immunostained cell preparations, one could also use a second color of fluorescence or differing antigen locations (cell surface vs. nuclear or cytoplasmic). Simultaneous two-color

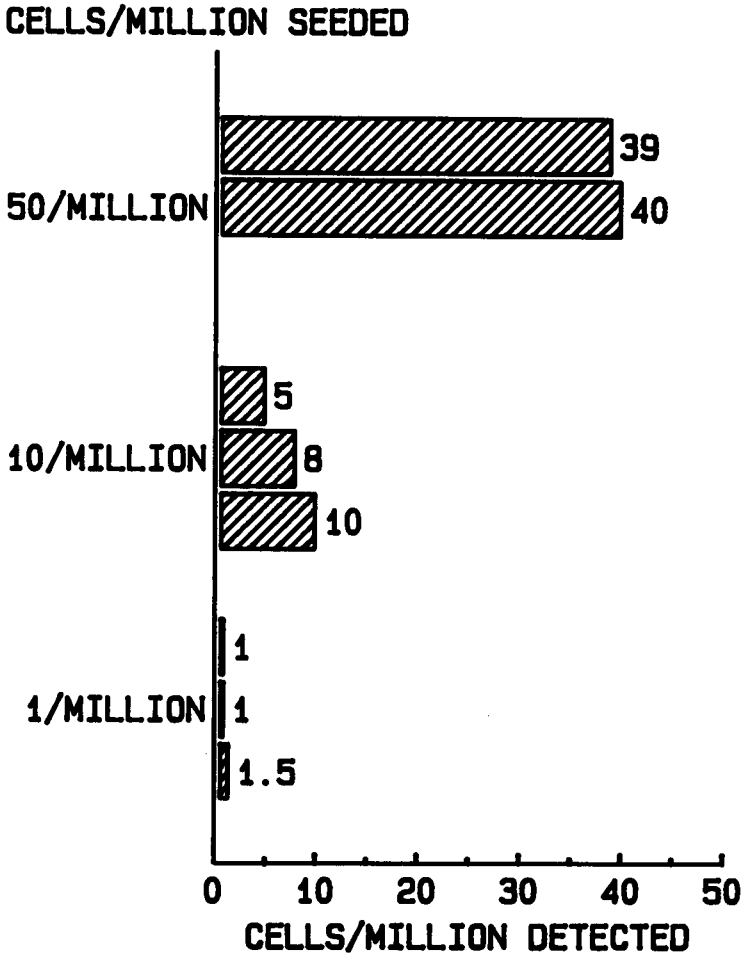

Figure 3. *Performance of system in detecting H342-marked leukemia cells seeded into marrow at various concentrations. Each bar represents the number of target cells detected per million marrow cells for one cytopreparation. For cells seeded at one per million, 2 million cells were analyzed.*

staining for terminal deoxynucleotidyl transferase and cell-surface antigens (27,28) is one example where such a two-color approach could be used to detect minimal disease.

The components of the system are all moderately priced, and with the cost of microcomputers and components decreasing almost constantly, the system is and will continue to be affordable. Using an IBM PC AT for the system allowed us to take advantage of the large amount of software available for the PC AT and similar microsoft disk operating system (MS-DOS) computer systems. Furthermore, software developed on the PC AT will be

useable with minor modifications both on the new generation of faster microcomputers that use the 80386 microprocessor (29) and with higher speed graphics hardware based on the new generation of graphics chips (30). Such increases in computer power should allow more and more automation to be programmed into the system, resulting in a decrease in the time required to analyze specimens. An increase in the speed of analysis will allow the system to be modified to allow quantitation of antigen density on rare cells of interest (31-34).

The system described here is already suitable for use in monitoring purging experiments, using H342-marked cells as a model system (23), and can be readily adapted to the analysis of immunostained clinical specimens. Employing the appropriate immunostaining reagents, the system should find use in other areas requiring rare-event analysis, including detection of minimal leukemic disease in blood or marrow (34) or quantitating infrequent normal cells such as hematopoietic progenitors in bone marrow.

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Eliminating Burkitt's Cells From Excess Bone Marrow With an Immunomagnetic Purging Procedure

V. Combaret, M. C. Favrot, B. Kremens, J. C. Laurent, I. Philip, and T. Philip

The immunomagnetic depletion (IMD) procedure used to deplete ex vivo an autograft of malignant cells obeys the same principle as some immunoassays or chromatography separation of proteins in liquid medium (1,2). However, the system is more complex when macroparticles such as malignant cells have to be eliminated from a viscous medium such as a cell suspension. The procedure's efficiency depends mainly on the chance for magnetic particles to meet the malignant cells and on the stability of the linkage of the beads to the malignant cells. The three steps of this linkage are reversible: 1) the adsorption of the antimouse immunoglobulin on the magnetic beads, 2) the reaction between the monoclonal antibody (MAb) and the antimouse immunoglobulin, and finally 3) the link between the antigen on the cell membrane and the MAb.

The IMD procedure was first described by J. Kemshead's group (3) for clinical applications such as an autograft in neuroblastoma. The method used for 32 patients in our institute has been proved to be nontoxic (4). However, though the method is efficient, large variations have been observed between individual manipulations, mainly owing to the weak and reversible linkage between the beads and the malignant cells. The major problem concerning the

assessment of the method in the neuroblastoma model is the lack of accurate detection (especially in culture assays) of residual neuroblasts. The Hoechst staining method described by Reynolds *et al.* (5) requires a computerized analysis to detect as few as 10^{-6} malignant cells (whereas the limit of detection with a visual microscope analysis is 10^{-4}). We have now refined the physical parameters of IMD in the Burkitt's lymphoma (BL) model. Indeed, a cell liquid culture assay enables us to detect as few as 10^{-6} BL cells, either from cell lines or from noncultured tumors, and the inhibition of BL cell growth is likely to reflect their full elimination (6; see also I. Philip *et al.*, "Using a Liquid Cell Culture Assay to Measure In Vitro Elimination of Burkitt's Cells From Bone Marrow," in this volume). In addition, results obtained in the same model with the complement lysis procedure could be used as a reference; therefore, the complementary effects of two immunologic procedures have been demonstrated (7,8; see also Favrot *et al.*, "Comparative Efficiency of an Immunomagnetic Purging Procedure and a Rabbit Complement Lysis for Eliminating Burkitt's Lymphoma Cells From Bone Marrow," in this volume).

MATERIALS AND METHODS

Normal bone marrow samples were obtained from healthy donors on preservative-free heparin, separated on a Ficoll gradient to obtain the mononuclear cell fraction, and irradiated with 50 Gy. Samples were contaminated with 1% BL cells from five different cell lines: Raji, Daudi, IARC BL_{99}, IARC BL_{93}, and IARC BL_2. The characteristics of these lines as well as the conditions of culture and viability have been previously described (9-11).

RFB_7 is a CD 20-like MAb and SB_4 (SANOFI) is a CD 19. These two IgM MAbs have been directly coupled to magnetic particles as described below. The MAb Y29/55 (mouse IgG2 subclass, 1mg antibody/ml) (Hoffmann-La Roche) was used at 1/200 final dilution, and B_1 and J_5 (both mouse IgG2 subclass and each 1-mg antibody/ml) (Coultronics) were each used at 1/50 final dilution (12).

Affinity-purified rabbit antimouse IgG (Biosys, B 12013/6091 or anti-mouse IgG + IgM) was coupled by physical adsorption on monodispersable 4.5-μm polystyrene microspheres (ME 450) containing magnetite (Dynabeads 14002, Dynal, Norway) (4-hour incubation at 4°C: 1 volume of phosphate-buffered saline [PBS]—IgG in 2 mg/ml; 3 volumes of particles—100 mg/ml in H_2O; 1 volume 0.5 M Na_2HPO_4 buffer) (pH 7.5). Beads were then washed with saline (0.9% NaCl) containing 1% PBS.

The IgM MAbs were directly coupled to beads by an 18-hour adsorption: 1 volume beads (30 mg/ml) in H_2O, 1 volume IgM MAb in 0.2 M PBS (150 μg protein/ml). Beads were then washed and kept as above.

Samples were resuspended at 20×10^6 cells/ml, incubated with MAbs at appropriate dilution for half an hour at 4°C, and washed twice before the incubation with beads in PBS medium without albumin. Mononuclear bone

marrow cell samples (10×10^6 cells/ml) were then incubated with 2-mg beads/ml for 30 minutes at 4°C under continuous slow rotation and passed through the magnetic system. The number of beads depends on the total number of cells to be treated, whether normal or malignant, and 2 mg of beads are then needed to clean samples containing less than 10% malignant cells.

The second step of the procedure involving the incubation with the beads and the magnetic separation was repeated twice (cells were then resuspended in appropriate medium for the freezing procedure).

Except for a few experiments in which malignant cells were detected with the Hoechst staining method (5), we used a liquid cell culture assay. After treatment, samples initially contaminated with 1% BL cells were grown in a liquid culture, and the percentage of growing BL cells at day 10 compared to a calibration curve, enabling us to quantify the number of residual BL cells after treatment. If no BL cell is detectable at day 10, samples are kept 2 more weeks in culture before we conclude that all BL cell growth has been inhibited, a finding usually correlated to the complete elimination of these cells in this assay (6; see also the above-named chapter by I. Philip *et al.* in this volume).

Mean results were then expressed in decimal logarithms of the initial ratio of malignant and normal cells divided by the final ratio after the purging procedure. In individual experiments, results are expressed as percentages of growing BL cells in the culture at day 10.

RESULTS AND DISCUSSION

As shown in Table 1, using the cocktail of three MAbs—B_1, Y29/55, and J_5—in this procedure enables us to eliminate 4-5 logs of malignant cells from samples contaminated with BL cells from five different cell lines, three having recently been established from our patients' tumor cells. In 11 of 22 experiments, malignant cells are fully eliminated. However, in three experiments,

Table 1. Efficiency of the Modified Procedure

Cell Line	Experiments (n)	Depletion (log)	Burkitt's Lymphoma Cell Growth Inhibition
Daudi	4	4	2/4
Raji	3	4	2/3
BL_{93}	5	5	5/5
BL_2	5	4[a]	2/5 (1 incomplete: 3 logs)
BL_{99}	5	4[a]	None (2 incomplete: 3 logs)

Note: Sample contamination was 1%. Burkitt's lymphoma cell elimination is expressed as a mean of three to five experiments on each line. Full elimination has been controlled at day 21 of culture.

[a]*Mean.*

we observed a partial failure of the procedure, having only a 2- to 3-log malignant cell elimination. These three failures could be owed to the use of a cocktail of three MAbs, two of them being irrelevant. Indeed, as shown in Table 2, in three of four experiments B_1 alone was more efficient than the cocktail of three MAbs, suggesting that Y29/55 and J_5 can inhibit, probably by competition, the attachment of the beads to the cell through the linkage of B_1 antimouse IgG.

As shown in Table 3, in preliminary experiments in which samples were

Table 2. Percentage of Burkitt's Lymphoma Cells at Day 10 of Culture

Cell Line	Treated Samples[a]		Calibration Curve[b]	
	B_1	Cocktail	10^{-5}	10^{-6}
BL_{99}	80	100	100	10
BL_2	0	60	100	80
	80	0	100	100
BL_{93}	0	45	60	50

Note: A comparison of the percentage of malignant cells at day 10 of culture in the treated samples, to that indicated by the calibration curve permits measurement of residual Burkitt's lymphoma cells after the purging. For example, for cell line BL_{93}, B_1 enables a full elimination of Burkitt's lymphoma cells, whereas in the calibration curve at 10^{-6}, malignant cells remain after the cocktail treatment (a 4-log elimination).

[a]One percent contamination before treatment.

[b]Untreated samples contaminated with Burkitt's lymphoma cells.

Table 3. Comparative Efficiency of IgM and IgG Monoclonal Antibodies in Direct and Indirect Methods

Cell Line	Experiments (n)	Indirect Method (log)	Direct Method (log)		Indirect Method (log)	
		B_1	RFB_7	SB_4	RFB_7	SB_4
BL_{99}[a]	3	2	1.5	1	—	—
BL_{93}[a]	2	1.5	1.5	1.3	—	—
BL_{99} or BL_{93}[b]	2	1.6	0.65	0.15	—	—
BL_{93}	2	1.75	1.25	0.9	2	1.5

Note: Evaluation was by the Hoechst staining method (samples were contaminated with 10% Burkitt's lymphoma cells).

[a]In the first two series of experiments, beads were used within 3 days of the monoclonal antibody coupling.

[b]In this experiment, beads were used within 18 days of the monoclonal antibody coupling.

treated once only and residual cells detected by the Hoechst staining method, two MAbs of the IgM subclass were tested, RFB_7 and SB_4. These MAbs are known to be very active in the complement lysis procedure and to recognize high-density antigens in BL cells. When directly coupled to the beads they were less efficient on cell line BL_{99} than was B_1 as used previously in the indirect method. In addition, the last experiment suggests that IgM coupling on the bead could be unstable. Such MAbs tested in an indirect method with a relevant antimouse immunoglobulin (IgG + IgM) (KPL, Gaithersburg, MD) had an activity similar to that of B_1. The IMD procedure has been tested in one case in which the patient's bone marrow was pathological, and it allowed as much BL cell elimination as the complement lysis procedure. Before purging 63% of the sample was contaminated with BL cells. At day 10 after purging with B_1, no BL cells were detected.

These results prompt two comments. First, IMD is a very efficient procedure that purges bone marrow with 1% or less BL cell contamination. Modifications of the procedure, initially described by J. Kemshead's group (3), even if minimal when compared to the whole concept (e.g., the Ficoll separation, the adjustment of the number of beads to the total number of cells in the suspension, and finally the double treatment of the suspension), significantly improved the BL cell depletion. Since such parameters are physical, we assumed that they would be valid for the neuroblastoma model. This assumption was confirmed by the results obtained by Seeger *et al.* (13) in the neuroblastoma model. This refined methodology was then used to purge grafts from 25 patients who had stage IV neuroblastoma, and in those samples in which neuroblastoma cells were detected before the purging procedure, we never noted any failure of the method as previously observed.

Second, the criteria for selecting relevant MAbs remain the main difficulty in IMD, and a common rule can certainly not be applied for tumor cells of different origins. In the model for Burkitt's lymphoma, results obtained with IgM MAbs directly coupled to beads are less reproducible than those obtained with IgG MAbs or the same IgM MAbs in the indirect method. Irrelevant MAbs can inhibit the activity of an optimal one. This activity is not strictly linked to the antigen density, even if B_1 recognizes on BL cells an antigen with a higher density than that recognized by Y29/55 and J_5. Indeed, these two MAbs, Y29/55 and J_5, had a good reactivity with the three lines in all experiments (microscopic analysis). It remains to be determined whether the antigen-antibody affinity is constant in all malignant cells and is the only criterion for MAb selection. If not, preclinical assays such as the one described here and elsewhere (see I. Philip *et al.* in this volume) would have to be performed for each patient when possible.

We stress here, once again, the difficulty of transferring the experimental model to the clinical situation, whatever purging method is chosen, and the need for accurately quantifying residual malignant cells before and after the purging for individual patients in clinical pilot trials before starting more extensive controlled or even randomized multicenter studies.

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Neuroblastoma

T. Philip and C. P. Reynolds, Chairmen

DR. T. PHILIP: I want to thank everybody first, and ask specific questions of each of the speakers. We'll begin with Dr. August. Any questions? Yes, Ross (Dr. Pinkerton).

DR. R. PINKERTON: Dr. August, you mentioned your plans to incorporate an ^{125}I-meta-iodobenzylguanidine in a future regimen. Was this intended to be a replacement for total body irradiation (TBI) or an adjunct to it and, if so, what exactly are your plans?

DR. C. AUGUST: It will probably occur before surgery so that there will be an interval of, I would guess, anywhere from a few weeks to a few months between that therapy and the ablative therapy and bone marrow rescue.

DR. PHILIP: Dr. Seeger, one point was not clear to me. In 10 of 17 patients you found tumor cells with immunologic screening and cytology was normal. Can you give us some details?

DR. R. SEEGER: Those patients all had bilateral aspirates and bilateral biopsies that were normal. They were all done at the same institution, UCLA, at the time of the autologous harvest.

DR. M. FAVROT: First, we do not so often find a difference between immunologic detection and morphology. What might the explanation be? Second, can you give us some objective criteria of what you call a tumor cell?

What is the role of computerized analysis to help us define objective criteria for tumor cells?

DR. SEEGER: As I understand the first question was how can we find so many marrows that are positive when you don't find so many positives? I think that is a function of how many cells you look at. I don't know how many you are looking at, but we look at at least 300,000 cells by monoclonal antibody and another 300,000 by neuron-specific enolase (NSE) staining. So that's a total of 600,000 cells. I think that is the first point. The second answer is that we do not call a cell malignant unless we are convinced morphologically and by staining that it is a malignant cell. We do occasionally see very small cells that look like lymphocytes that stain with this cocktail for antibodies. Those cells are not called tumor cells.

DR. K. DICKE: I have a question for you and for the other speakers. Basically, listening as an outsider to neuroblastoma, what is really striking to me is that in your studies you come up with prognostic factors that enable you to do bone marrow transplants in prognostically poor patients. Why are you the only one who is doing that? Is there a discrepancy between interpretation of prognostic factors?

DR. SEEGER: I think those of us working in neuroblastoma would take that as a compliment. I think many of us feel very strongly that even with transplants we will be able to define prognostically different groups and that is why we are continuing to use these factors. We have had a long-standing interest, at least I have, in prognostication, and I think it will be useful.

DR. DICKE: Dr. Philip, what is your idea about it?

DR. PHILIP: I agree with what you just said and my comment would be that it is true that the Seeger study is a very important one because it is the first randomized study in which the question has been asked prospectively. But it is a first step; not everything can be solved by a randomized study. We know that high-dose melphalan should be included in our next study, and we also know that it may have a positive effect on disease-free survival. I think that the prognostic factors will still be valid even if we are able to increase the disease-free survival rate.

DR. DICKE: That means that you plan to include these prognostic factors in your next study?

DR. PHILIP: Certainly, 2 weeks ago we had a meeting on prognostic factors.

DR. SEEGER: That is an important point. We had a meeting with most of the people in the world involved in neuroblastoma, 2 weeks ago, and arrived at recommendations that are standard recommendations for staging and response rate criteria. We anticipate another meeting in about a year that will incorporate many of these new prognostic factors which can then be used

worldwide for studies. Then I can compare my studies to both Thierry's (Dr. Philip) and Dr. Berdini's.

DR. PINKERTON: In stage IV neuroblastoma there are trends of prognostic factors. However, the overall survival of the classically defined stage IV over a year is so abysmal that we are, at the moment, forced to apply megatherapy to that group, and maybe in the future we will start subdividing.

DR. PHILIP: In summary, the point is that we want to find the poor stages I, II, and III and the good stage IV. Okay? The second point, is that we are facing another problem, being that when we began these studies, the control curves were very poor, in the range of 5%. It appears now that the control curve, the ENSG reference curve without bone marrow transplantation, is more in the range of 20% long-term survival. Again, we very logically defined our objective at the beginning with a control curve of 5% and we are now making interpretation of the results with a control curve which is 20%.

I think that we can close this part of the discussion and move on to Dr. Reynolds' opinion about Marie's (Dr. Favrot) question concerning the role of a computerized analysis for detection of tumor cells.

DR. C. REYNOLDS: I sort of briefly skimmed over the idea of digital image microscopy and I mainly just want to introduce the topic in case someone wants to ask specifics, we can go into it. The basic concept there is that we can scan through and localize cells that are likely to be positive, go back and focus on them, and exclude them as false-positives with a variety of things. There are a lot of algorithms that people have developed for analyzing leukemia cells or other types of cells by nucleocytoplasmic ratio and other morphologic criteria. And of course all those can be incorporated into the system because you are not trying to analyze each of the 2 million cells with the computer. You are only scanning and picking up things that are bright for common acute lymphoblastic leukemic antigen (CALLA) or bright for NSE and anticell-surface monoclonal antibodies for neuroblastoma. Whatever your original scan is on, then you can have the computer go back and let the user confirm that himself (with his brain), or the computer can take the image in again and rework it to give you back a lot of criteria that would allow you to exclude or include it as a tumor cell. All that, of course, is future development. The way the system is designed and implemented immunologically now, if I can ever get in the laboratory to do it, we are going to be looking at two to three different antibodies. For instance, in leukemia we look at CALLA on the cell surface and TdT (terminal deoxynucleotidyl transferase) for nuclear antigens; for neuroblastoma we will be looking at a cocktail of cell-surface monoclonal antibodies. One can also use quantification of this because there is a 256 Gy-level capability on it. So you can measure quantitative differences in amounts of antigens to help you discriminate a malignant from a normal cell. And finally, I will just add that the system is not as intensive in maintenance or user/operator skills as a flow cytometer, by any nature, and it is not nearly as

expensive, so I think that everybody who is doing marrow purging could actually have this in their laboratory.

DR. FAVROT: I have one quick question regarding the TdT stain. So these cells are always stained on the cytospin slides or are they stained in suspension with this antibody?

DR. REYNOLDS: The technique for simultaneous two-color staining involves staining of the viable cells in suspension for cell-surface antigens, with biotinylated J5, and then cytospinning the cells, fixing, and then staining with directly fluoresceinated anti-TdT, and at the same time adding phycoerythrin-avidin to develop the cell-surface antibody. But variations of that technique work as well.

IV. Breast Cancer

High-Dose Chemotherapy Intensification and Autologous Bone Marrow Transplantation With Emphasis on Breast Carcinoma

Gary Spitzer, Aman Buzdar, Gabriel Hortobagyi, Leonard Horwitz, Sundar Jagannath, Kathleen Smith, and Karel Dicke

The treatment of stage IV breast carcinoma has been static since the introduction of doxorubicin (Adriamycin) into combination chemotherapy protocols about 10 years ago. Doxorubicin combined with alkylating agents, antimetabolites, vinca alkaloids, steroids, or endocrine manipulation generates an overall response rate of approximately 70% to 80% and a complete remission (CR) rate of only about 20% to 30% (1-6). Unfortunately, few of these CRs are durable (7). Breast cancer without the presence of estrogen receptors appears to have a virulent, aggressive course; survival frequently is only for about 12 months (4). Alternative strategies such as the use of alternating non-cross-resistant combination chemotherapy regimens, new drugs, like mitoxantrone, and biologics do not seem to have affected the rate and duration of response of patients with hormonally unresponsive breast cancer (1).

Several recent analyses suggested that the response rate in stage IV breast cancer patients and the freedom from relapse with use of adjuvant chemotherapy for stage II disease are related to dose intensity, regardless of the therapy program (8-12). High-dose chemotherapy studies in relapsing breast

cancer patients have shown high response rates. As expected, because of the advanced stage and chemotherapy-resistant nature of these patients' illness, the responses have been short (13). Other investigators, who examined high-dose combination alkylating agent therapy with cisplatin as initial therapy in a small group of premenopausal women with stage IV disease, documented an almost 100% response rate and an approximately 50% CR rate (14). It is not yet possible to evaluate the durability of these responses.

Our approach to therapy for nonhematologic neoplasms is to use high-dose chemotherapy early in the treatment strategy as an intensification after three to six courses of initial standard induction chemotherapy. This approach allows the use in overall treatment of such important drugs as doxorubicin, drugs that cannot be escalated to any significant degree in the bone marrow transplant program because of their significant provocation of mucositis (15-18). With this approach, we increased the tumor response in an elderly group of patients with small cell bronchogenic carcinoma and frequently associated lung and heart disease. Furthermore, 6 of 32 patients treated with this high-dose therapy were disease-free 4 years later (19).

To increase the intensity of this approach, we introduced cisplatin therapy into the cyclophosphamide/etoposide combination, and here describe the response and preliminary progression-free survival of 30 patients with estrogen receptor-negative metastatic breast carcinoma and other nonhematologic malignancies treated with this strategy. Furthermore, in the last 15 patients studied, a randomization was performed to define more accurately the necessity of autologous bone marrow transplantation (ABMT) for high-dose intensification studies in patients with minimal previous chemotherapy.

MATERIALS AND METHODS

Patient Population and Supportive Care

Thirty patients received one or two courses of high-dose CVP: 4.5 g/m^2 of cyclophosphamide, 750-900 mg/m^2 of etoposide (VP-16-213), and 120-150 mg/m^2 of cisplatin (platinum) as intensification therapy after three or four courses of standard-dose induction (23 patients) or for relapse (7 patients). The patients' median age was 47, with a range of 29 to 63 years. The first 15 patients registered on this study all underwent ABMT. The last 15 patients were randomized to receive CVP with or without ABMT. All 15 patients in the randomized study had marrow aspirated and stored, and 8 were scheduled for ABMT. Patients who were randomized not to receive autologous bone marrow and whose granulocytes had not recovered to 500/mm^3 by day 28 after chemotherapy received a reinfusion of marrow. In patients scheduled for ABMT, marrow was infused 2 or 3 days after the third and last day of high-dose chemotherapy. The details of marrow collection, storage, and thawing were described previously (19). Patients were prehydrated and continuously hydrated with approximately 5 liters per day of intravenous fluids until 1 day

after completion of chemotherapy, to diminish both bladder toxicity of cyclophosphamide and the nephrotoxicity of cisplatin. The patients' fluid intake and urine output were carefully monitored, and appropriate diuresis was instituted to maintain at least 4 liters of urine output daily. Patients randomized to receive no bone marrow were routinely housed in a germ-free isolation unit; the others were treated in a private hospital room with no special precautions. Appropriate antibacterial therapy was given when patients were febrile (higher than 101°F). Irradiated blood products were administered when needed. Nonresponsive, documented infections were treated with leukocyte transfusions.

RESULTS

Tumor Response

To date, 12 women with estrogen receptor-negative or hormonally unresponsive breast cancer (all at stage IV, but several with associated inflammatory local disease) have undergone treatment intensification. The range and median disease-free period were 0 to 180 months and 11 months, respectively. The most prominent sites of involvement were the lung and mediastinal lymph nodes. Most patients received initial doxorubicin plus cyclophosphamide therapy for at least three and as many as six courses before intensification therapy. Prior to intensification, 2 patients (17%) had achieved CR and, following intensification, 5 of the 10 remaining patients achieved CR for an overall CR rate of 58%; the overall response rate was at least 92%. All patients with evaluable disease showed further tumor shrinkage with intensification. The response rate and progression-free survival at this early stage are listed in Table 1.

Among 10 patients with small cell bronchogenic carcinoma, 8 received CVP as intensification therapy and 2 as therapy for relapse. Of the 6 patients with evaluable disease at the time of intensification, 5 showed a conversion to CR; the overall CR rate to induction and intensification therapy in patients evaluable for response was 9 of 10 (90%) (Table 2).

Three other patients (one with lymphoepithelioma, one with sarcoma, and

Table 1. Response and Progression-Free Survival in Breast Cancer

Induction	Intensification	Progression-Free Survival (mo)
2 CR	2 CR	4+, 9
7 PR	4 CR, 3 > PR	ED, 3+, 3+, 4+, 4+, 4, 7
3 SD	? PR, 1 PR, CR	1+, 3+, 4+

Abbreviations: CR, complete response; PR, partial response; ED, early death; SD, stable disease.

Table 2. Response and Progression-Free Survival in Small Cell Bronchogenic Carcinoma Patients

Induction	Intensification	Progression-Free Survival (mo)
3 CR	3 CR	7, 13+, 20+
3 PR	2 CR, 1 PR	2+, 5+, 12+
1 surgically NED	Still NED	9+
1 SD	1 CR	9+
2 relapse	2 CR	4+, 6

Abbreviations: CR, complete response; PR, partial response; NED, no evident disease following surgery; SD, stable disease.

one with non-oat cell carcinoma) received intensification therapy. Their progression-free survival has been 14, 6+, and 1+ months. Five patients who had soft tissue sarcoma, squamous cell carcinoma of the lung, gastric carcinoma, large cell lymphoma, and adenocarcinoma of the lung were treated upon relapse; one died early, and progression-free survivals in the other four lasted only 1, 2, 2, and 4 months.

Toxicity

Hematologic toxicity in these patients is shown in Tables 3 and 4. Time to granulocyte recovery for 100, 500, and 1,000/mm^3 was identical for first and second courses, the median number of days being 19, 21, and 23, respectively, from day 1 of chemotherapy. In the second course, time to platelet recovery to 50,000/mm^3 and 100,000/mm^3 was slower than in the first: 23 compared with 20 and 28 compared with 21 days, respectively. Among the 15 patients in the

Table 3. Days Until Neutrophil Recovery Following Start of High-Dose CVP Regimen

First Course			Second Course		
>100/mm^3	>500/mm^3	>1,000/mm^3	>100/mm^3	>500/mm^3	>1,000/mm^3
19 (10-26)[a]	21 (12-34)	23 (14-39)	19 (14-52)	22 (12-54)	23 (12-56)

Abbreviation: CVP, cyclophosphamide, VP-16-213 (etoposide), platinum (cisplatin).
[a]*Median and range.*

Table 4. Days Until Platelet Recovery Following Start of High-Dose CVP Regimen

First Course		Second Course	
>50 × 10^3/mm^3	>100 × 10^3/mm^3	>50 × 10^3/mm^3	>100 × 10^3/mm^3
20 (15-32)[a]	22 (17-28)	24 (12-51)	28 (18-60)

Abbreviation: CVP, cyclophosphamide, VP-16-213 (etoposide), platinum (cisplatin).
[a]*Median and range.*

randomized study, the ABMT and non-ABMT patients experienced no difference in recovery of granulocytes and platelets after the first course of intensification. Recovery to 500 granulocytes/mm^3 is shown in Figure 1. In four patients in the randomized study, hematopoietic recovery was not evaluable during the second course because one patient died early, two were removed from the study because of lack of response to chemotherapy during the first course, and the fourth patient was not yet evaluable. All five remaining patients randomized to undergo ABMT recovered rapidly during the second course. Two of the six remaining patients who were not to undergo ABMT had delayed granulocyte and platelet recoveries and required reinfusion of bone marrow on days 33 and 42 for this reason (Fig 2).

During 52 courses of treatment evaluated so far in the 30 patients, only 12 documented infections have occurred (3 of pneumonia, 2 of the anorectal region, 6 septic, and 1 subcutaneous), and there were 19 episodes of fever of unknown origin (36%). Patients in about 40% of the courses were without fever, and in 23% of the courses no infection occurred (Table 5).

Nonhematologic Toxicity

The most common toxic reactions were nausea and vomiting, which were controlled acceptably by combinations of antiemetic and steroid drugs. Most important, the treatment caused minimal stomatitis. For two patients, however, delayed neurotoxicity has been quite incapacitating.

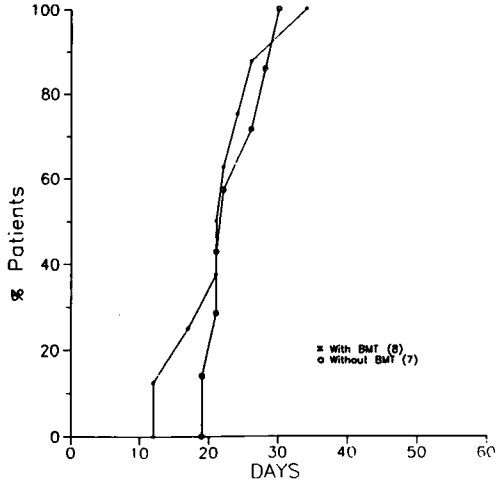

Figure 1. *Percentage of patients recovering absolute granulocyte counts of 500/mm^3 after course 1 of cyclophosphamide, etoposide, and cisplatin. *, with bone marrow transplantation (8 patients); ○, without bone marrow transplantation (7 patients).*

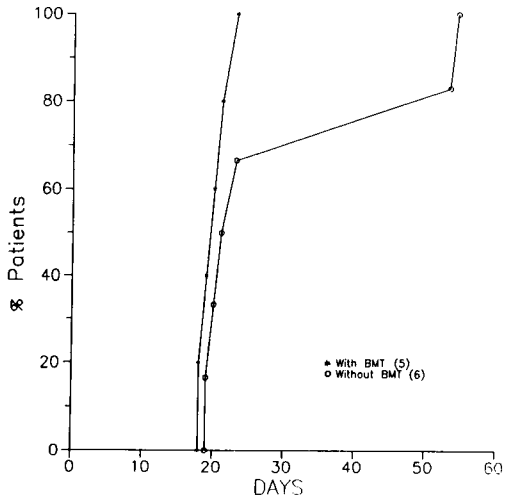

Figure 2. *Percentage of patients recovering absolute granulocyte counts of 500/mm³ after course 2 of cyclophosphamide, etoposide, and cisplatin. *, with bone marrow transplantation (5 patients); O, without bone marrow transplantation (6 patients).*

Table 5. Nonhematologic Toxicity

Type of Illness	Percentage of Patients (n = 30)
Nausea and vomiting usually grade 2	90%
Stomatitis grade 1	36%
Diarrhea	54%
Bladder	8%
Neurotoxicity (2 episodes of blurred vision, 2 episodes of peripheral neuropathy)	8%
Cardiotoxicity (1 episode)	2%

DISCUSSION

We have described a combination of drugs that, when administered at moderate to high doses, was associated with acceptable toxicity, including no life-threatening extramedullary toxicity. Because of the drugs' minimal erosive gastrointestinal toxicity, predisposition to bacteremia and subsequent sepsis as a result of intestinal organisms is limited. This combination of drugs universally induces absolute granulocytopenia of less than 100/mm³ and thrombocytopenia of less than 20,000/mm³. At present, we are studying the influence of bone marrow infusion on hematopoietic recovery after high-dose CVP. Prelimi-

nary data suggest that a small proportion of patients require bone marrow infusion during the second course of therapy to prevent a delay in hematopoietic recovery.

We have used this drug combination to intensify treatment after variable initial induction therapy with standard drugs for several diseases. Judging by the response rate to individual drugs at normal doses among patients with small cell bronchogenic carcinoma, the drug combination seems to be an appropriate choice for this disease. The high complete response rate in small cell bronchogenic carcinoma is, therefore, no surprise; though numbers are small, the complete response rate could be superior to the 70% response rate we obtained with high-dose cyclophosphamide/etoposide intensification in our earlier studies (19).

The use of this combination for breast cancer is more controversial. The response rates to cisplatin and etoposide as individual drugs are quite controversial and usually but not invariably disappointing (20-22). A major reason for incorporating cisplatin in this program was, however, its known synergism with etoposide and other drugs in a number of experimental animal models, and the increasing number of reports of synergism of cisplatin with etoposide and other drugs in a number of human tumors. The nearly 60% CR rate in estrogen receptor-negative breast cancer and the close to 100% response rate is equivalent to that of other small studies of high-dose chemotherapy for this disease (14). This patient group was, however, highly selected and excluded patients with obvious progression before treatment intensification. We are evaluating our long-term experience of the response and survival of an equivalent group treated with conventional therapy. The CVP combination is not associated with veno-occlusive disease, pulmonary toxicity, and significant loss of performance status, unlike the combination high-dose regimens using alkylating agents for this disease (23).

The dosages described here are still at the first level of investigation, however, while we investigate the question of ABMT. Once resolved, we will further escalate the regimen shown in Table 6 to determine whether the maximum tolerated doses should be higher than those currently used and whether, at these maximum tolerated doses, the CR rate will be even higher.

The issue of the need for ABMT is not purely an academic question but is related to the high frequency of bone and bone marrow involvement in breast cancer (24,25). In the future, we believe that this treatment for breast cancer will

Table 6. Phase I Study of High-Dose Cyclophosphamide, Etoposide, and Cisplatin

Cyclophosphamide	1.5 g/m^2 × 3	1.5 g/m^2 × 3	1.75 g/m^2 × 3	2.0 g/m^2 × 3
Etoposide	250 mg/m^2 × 3	300 mg/m^2 × 3	400 mg/m^2 × 3	500 mg/m^2 × 3
Cisplatin	40 mg/m^2 × 3	50 mg/m^2 × 3	60 mg/m^2 × 3	60 mg/m^2 × 3

require methods of detecting minimal numbers of contaminating tumor cells with, possibly, the use of monoclonal antibodies conjugated to toxins or immunomagnetic methods to eliminate clonogenic breast cancer cells (26-28).

The results from our and other groups' studies of high-dose chemotherapy for early breast cancer suggest that such approaches generate high CR rates; if we can improve these response rates further, we may be able to keep some patients free of disease for several years. If a proportion of patients with stage IV disease are rendered free of disease by this treatment method, the regimen could then be moved to an even earlier stage of disease to treat subsets of patients with stages II and III disease. Potential patient categories could be those with inflammatory disease and residual pathologic documentation of disease following induction doxorubicin combination chemotherapy or stage II patients with estrogen receptor-negative tumors and with more than 10 involved nodes. Because of the large numbers of patients required for accurate evaluation of response and response duration, and the long-term follow-up needed by early-stage patients, initial studies should focus primarily on estrogen receptor-negative patients with stage IV disease. These patients have had only brief survival spans, so that improvements in their median survival and disease-free survival rates will be rapidly seen.

ACKNOWLEDGMENT

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Strategies in the Treatment of Breast Cancer With Intensive Chemotherapy and Autologous Bone Marrow Support

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The treatment of metastatic breast cancer is currently disappointing. Despite the availability of more than 15 active chemotherapeutic agents and numerous combinations, no curative regimen has been developed. Further, notwithstanding considerable effort and many clinical trials, the improvement in survival over the past 20 years has been at best limited. However, several leads have appeared over the past decade. First, convincing evidence for a dose-response relationship has been demonstrated in the treatment of breast cancer, both in the adjuvant and advanced disease situations. This relationship appears to hold also when doses are escalated beyond the conventional range and the patient is supported with autologous bone marrow (1). Second, regimens that cannot cure or even produce a high complete response rate (e.g., the cyclophosphamide, methotrexate, and 5-fluorouracil regimen [CMF]) in metastatic disease, have been shown to improve survival when used as adjuvant treatment (2).

Simultaneously, syngeneic and allogeneic transplantation with intensive chemoradiation therapy has been shown to be curative for patients with selected leukemias and lymphomas. Again, fundamental to the success of these approaches was the recognition that treatment of early disease was often far more effective and less toxic than the same therapy used as a last resort.

Interest has been renewed recently in the treatment of breast cancer and other solid tumors using intensive chemotherapy, and particularly intensive combination chemotherapy with autologous bone marrow support (ABMS). Our group has focused on this disease, attempting to evaluate therapeutic regimens systematically based on biochemical and biological properties and modifications of these regimens targeted at breast cancer.

CONCEPTS

The dose-response curve for chemotherapeutic agents in the treatment of breast cancer is steep. Bonadonna and Valagussa (2) demonstrated a major impact of dose on the treatment of metastatic breast cancer, and they extended these findings to the adjuvant treatment setting. When Hryniuk and Bush (3) analyzed treatment results from a number of clinical trials employing CMF and cyclophosphamide, Adriamycin (doxorubicin), and fluorouracil (CAF), they demonstrated a major influence of dose intensity on the therapeutic outcome. The use of intensive chemotherapy with ABMS resulted in objective response rates in resistant disease in excess of what would have been generally expected (4-6). These observations provide encouragement for the use of high-dose regimens in treating patients for breast cancer.

Combination chemotherapy was demonstrated to be more effective than single agents in the treatment of breast cancer (7). This observation is consistent with treatment results for other diseases and predicts that, at high doses, the use of combinations of agents is more likely to provide a therapeutic advantage than the use of single drugs. Table 1 lists the response rates in advanced breast cancer for several drugs used as single agents in conventional doses. Dose escalation with bone marrow support was undertaken with several of these agents and the maximum tolerated dose established. Unfortunately, little information exists on the objective response rates obtained at high doses with these drugs in patients with breast cancer.

It is relevant to high-dose combination chemotherapy efforts that the nonmyelosuppressive dose-limiting toxicities encountered at high doses differ among selected agents (8). This finding offers the opportunity of combining several agents with differing nonmyelosuppressive toxicity using ABMS. When this method was tested in a phase I trial of combination

Table 1. Single Agents Useful in Treatment of Breast Cancer

Drug	Standard-Dose Response Rate Percentage[a]	With Bone Marrow Support: Maximum Tolerated Dose (mg/m^2)	With Bone Marrow Support: Organ Affected by Side Effects
Doxorubicin	35	NA	
Nitrogen mustard	35	33	CNS
Cyclophosphamide	34	7,500	Cardiac
Mitolactol C	29	ND	
Mitomycin C	24	60	VOD
Methotrexate	23	NA	
Streptonigrin	23	ND	
Fluorouracil	21	NA	
Vindesine	20	ND	
Melphalan	19	240	GI
Altretamine	17	ND	
Triethylenethiophosphoramide	17	1,575	GI, CNS
Carmustine	16	1,000	Hep/pulm
Cisplatin	15	240	Renal
Vincristine	14	NA	
Mitoxantrone	13	NA	

Abbreviations: NA, not amenable to dose escalation with bone marrow—synergy with other agents may make use in combination relevant; ND, maximum tolerated dose with bone marrow support not yet determined; cardiac, hemorrhagic myopericarditis; GI, gastrointestinal—enterocolitis, stomatitis; VOD, veno-occlusive disease; hep, toxic hepatitis; pulm, pulmonary fibrosis.

[a]*Response rates from references 9-11 or pooled from several phase II trials.*

alkylating agents (6), the results demonstrated that 1) several alkylating agents can be combined at full or nearly full doses before dose-limiting major organ toxicity occurs, although agents cannot be combined with impunity; 2) the actual toxicities encountered in combination differ from those predicted for individual agents at high doses (12); and 3) frequent and rapid responses can occur even in patients with resistant disease. Responses were especially frequent and rapid in patients with advanced breast cancer, although the duration of response was in general short.

These results stimulated us to evaluate high-dose combination cyclophosphamide, cisplatin, and carmustine with ABMS in the treatment of patients with estrogen receptor-negative, measurable metastatic breast cancer who had not received chemotherapy for metastases. In this review we compare these treatment results to our current results in patients who were and were not previously treated with conventional chemotherapy for advanced breast cancer.

METHODS

Patient Population

Thirty-three patients with metastatic breast cancer were treated with high-dose combination alkylating-agent regimens using ABMS. Patients were considered eligible if they had measurable metastatic disease and no evidence of bone or bone marrow involvement at the time of bone marrow harvest. Twenty-one patients were treated with a fixed dose and schedule of high-dose combination cyclophosphamide, cisplatin, and carmustine as their initial chemotherapy for metastatic breast cancer (13). One patient included in this series was treated at the Dana-Farber Cancer Institute by the senior author in the initial phase I trial (6). Twelve patients who had been previously treated with chemotherapy for metastatic breast cancer were treated with high-dose combinations of cyclophosphamide, cisplatin, and either melphalan or triethylenethiophosphoramide (thio-TEPA).

Bone Marrow Harvest

Patients' bone marrow was harvested under general anesthesia from the bilateral posterior iliac crest and cryopreserved as described elsewhere (14). The bone marrow was not treated with drugs or monoclonal antibodies. Marrow was thawed rapidly when needed at 37° C and infused without further treatment.

Treatment Programs

The treatment regimens used were detailed elsewhere (6,14-16) and are outlined in Table 2. Cyclophosphamide was administered as an intravenous

Table 2. Treatment Regimens

	Days from Bone Marrow Infusion						
Drug Regimen	-6	-5	-4	-3	-2	-1	0
Cyclophosphamide, cisplatin, and carmustine							
Cyclophosphamide (1875 mg/m²/day)	x	x	x				
Cisplatin (55 mg/m²/day)[a]	x	x	x				
Carmustine (600 mg/m²)				x			
Cyclophosphamide, cisplatin, and melphalan							
Cyclophosphamide (1875 mg/m²/day)	x	x	x				
Cisplatin (60 mg/m²/day)	x	x	x				
Phenylalanine mustard (40-150 mg/m²)			x				
Cyclophosphamide, cisplatin, and thio-TEPA							
Cyclophosphamide (1875 mg/m²/day)	x	x	x				
Cisplatin (55 mg/m²/day)	x	x	x				
Thio-TEPA (300-450 mg/m²)			x				

[a] *Cisplatin was given as a continuous infusion.*

infusion over 1 hour, cisplatin as a continuous infusion over 3 days, and carmustine intravenously at 5 mg/m^2/minute. Thio-TEPA and melphalan were given as intravenous infusions over 2 hours. The pharmacokinetics of these agents were measured and reported elsewhere (15-17).

Supportive Care

Patients were treated in simple reverse isolation using a low-bacterial, low-fungal-content diet. Their hematocrit was maintained at more than 42%, and platelets were transfused to maintain a platelet count higher than 20,000/mm^3.

RESULTS

High-dose combination alkylating-agent therapy with ABMS resulted in frequent and rapid regressions of measurable breast cancer lesions in the treated patients. Despite the similar frequency of objective tumor regression in patients who had or had not received previous chemotherapy for metastatic breast cancer, the two groups differed in the frequency of complete response and in the time to treatment failure. Table 3 shows the objective response rates in treated patients. The overall response rate was similar among patients who received high-dose combination alkylating agents as initial therapy or after previous treatment with standard chemotherapy. The frequency of complete response was substantially higher, however, among patients who were treated with the combination of cyclophosphamide, cisplatin, and carmustine as their initial treatment for metastatic disease.

Figures 1 and 2 show the time to treatment failure for all responding

Table 3. Treatment Results of High-Dose Chemotherapy and Autologous Bone Marrow Support

Disease Status Response Treatment Program[a]	No. Treated/ No. Evaluable for Response	% CR + PR	% CR
No previous chemotherapy (88 days) CPA, cisplatin, and carmustine	21/16	14	50
Previous chemotherapy CPA, cisplatin, and phenylalanine mustard (78 days) CPA, cisplatin, and thio-TEPA	12/9	7	33

Abbreviations: CR, complete response; PR, partial response; CPA, cyclophosphamide.

[a]*Treatment programs described in Table 2.*

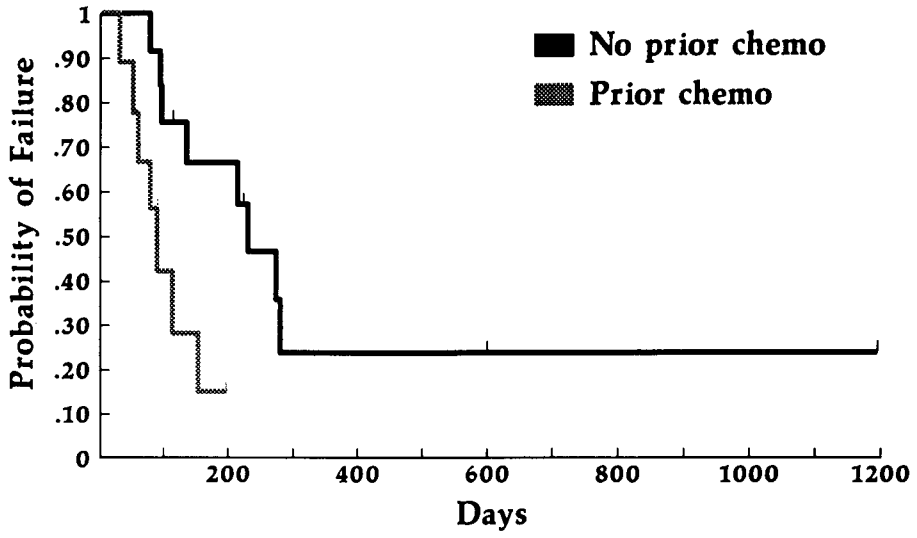

Figure 1. *Time to treatment failure for patients receiving high-dose combination alkylating agents as initial chemotherapy for metastases or after previous treatment with standard chemotherapy.*

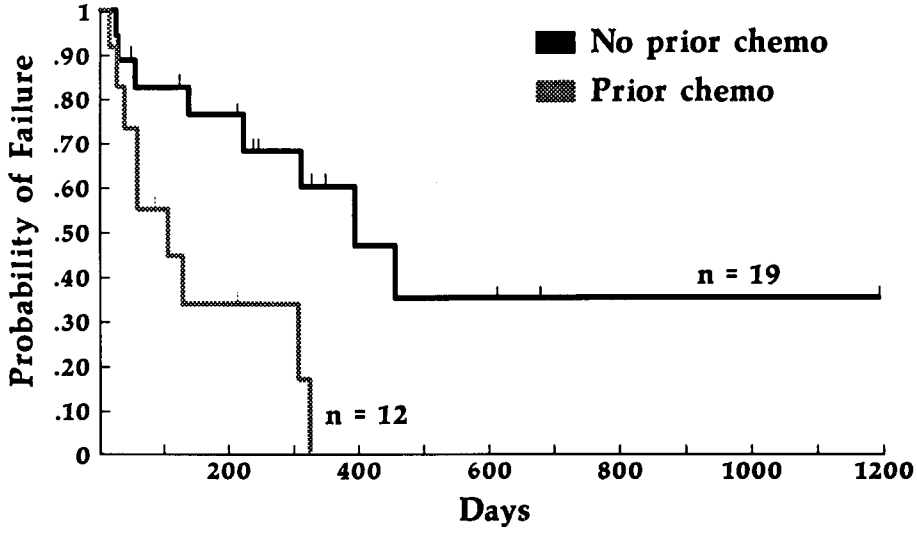

Figure 2. *Duration of survival from time of transplantation of all patients receiving high-dose combination alkylating agents as initial chemotherapy for metastatic disease or after previous treatment with standard chemotherapy.*

patients and survival for all treated patients. The median time to treatment failure for patients who had received previous chemotherapy was shorter than for patients who had received no chemotherapy (88 versus 225 days; *P*, .014). Similarly, the median survival for all treated patients was longer for those who had received no previous chemotherapy compared with previously treated patients (390 versus 102 days; *P*, .004).

The toxicity associated with high-dose combination alkylating-agent therapy is substantial. Among the 33 treated patients, seven therapy-related deaths occurred—an overall treatment-related mortality rate of 21%. Toxicity seems to be increased by a patient's reduced performance status before therapy, extensive previous therapy, especially with doxorubicin and radiation, and the presence of bulk disease at the start of therapy. Late opportunistic infections occurred in seven patients, perhaps related to inversion of the T-cell subsets that have persisted beyond 1 year from therapy (18) (unpublished data).

DISCUSSION

Intensive chemotherapy with ABMS is capable of producing a high objective response rate in patients with metastatic breast cancer. Although the regimen may produce regression of disease in patients who have received previous chemotherapy, the duration of their response is short and therapy-related complications are frequent. When used as initial treatment for metastatic disease, frequent, rapid, and complete responses occur. Relapse remains common, however, the median response duration being 7.5 months. Relapse occurs most often (according to data not presented here) at pretreatment sites of bulk disease, suggesting that additional surgery or radiation therapy may prove of benefit. The difference in time to treatment failure (Fig 1) between patients having received previous chemotherapy or not for metastatic disease most likely represents differences in the intrinsic resistance of extensively treated disease. The different treatment programs (cyclophosphamide, cisplatin, and carmustine compared with cyclophosphamide, cisplatin, and melphalan or thio-TEPA) may, however, be less effective.

The major limitation of the therapeutic approach described here seems to be the timing of treatment. Treatment of patients with resistant, relapsed acute leukemia or lymphoma with intensive chemoradiation therapy and marrow transplantation produced few long-term survivors and a high toxicity rate. When this therapy was applied during remission, however, especially first remission, more patients were cured and the toxicity became less pronounced. The breast cancer patients described here represent patients in first relapse and resistant relapse.

To be more effective, this therapy needs to be attempted at earlier or at least during more favorable disease states. Table 4 lists the extent of breast

Table 4. Considerations in Optimal Timing of High-Dose Chemotherapy and Autologous Bone Marrow Support in Breast Cancer Patients

Transplantation Time	Relevant Breast Cancer Disease Status	Standard Therapy		Comments
		5-Yr Disease-free Survival	5-Yr Survival	
Presentation	? stage III	< 30%	40%	Bulk of disease will require combined-modality therapy
First CR	Adjuvant therapy after primary surgery or radiotherapy >10 nodes	< 20%	20%	Situation in which significant impact most likely
First relapse	Development of metastases or presentation with stage IV disease	< 5%	< 10%	Bulk disease already present, appropriate place for phase II test of regimen
Second CR	CR after induction chemotherapy or combined-modality therapy	< 5%	< 10%	Current standard treatment will induce CR at only about 20% rate
Second or later relapse	Failure of chemotherapy	<< 5%	< 5%	Resistant disease; experience useful for developing new treatment programs

Note: Survival data refer to premenopausal patients with estrogen receptor protein-negative disease.

cancer disease in comparable terms to those used for marrow transplantation in leukemia. There are two disease settings in which intensive chemotherapy regimens for breast cancer is most likely to have a favorable therapeutic-toxic ratio: 1) the high-risk patient with adjuvant disease and 2) the patient who can be rendered free of disease after development of metastases. The former situation (the equivalent of first complete remission) can be approached directly. Patients who are found to have cancerous involvement of 10 or more lymph nodes at the time of primary surgery for breast cancer have a poor prognosis despite routine adjuvant chemotherapy. In this setting, the use of intensive chemotherapy with bone marrow support with or without additional standard chemotherapy is an attractive and testable therapeutic approach. The latter situation (treatment of patients rendered disease-free by standard approaches after first relapse) is more complex and less easily analyzed. Currently available therapeutic regimens produce complete responses in only about 7% to 20% of treated patients. This severely limits the applicability of this approach and would subject patients to induction chemotherapy, which may produce tumor resistance and host toxicity. Sites of pretreatment bulk disease will likely require additional surgery or radiation therapy. Nonetheless, the setting is a frequent clinical problem, and investigation of intensive therapy approaches is warranted.

The optimal intensive therapeutic program for the treatment of breast cancer remains to be defined. The availability of multiple non-cross-resistant agents with nonoverlapping, nonmyelosuppressive toxicities offers the possibility of constructing novel combination programs. These new regimens will have to be tested carefully in comparable disease settings to establish the relative therapeutic efficacy and comparative toxicity of different programs. With continued study, the toxicity of treatment programs may be reduced either by development of better supportive care, patient selection, or modification of the treatment approach.

Bone marrow contamination with malignant cells is likely to be a frequent problem in breast cancer. The frequency of bone or bone marrow involvement with neoplasia is in excess of 40%. As therapeutic programs improve, the likelihood of relapse from contaminated bone marrow will increase, and laboratory investigations aimed at determining the frequency and magnitude of malignant involvement are needed.

In summary, high-dose combination alkylating-agent therapy is capable of producing frequent and rapid complete responses in breast cancer patients when used as initial chemotherapy for metastases. Systematic, targeted trials at earlier disease states are the next step in establishing the value of this approach.

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Phase I and II Studies of High-Dose Alkylating Agents in Poor-Risk Patients With Breast Cancer

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Bone marrow transplantation offers the technical opportunity of using high doses of chemotherapeutic regimens for patients who have a poor prognosis because of refractory cancers or other conditions (1,2).

For several tumors, the antitumoral effects of alkylating agents are related to a clear dose-response relationship (3); in conventional chemotherapy the main dose-limiting toxicity of these agents is their hematologic toxicity. For these reasons, high doses of alkylating agents alone or in combination are frequently selected for the conditioning regimens of cancer patients eligible for bone marrow transplantation. In breast cancer, despite the development of new strategies, patients with metastatic diseases or inflammatory breast cancers are generally considered not curable (4). The use of high doses of alkylating agents followed by autologous bone marrow transplantation (ABMT) may, however, modify the adverse natural history of this disease in selected patients known to have the worst prognosis.

We report here phase I and II studies of high doses of melphalan alone or

in combination with other alkylating agents, followed by ABMT in 11 patients with metastatic or inflammatory breast cancer.

PATIENTS AND METHODS

Patients

Eleven women with a median age of 40 years (range, 26-54 years) entered the study. All patients had breast cancer with a very poor prognosis: four patients had a history of secondary metastatic disease resistant to conventional (doxorubicin, cyclophosphamide, 5-fluorouracil) and second-line chemotherapies. Three patients (numbers 116, 119, and 137; Table 1) had inflammatory breast cancer and had developed thoracic skin involvement during their chemotherapy. Four patients had inflammatory breast cancer and were in complete (three patients) or partial remission (one patient) since their first therapy course.

Patients with visceral metastasis received ABMT a median of 37 months after the diagnosis and 12 months after the development of metastasis. The other patients were treated a median of 8 months after diagnosis (range, 6-14 months).

Overall, nine patients had undergone mastectomy, seven had received extensive radiotherapy, and all had been treated with chemotherapy consisting of doxorubicin, cyclophosphamide, and fluorouracil (median, 10 courses; 3-15 courses).

Methods

Conditioning Regimens

Melphalan was used in each case at a dose of 140 mg/m^2 given as an intravenous bolus injection during hyperhydration. Four patients received only this regimen (5).

Cyclophosphamide and melphalan were used in five patients; cyclophosphamide (60 mg/kg/day) was given on days -5 and -4 and melphalan (140 mg/m^2) on day -2.

Mitoxantrone was combined with cyclophosphamide and melphalan for two patients and given at a dose of 12 mg/m^2 on days -7 and -6.

Autologous Marrow Transplantation

Unpurged marrow transplantation was used after a minimum of 2×10^8 medullary cells/ kg were collected and screened with cytologic and histologic assays for the absence of marrow involvement by cancer. Fresh marrow was used in three patients (numbers 03, 12, and 33; Table 1) treated with melphalan alone after marrow storage at room temperature for 24 hours. All other patients received dimethyl sulfoxide-cryopreserved autologous bone marrow.

Table 1. Clinical Data

Patient No.	Age (yr)	Initial Staging	Previous Therapy: Surg/Radio/ 1st-line chemo[a]/ 2d-line chemo	Disease Duration (mo) (Metastatic Disease)	Disease Sites	Conditioning Regimen[b]	Response	Response Duration (mo)	Outcome (Nov. 1986)
03	29	T2N+	+ / + / + / +	65 (19)	Lungs, liver, skin	Melphalan	mR	—	Dead, 21 days, encephalitis
12	35	T3N+	+ / + / + / +	21 (12)	Lungs, lymph nodes, skin	Melphalan	mR	—	Dead, 17 days, heart failure, resp. distress
33	44	T2N+	+ / + / + / +	40 (3)	Lungs, pleura	Melphalan	CR	24	Dead, relapse, 26 months
46	44	T3N+	+ / + / + / +	35 (12)	Liver	Melphalan	Stable disease	3	Dead, relapse, 8 months
116	54	T3N+PEV3	+ / + / + / +	10	Skin	Cy, melph	CR	6	Alive in relapse, >14 months
119	49	T3N+PEV3	+ / − / + / −	11	Skin	Cy, melph	CR	8	Alive in 2d CR, >13 months
129	48	T3N+PEV3 Bilat.	+ / + / + / +	14	—	Cy, melph, radiotherapy	NE	5	Dead, relapse, 9 months
137	40	T4N+PEV3 Bilat.	+ / − / + / −	6	Skin, lymph nodes	Cy, melph, radiotherapy	CR	3	Alive in relapse, >7 months
141	39	T3N+PEV3	+ / − / + / −	8	—	Cy, melph, radiotherapy	NE	—	Alive in CCR, >6 months
155	26	T3N+PEV3	− / + / + / −	8	—	Mito, Cy, melph	NE	—	Alive in CCR, >2 months
157	38	T3N+PEV3	− / − / + / −	6	Breast	Mito, Cy, melph, radiotherapy	PR	—	Alive, >1 month

Abbreviations: mR, minimal response; CR, complete remission; NE, not evaluable; PR, partial remission; CCR, continuous complete remission.

[a] *Surg, mastectomy; radio, radiotherapy; 1st-line chemo, doxorubicin, cyclophosphamide, fluorouracil.*

[b] *Conditioning regimens: melphalan, 140 mg/m² × 1; Cy, melph, 60 mg/kg × 2 cyclophosphamide and 140 mg/m² × 1 melphalan; mito, Cy, melphalan, 12 mg/m² × 2 mitoxantrone, 60 mg/kg × 2 cyclophosphamide, and 140 mg/m² × 1 melphalan.*

Supportive Care

Patients were housed in single rooms in reverse isolation. They received irradiated blood products (15 Gy) to maintain their platelet count at more than 20,000/mm^3 and their hematocrit at more than 30%. If they became febrile, they were treated promptly with broad-spectrum antibiotics, followed by empiric i.v. amphotericin B if the fever persisted more than 48 hours. Parenteral nutrition was given when the patient's oral intake was less than 50% of the standard caloric requirement.

Evaluation of Extrahematologic Toxicity

Mucositis was considered as mild if the patient complained of soreness during meals, moderate if the patient was unable to eat, and severe if the pain required narcotics. Diarrhea was coded as mild for watery stools of less than 500 ml/day, moderate if diarrhea was more than 500 ml with pain, and severe when associated with hemorrhage.

RESULTS

Antitumoral Response

Two patients died during the first month after ABMT. Six patients were evaluable: four had a complete response, one a partial response, and one had stable disease. Median duration of responses was 5.5 months (range, 3-24 months).

Overall, of the nine patients evaluable after 1 month, six experienced disease progression, and three are in continuous complete remission with a short follow-up. Among the six patients who relapsed, two had metastatic cancers that progressed, and four patients had a thoracic recurrence, with skin and subcutaneous involvement.

Hematologic Toxicity

As Table 2 shows, all patients had evidence of engraftment, granulocytopenia (fewer than 500/granular leukocytes/mm^3) lasting a median of 14 days (range, 8-37 days), and thrombocytopenia (fewer than 50,000 platelets/mm^3) a median of 27 days (range, 20-50 days).

Extrahematologic Toxicity

Two patients with multiresistant metastasis died early of viral infection and heart failure. Moderate or severe mucositis occurred in 6 of the 11 patients, and moderate or severe diarrhea in 4 of the 11 patients. Extrahematologic toxicity was neither more frequent nor more severe in patients receiving additional alkylating agents in addition to melphalan. One patient, conditioned with mitoxantrone, cyclophosphamide, and melphalan, developed renal failure hours after the marrow infusion; his renal function returned

Table 2. Toxicity

Patient No.	Mucositis[a]	Nausea[a]	Diarrhea[a]	Other	Days with Parenteral Nutrition	Bacteremia	Days of		
							Gr/mm³<500	Plts/mm³<50,000	Hospitalization
03	3	2	3	—	10+	—	16	—	21+
12	2	1	3	—	8+	—	17	—	20+
33	1	1	—	—	19	Staph	15	20	32
46	3	1	—	—	14	Staph	14	28	36
116	2	3	2	—	16	—	8	35	27
119	2	2	2	—	12	—	14	26	40
129	—	3	—	—	24	Strepto	11	24	40
137	2	2	1	—	21	—	13	30	22
141	1	1	1	—	19	—	17	22	20
155	1	2	1	Renal failure	35	—	37	50	44
157	—	2	1	—	17	—	14	30	30

[a]*Numbers pertain to degree of severity: 1 = mild, 2 = moderate, and 3 = severe.*

Abbreviations: Gr, granulocytopenia; Plts, platelets; staph, staphylococcal; strepto, streptococcal.

to normal after 1 month of dialysis. The etiology was not documented but the problem may have been caused by dimethyl sulfoxide toxicity.

DISCUSSION

In this phase I and II study we showed, as others have done (6,7), that high doses of alkylating agents followed by ABMT were able to induce a high response rate with acceptable toxic effects, in patients with advanced breast cancer.

Transplantation-related deaths were observed in the first two patients whose performance status was extremely poor at that time and who had a long history of multiresistant disease. Despite intensification of the conditioning regimen by the addition of other alkylating agents to melphalan, the next patients' tolerance of the procedure was quite acceptable; these patients were treated sooner after the diagnosis, and they had a good performance status.

Although the response rate was exceptionally high for these patients (five objective responses among six patients), the short duration of these responses was generally disappointing. Local recurrences were a major cause of failure in the patients treated early in the evolution of inflammatory breast cancer, which suggests that these patients may need adjuvant extensive local-regional radiotherapy.

Our current data invite us to use high doses of a combination of alkylating agents and ABMT early in the disease course of selected patients with inflammatory breast cancer, in association with extensive radiotherapy.

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High-Dose Chemotherapy and Autologous Bone Marrow Transplantation in Breast Cancer: The Beth Israel-Dana-Farber Cancer Institute Experience

Karen Antman, Paul Eder, W. David Henner, Beverly Teicher, Tom Shea, Anthony Elias, Sue Schryber, Bob Siegel, Lowell Schnipper, and Emil Frei III

While allogeneic bone marrow transplantation (BMT) currently results in a disease-free survival rate of 50% in patients with acute myelogenous leukemia in first remission, and autologous bone marrow transplantation (ABMT) and allogeneic BMT for lymphoma has yielded a disease-free survival rate of 20-50% (1-3), the role of high-dose therapy with ABMT has only recently been scrutinized in the treatment of solid tumors. Selected neuroblastomas can apparently be cured, and there are possible cures of small cell lung cancer, gliomas, and Ewing's sarcoma (4). The major current limitation in the effective bone marrow transplantation of solid tumors is the lack of an optimal preparative regimen for cytoreduction for the more common adult solid tumors.

Criteria for successful ABMT include a malignancy responsive to cytoreductive therapy; an effective cytroreductive regimen whose limiting toxicity is bone marrow failure; transplant early in the course of the disease when there is minimal tumor burden and the least probability of resistance; and

finally, a source of bone marrow stem cells free of clonogenic tumor cells (5). Patients with breast cancer frequently have bone marrow that is uninvolved by metastatic tumor. However, ABMT for breast cancer is generally considered by the clinician only when patients have not responded to multiple regimens.

The solid tumor autologous marrow program (STAMP) was organized to integrate an experimental laboratory and clinically based program in the rational design and systematic evaluation of an effective ablative regimen for solid tumors based on preclinical observations, pharmacology, and clinical observations. Alkylating agents were chosen for emphasis in this program because of their steep dose-response curves, non-cross-resistance, broad clinical activity, therapeutic synergy, varying nonhematologic toxicity, and lack of cell-cycle specificity.

The initial study evaluated a combination of cyclophosphamide, cisplatin, and BCNU (carmustine) (STAMP I). The maximum tolerated doses (MTDs) were 5625 mg/m^2, 165 mg/m^2, and 600 mg/m^2, respectively. The toxicity of the three drugs in the combination was nonoverlapping and, therefore, the drugs could be combined at almost 100% of the MTD of each agent individually. Of 17 patients with breast cancer treated on the phases I and II protocol, 14 were treated at the MTD. Two patients had had no prior chemotherapy. Two had failed within 6 months of completing adjuvant chemotherapy and 13 had failed prior chemotherapy. Twelve either had less than a partial response on initial therapy or progressive disease on secondary therapy. Of 17 patients, 16 were estrogen receptor protein (ERP) negative. All were premenopausal. One patient had no measurable disease at the time of transplant. Of the 16 evaluable patients with breast cancer, 6 responded completely (38%) and 8 partially (response rate, 88%) (7).

Responses in breast cancer were unusually rapid, with a median of 11 days to partial response (PR) and 12 days to complete response (CR) (7). Five of six complete responders had met the criteria for a PR by day 7. Because of the promising results in breast cancer in the STAMP I study, a second high-dose regimen was designed to combine single agents with known clinical activity in breast cancer with potentially less toxicity.

Preclinical laboratory studies in MCF7 human breast cancer cells document a steep dose-response curve for triethylenethiophosphoramide (thio-TEPA) and 4-hydroperoxycyclophosphamide (4-HC) which can be measured over 4 logs. When the dose of thio-TEPA was held constant at 10, 50, and 200 μm and the dose of cyclophosphamide increased from 1 to 100 μm, substantial synergy was observed. When the concentration of 4-HC was constant in three experiments at 5, 25, and 50 μm and the dose of thio-TEPA varied from 1 to 200 μm, synergy was most impressive at the highest doses of 4-HC.

When mice bearing C3H breast adenocarcinoma were treated with varying doses of thio-TEPA and cyclophosphamide, survivals were longest with a 30 mg/kg/day dose of cyclophosphamide and 1 mg/kg/day of thio-

TEPA. Larger ratios of thio-TEPA to cyclophosphamide resulted in unacceptable toxicity (Dan Griswald, 1986; personal communication).

In the clinic cyclophosphamide as a single agent can be given at 7.5 g/m^2 without ABMT support with dose-limiting perimyocarditis. Thio-TEPA (1500 mg/m^2) as a single agent with ABMT resulted in dose-limiting neurotoxicity and mucositis (8; unpublished data).

Thus, cyclophosphamide and thio-TEPA in combination appear to be synergistic in the laboratory setting. Both are active at standard doses in breast cancer. The MTDs for both agents are established with and without autotransplant and the organ toxicities at high doses are nonoverlapping—myopericarditis for cyclophosphamide and neurotoxicity and mucositis for thio-TEPA.

Our current regimen (STAMP III) combines cyclophosphamide at 6 g/m^2 with escalating doses of thio-TEPA. In 20 patients with various tumors, there has been one death owed to infection at the second dose level (360 mg/m^2 of thio-TEPA). Diarrhea, rash, and mucositis appear dose related and become dose limiting at 900 mg/m^2 of thio-TEPA.

Response occurred in one of three patients with breast cancer treated at thio-TEPA doses less than 400 mg/m^2 and five of five patients treated at doses greater than 400 mg/m^2. Pharmacokinetic studies of thio-TEPA for the 4-day continuous infusion revealed two-fold variations in the area under the curve among the patients at each dose level. Thio-TEPA levels during the infusion for the 180, 500, and 900 mg/m^2 dose levels have been 0.7, 1.9, and 5 μm, respectively.

We have begun a study of four cycles of induction with continuous infusion high-dose doxorubicin, methotrexate, and 5-fluorouracil, followed by intensification with cyclophosphamide and thio-TEPA in patients with untreated stage IV breast cancer.

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Immunodetection of Breast Carcinoma Cells in Human Bone Marrow Using Multiple Murine Monoclonal Antibodies and Flow Cytometry

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Breast cancer is one of the malignancies that frequently spread to bone and bone marrow. Detecting tumor in marrow from patients with apparently early-stage disease could prompt more aggressive adjuvant therapy. Preliminary reports from London and Milan suggest that marrow may be contaminated in stage I disease in as many as 28% of patients (1,2). Methods employed to date, however, have made it difficult to confirm the epithelial nature of the cells detected with multiple monoclonal antibodies. As a result, patients with positive bone scans have been excluded from autologous bone marrow transplantation programs out of concern that their marrows might be subclinically contaminated with malignant cells. A sensitive and specific technique for detecting residual cells might permit more rational selection of transplant candidates. An assay for detecting tumor cells might also be used to monitor the elimination of malignant breast cancer cells following treatment ex vivo with various cleansing techniques.

SELECTED MURINE MONOCLONAL ANTIBODIES REACT WITH HUMAN BREAST CANCER

A group of monoclonal antibodies has been developed against fresh breast cancer specimens and breast tumor cell lines (3). These reagents recognize at least 12 different antigen families, ranging in molecular weight from 42,000 to more than 300,000. Substantial heterogeneity has been observed in the phenotype of any single tumor, but many of these antigen families are expressed by at least a few cells from a majority of human breast cancers. Among 20 primary breast cancers phenotyped with monoclonal reagents, only 2 expressed the same combination of antigens (Boyer *et al.*, unpublished data). When used in combination, five antibodies were capable of binding to at least some cells in each of 15 tumors. More than 95% of the cells were stained in 12 of the 15 tumors when the biotin-avidin immunoperoxidase technique was used.

A TECHNIQUE FOR DETECTING BREAST CARCINOMA CELLS IN HUMAN BONE MARROW

In recent studies using immunofluorescence, a total of 123 distinct murine monoclonal antibodies reactive against surface membrane antigens of breast cancer cells were screened for their ability to bind to normal human bone marrow mononuclear cells (Leslie *et al.*, unpublished data). From the 27 antibodies that lacked reactivity against human bone marrow, a group of 5 was selected based upon their additive binding to tumor cells under direct and indirect immunofluorescence. Breast cancer cells from effusion fluids or established cell lines were mixed with human bone marrow at dilutions of from 1:10 to 1:10,000. Cells were incubated for 1 hour at 4°C with directly fluoresceinated 2G3, 317G5, 260F9, 741F8, and 9C6 antibodies in the presence of 0.02% sodium azide. After washing, 10^6 cells were evaluated for green fluorescence using an Epics 753 flow cytometer. Antigens recognized by each of the antibodies were present in a sufficiently high copy number that additive fluorescence was observed when combinations of two antibodies were evaluated.

When mixtures were prepared using human bone marrow and cells from the CAMA-1 breast carcinoma cell line or from effusion fluids, 1 cell in 10,000 produced a statistically significant signal when compared to the background staining associated with normal human bone marrow. The counting efficiency was only approximately 60%, and a linear correlation was obtained over approximately 4 logs between the number of tumor cells added and the number of tumor cells detected.

If additional control studies do not document extreme variability in backgrounds between different marrow samples, these techniques promise to permit detection of tumor cells at very low levels. In addition, tumor cells

residing within marrow can be sorted and examined by standard morphologic techniques or by staining with additional antibodies. A linear correlation between tumor cells added and tumor cells detected could permit monitoring of micrometastatic disease.

CONCLUSION

In a model system, flow cytometry has detected 1 breast tumor cell in 10,000 bone marrow cells after incubation with a mixture of five directly fluoresceinated murine monoclonal antibodies. The number of tumor cells detected was proportional to the number of tumor cells added over a range of 4 logs.

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Autologous Bone Marrow Transplantation for Metastatic Breast Carcinoma

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The treatment of hormone receptor-negative metastatic breast cancer in premenopausal women remains unsatisfactory. Although most patients respond to aggressive combination chemotherapy, the median duration of response is only 6-12 months, and survival after failure of "first-line" combinations is usually short (1). Complete remissions (CRs) are infrequent, even with the most aggressive regimens (1). Animal studies and preliminary clinical trials in humans, reviewed by Frei and Canellos (2), suggest that higher doses of cytotoxic agents may produce meaningful increases in tumor-cell killing. Peters hypothesized that combinations of non-cross-resistant alkylators with minimal overlapping toxicities might further enhance tumor cytoreduction (3), and a recent trial employing autologous bone marrow support demonstrated encouraging results (4). However, the relative contribution of individual drugs in these combinations has not been studied in detail.

In 1983, we began a clinical trial to determine the effect of high-dose single alkylating agents on efficacy and toxicity as they were added to a combination regimen for patients with metastatic breast carcinoma. The

initial six patients were treated with high-dose cyclophosphamide alone and the next four with the combination of cyclophosphamide and BCNU (carmustine). Both groups of patients received cryopreserved nonpurged autologous marrow cells following completion of drug therapy.

PATIENTS AND METHODS

All patients gave written informed consent to participate in the study after it had been approved by the Institutional Review Board. Since the inception of the trial, 30 premenopausal women who either had metastatic breast carcinoma or were at high risk for relapse of stage II or III tumors have undergone bone marrow procurement. Two patients were excluded because of tumor contamination of the harvested marrow despite normal preharvest marrow biopsies. Two others died of metastatic disease before marrow could be transplanted. Of 26 patients eligible for the trial, 10 have received transplantations thus far. The clinical characteristics of these patients are shown in Table 1. Ages ranged from 37 to 53 years (median, 40 years). All tumors were estrogen receptor (ER)- and progesterone receptor (PgR)-negative, except for one in a woman with an ER-, PgR+ carcinoma that had failed to respond to hormonal manipulation. All had measurable metastases, and all but one had visceral disease. Patient number 4 with only skin and soft tissue metastases had extensive chest wall inflammatory carcinoma that had progressed despite local radiotherapy and combination chemotherapy. Three of the patients had a poor performance status (3-4 on the Zubrod scale). Seven had had prior radiotherapy. All 10 had received chemotherapy prior to bone marrow harvesting, and only one had not been previously treated with standard doses of cyclophosphamide. Five of the 10 had had

Table 1. Pretransplant Clinical Characteristics

Patient	Age	PS	Prior Therapy	Disease Sites
1	41	1	R[a], Mp[a]	Pleura, lung, nodes
2	47	2	R[a], CMF[a]	Pleura
3	37	0	R[a], CMF[a], CAF	Lung
4	53	1	R, CMF, Pt, A	Chest wall
5	39	4	R[a], CMF[a], CAPt, Vb	Liver
6	53	1	T, Ma, CMF, A	Lung, bone
7	39	3	CAF[a], C, R, Vb	Lung, liver, brain
8	37	2	CMF[a], CAF[a], R	Lung, bone, brain
9	40	0	CMFPr[a]	Lung, nodes
10	39	3	CAF[a]	Lung, bone, nodes

[a]*Adjuvant therapy.*

Abbreviations: PS, performance status (Zubrod); R, radiotherapy; Mp, melphalan; C, cyclophosphamide; M, methotrexate; F, 5-fluorouracil; A, Adriamycin; Pt, cisplatin; Vb, vinblastine; Pr, prednisone; T, tamoxifen; Ma, megestrol acetate.

only adjuvant chemotherapy, but most of these had developed clinical evidence of metastases while on or within 12 months of completing therapy.

Bone marrow was harvested only after bilateral iliac crest bone marrow biopsies demonstrated at least a 40% cellularity and absence of metastatic tumor. Marrow cells were obtained by multiple aspirations of the posterior and occasionally the anterior iliac crests. Nucleated cells were concentrated using a Haemonetics Model 30 Cell Separator and frozen in autologous plasma and 10% dimethyl sulfoxide at a controlled rate of 1°C/minute to -80°C, then stored in the liquid phase of a liquid nitrogen freezer. The concentration step resulted in a loss of approximately 25% of mononuclear cells but retention of more than 90% of the granulocyte-macrophage colony-forming cells. Final concentrated cell yields ranged from $1.31\text{-}3.5 \times 10^8$/kg body weight; the mean was 2.37×10^8/kg.

The first six patients were treated with cyclophosphamide alone in a scheduled dose of 50 mg/kg/day for 4 days. One patient in this group, however, developed gross hematuria after a total of 150 mg/kg and the fourth dose was omitted. Patients 7 through 10 received 160 mg/kg of cyclophosphamide over 4 days plus 300 mg/m^2/day of carmustine on the first 3 days of the cyclophosphamide. The cryopreserved autologous bone marrow cells were rapidly thawed in a 37°C water bath and infused approximately 36 hours after the final dose of cyclophosphamide.

Patients were treated in private rooms under mask and hand-washing isolation. Laminar air flow was not used. Broad-spectrum antibiotics, usually mezlocillin and gentamicin, were initiated when a patient's temperature reached 38.5°C. Vancomycin was added for documented or suspected gram-positive infection. Amphotericin B was initiated for documented fungal infection or for unexplained fever persisting after 72-96 hours of broad-spectrum antibiotics. Prophylactic platelet transfusions were given when the platelet count fell to less than 30,000. All blood products were irradiated, and cytomegalovirus-negative products were routinely used for patients whose sera were cytomegalovirus-negative.

RESULTS

All patients became profoundly leukocytopenic, with circulating WBC counts of less than 100/mm^3, by day 2 after autografting, and all developed fever requiring antibiotics. Platelet support was also required for all patients. Median time for recovery to greater than or equal to 1000 WBCs/mm^3 was 14 days postautograft and for recovery of a self-sustaining platelet count of 50,000, 21 days. Hematopoietic recovery time did not correlate with the number of marrow cells infused. There was no difference in time to granulocyte recovery between the group treated with cyclophosphamide alone and those treated with the combination of cyclophosphamide and carmustine, but platelet recovery occurred later in the combined alkylating

agent group. When patients treated at our institution with the same regimens for other malignancies are included in the analysis, the median times to platelet recovery of 12 and 31 days for cyclophosphamide alone versus cyclophosphamide and carmustine, respectively, are significantly different ($P < .01$) (Table 2). Two patients died before reaching a platelet count of 50,000, one at day 14 and one at day 81, although the latter patient did not require platelet support after day 30. The one transplant-related death in this series, from pneumonia and presumed sepsis on day 14, occurred in the patient whose pretransplant performance status was 4.

Most of the patients had transient elevations of liver enzymes early in the posttransplant period, but none developed significant hepatic dysfunction or the clinical syndrome of veno-occlusive disease. Two patients, however, did develop symptomatic posttransfusion hepatitis after they were discharged from the hospital. One patient had serologically proven hepatitis B and one had non-A, non-B, both demonstrated by biopsy. No patient experienced significant myocardial, pulmonary, or renal decompensation attributable to the drug regimen. However, two women who had previously received chest-wall radiotherapy developed transient signs of pericarditis. Although neither patient had evidence of hemodynamic compromise, one required corticosteroids for relief of chest pain. Mucosal toxicity was mild and manifested chiefly as diarrhea. Total duration of hospitalization for the nine patients who survived the procedure ranged from 19 to 40 days (median, 22 days).

Tumor response data are shown in Table 3. Of the six patients treated with cyclophosphamide alone, four had objective tumor regression, with responses lasting from 4 to 15 months. Patient 2, who had extensive pleural metastases and scarring from previous sclerotherapy, had a slightly less than 50% reduction in pleural tumor mass documented by completed tomography, listed as a minor response. Her unmaintained response of 15 months, though, was the longest in the group. Interestingly, her disease recurred locally, and rebiopsy revealed ER+, PgR+ adenocarcinoma, whereas her tumor had been identified as receptor negative on two occasions pretransplant. Recurrent disease was manifested as progression at sites of previous metastases in each of the responders, although patient 3 developed brain metastases concomitantly.

Table 2. Time to Recovery Posttransplant

Cytoreductive Regimen	No. of Patients	Median Days to WBC ≥ 1000	Median Days to Plts ≥ 50,000
Cy200	8	13 — NS[a]	12 — $P < .01$[a]
CyBCNU	10	16	31

[a]Wilcoxon rank sum for unpaired samples.

Abbreviations: WBC, white blood cells; Plts, platelets; Cy200, cyclophosphamide (200 mg/kg); CyBCNU, cyclophosphamide (160 mg/kg) plus BCNU (carmustine) (900 mg/m²); NS, not significant.

Table 3. Treatment Results

Patient	Therapy	Response	Duration (mo)	Survival (mo)
1	Cy150	PR	4	18
2	Cy200	MR	15	19
3	Cy200	PR	8	20
4	Cy200	PR	3	9
5	Cy200	NE	—	0.5
6	Cy200	NR	—	2.5
7	CyBCNU	PR	2	4
8	CyBCNU	NR	—	2
9	CyBCNU	CR	7	9+
10	CyBCNU	CR	2	3

Abbreviations: Cy150, cyclophosphamide (150 mg/kg); Cy200, cyclophosphamide (200 mg/kg); CyBCNU, cyclophosphamide (160 mg/kg) plus BCNU (carmustine) (900 mg/m²); CR, complete response; PR, partial response; MR, minor response; NR, no response; NE, not evaluable.

Because no CRs were achieved by the first six patients, cyclophosphamide was reduced 20% and carmustine was added for patients 7 through 10. Three of the four responded, and two had clinical CRs. However, only patient 9 had a response of significant duration, and she relapsed with multiple brain metastases after 7.5 months. The other two patients relapsed locally after short responses. In the entire series, six of the seven responders relapsed locally, suggesting inadequate tumor cytoreduction rather than a contribution by tumor-contaminated autologous marrow.

For the most part, response durations were disappointingly short. The median duration of response was 4 months, and only three patients had unmaintained responses in excess of 6 months.

Neither the type of pretransplant therapy nor the performance status seemed to influence response rate in this small group of patients. Four of the five patients who had previously received only adjuvant chemotherapy responded, including both of those achieving CR, while three of the five patients who had failed standard-dose combination chemotherapy for measurable metastases achieved PRs with the high-dose regimens. Four of five with a pretransplant performance status of 0 or 1 responded, while three of five responded whose performance status was 2 to 4. Nevertheless, transplant-related morbidity was greater in the patients with a poor performance status.

DISCUSSION

Preliminary evidence suggests a steep dose-response relationship for cytotoxic chemotherapy for metastatic breast cancer (3,4). The technique of stem-cell autografting provides for the testing of this hypothesis without the dose-limiting toxicity of irreversible marrow suppression. Studies using this

approach in metastatic breast cancer have been limited, as reviewed recently by Souhami and Peters (5). The largest trials have employed either single agents not usually used as treatment for metastatic breast cancer (6-8) or combinations of multiple alkylating agents (4). The latter approach seems to be the most encouraging, resulting in a significant number of CRs. However, the relative contributions of the individual agents have not been addressed. The preliminary trial reported here demonstrates that high-dose cyclophosphamide alone may produce responses in patients with breast cancer refractory to standard doses of the same drug but is unlikely to result in a significant number of CRs.

Since survival benefit often depends largely on attainment of CR, a second drug was added to the treatment regimen in this trial. Even though carmustine in phase II studies has only modest activity against breast carcinoma, its addition to cyclophosphamide has resulted in CRs in two of four patients treated thus far. Although more patients are needed for confirmation, our results support the hypothesis that multiple high-dose alkylating agents may be additive or even synergistic in the treatment of metastatic malignancies.

The toxicity of this aggressive approach, though significant, was tolerable in the patients with good pretransplant performance status. The treatment cannot be recommended for those with significant functional impairment.

The shorter platelet recovery time of patients receiving cyclophosphamide alone suggests that endogenous marrow recovery played a significant role in hematopoietic reconstitution and supports the studies of Smith *et al.* (9) indicating that autografting is unnecessary with this regimen. However, the platelet recovery time of patients treated with cyclophosphamide plus carmustine was identical to that of patients at our institution autografted after marrow-lethal doses of cyclophosphamide and total body irradiation, suggesting the need for stem cell rescue.

Although this preliminary study resulted in a high response rate, the median duration of response was disappointingly short. The predominance of local recurrences in relapsing patients indicates that the high-dose drug regimens employed here failed to eradicate endogenous tumor. Since the drug doses used are near the limit of their nonhematologic tolerance, it is likely that either additional drugs or alteration of the timing of the procedure will be necessary to achieve more meaningful disease control.

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Detection of Occult Breast Cancer Cells in Cultured Pretransplantation Bone Marrow

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A major criterion of eligibility for treatment involving high-dose therapy followed by transplantation of previously harvested, unpurged autologous bone marrow is that the patient's marrow be free of tumor cells at the time of harvest. The accepted method of evaluating bone marrow for the presence of malignant cells is the light-microscopic examination of bone marrow aspirate and biopsy specimens (1). Recent studies have indicated that this approach is not always adequate, however, since histologically normal marrow has been found to harbor malignant cells detected by chromosomal analysis, monoclonal antibodies, and culture techniques (2-8). In our application of culture techniques to marrow harvested from patients undergoing autologous bone marrow transplantation (ABMT) for cancer, we have demonstrated occult tumor cells in the histologically tumor-free marrow of five of 20 consecutive patient samples; the patients had either non-Hodgkin's lymphoma or solid tumors, including breast cancer (8). These culture techniques are particularly suited to examining the harvested "normal" marrow of patients with breast cancer because several monoclonal antibodies are available that recognize breast tumor cells with high efficiency (9). In this report we describe the application of culture methods to detect occult tumor cells in marrow harvests from nine consecutive patients with breast cancer and no histologic evidence of marrow metastases.

MATERIALS AND METHODS

Bone Marrow Harvest

If normal bilateral iliac crest bone marrow aspirate and biopsy specimens were free of metastatic tumor and the patient met all other eligibility requirements, marrow was aspirated from the posterior iliac crests while the patient was under general anesthesia. Approximately 2×10^8 nucleated cells per kilogram of body weight were collected and placed in heparinized Hanks' balanced salt solution (HBSS). The entire marrow specimen was filtered sequentially through stainless steel mesh of 0.307-mm and 0.201-mm wide openings to remove particulate material.

Culture Techniques

Cells, fat, and particulate material collected during the filtering process were scraped from the screens and placed in HBSS. Particles were allowed to settle for 5 minutes at room temperature. Material in suspension was layered onto lymphocyte separation medium (LSM®, Litton Bionetics, Charleston, SC) and centrifuged at 400 × g for 20 minutes. The cells were washed by centrifugation (400 × g for 7 minutes) and then resuspended in Tris-buffered ammonium chloride for 5 minutes to lyse mature red blood cells. Medium containing fetal bovine and horse sera was added to each tube and the suspensions were washed again. Cells were cultured using a modified method described by Coulombel *et al.* (10) using RPMI 1640 medium supplemented with 10% horse serum, 10% fetal bovine serum, 10^{-5} M 2-mercaptoethanol, 10^{-6} M hydrocortisone sodium succinate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Flasks containing 2×10^7 cells were incubated for 7 days at 37° C in 5% CO_2 in air, at which time the flasks were transferred to an incubator at 33° C for the remainder of the study.

Marrow Colony-Forming Unit, Granulocyte, Erythrocyte, Monocyte, Megakaryocyte (CFU-GEMM) Assay

Adherent cells from long-term marrow cultures established as above were removed using trypsin-EDTA and washed. The cells (6×10^5 per plate) were cultured in GEMM medium with 0.3% Bacto-agar in the presence of two units of erythropoietin. The GEMM medium was composed of McCoy's 5A medium supplemented with amino acids, 1% phytohemagglutinin-stimulated lymphocyte-conditioned medium, and 10% fetal bovine serum. Plates were cultured at 37° C in 5% CO_2 in air for 14 days. Colonies and clusters were examined and enumerated, using an inverted microscope. If apparently abnormal cells or unusual colonies were noted in the GEMM assays or primary cultures, cytospin preparations were made for cytologic and immunocytochemical analyses from replicates of the original culture flasks.

Cytologic and Immunocytochemical Techniques

Cells obtained directly from the harvest screens and cells harvested from the long-term cultures were spun onto precleaned glass slides using a Shandon Cytospin centrifuge (Shandon Southern Instruments, Inc., Sewickley, PA). The slides were air dried and stained with Wright's stain. Additional slides were fixed for 2 minutes in ice-cold acetone and stained with an avidin-biotin immunoperoxidase technique using antibodies to alpha-lactalbumin or epithelial membrane antigen (EMA), antibodies #A579 and #M613 (Dako Corporation, Santa Barbara, CA), respectively.

RESULTS

The results of applying culture techniques to detect occult tumor cells in marrow harvests from nine consecutive patients about to undergo high-dose therapy plus ABMT for breast cancer are summarized in Table 1. All of these samples were obtained from patients whose marrow was considered histologically free of malignant cells. Four of the nine samples showed no evidence of occult tumor cells by any of the methods employed. However, four other samples showed abnormal-appearing cells or colonies in Wright's stained plates of the human pluripotent hematopoietic colonly-forming units (CFU-GEMM) assay. Of particular note were isolated cells or large colonies containing cells with a round or oval acidophilic, euchromatic nucleus and occasional prominent nucleoli. All cultures yielding such cells were sampled

Table 1. Detection of Occult Tumor Cells in Cultured Marrow Harvests From Breast Cancer Patients

Harvest ID #	Occult Tumor Cells Suspected/Method	Total Weeks in Culture Before Detection	Cytospin Confirmation	
			H & E Stain	EMA/α-Lactalbumin Immunocytochemistry
4	No/—	—	No	No
14	Yes/GEMM assay	6	Yes	Yes
19	Yes/GEMM assay	14	Yes	Yes
25	Yes/GEMM assay	6	No	No
49	No/—	—	No	No
50	Yes/GEMM assay	2	Yes	Yes
54	No/—	—	No	No
141	No/—	—	No	No
142	Yes/Culture	4	No	? Yes (see text)

Abbreviations: H & E, hematoxylin and eosin; EMA, epithelial membrane antigen; GEMM, granulocyte, erythrocyte, monocyte, and megakaryocyte colony-forming unit.

and cytospin preparations obtained for staining with hematoxylin and eosin and monoclonal antibodies. These techniques confirmed the presence of tumor cells in three of the four samples and indicated that the fourth, although yielding unusual cells in the CFU-GEMM assay, did not appear to contain tumor cells.

This result indicates that at least 33% (3/9) of the histologically normal marrows harvested from patients with breast cancer contained occult tumor cells. These tumor cells were not evident in cytospin preparations of the material scraped from the screens and used to initiate the cultures, indicating that the culture time may have been important for amplifying the frequency of tumor cells in the samples. We did not routinely assay all the samples at earlier time points, thus our results may not give a true indication of the average time needed to detect tumor cells.

The last marrow harvest in the series (#142, Table 1) grew abnormal cells in the primary cultures. A routine cytospin preparation revealed two EMA-positive cells. This preparation contained large numbers of histiocytes that showed a slight diffuse cytoplasmic reaction, but none were clearly positive. In contrast, the two EMA-positive cells were clearly positive and possessed morphological features consistent with adenocarcinoma.

DISCUSSION

These findings demonstrate that culture techniques can be used to amplify and consequently detect occult tumor cells in marrow harvests from candidates for ABMT. Our results suggest that at least 33% of the marrows harvested from breast cancer patients that have been judged free of tumor cells by conventional histologic analysis nevertheless contain occult tumor cells. Although this estimate is preliminary because of the small sample size, our observation emphasizes that in a significant proportion of patients, breast cancer is a systemic rather than a regional disease and must be treated as such.

Our culture techniques often require a period of several weeks to detect occult tumor cells, which would not be a disadvantage for patients who are referred early in the course of their disease or in clinical remission. Their harvested marrow is frozen and stored for weeks to months prior to beginning therapy, allowing adequate time for evaluation in culture.

Several factors may have contributed to the success of our long-term culture system in detecting occult tumor cells in bone marrow. The initial material used to establish the cultures is obtained from the screens used to filter the entire marrow harvest. Thus, very small aggregates of metastatic tumor cells, which would be sampled infrequently and by chance using conventional histologic methods, may be detected by our technique. Adequate sampling is not the only advantage gained in our culture system, however, since examination of cytospin preparations of the harvest material

obtained from the filter screens usually has failed to reveal the presence of tumor cells. This suggests that the loss of terminally differentiated cells, and enhanced tumor-cell growth provided by the culture system, are also important advantages.

Because of the low number of samples and the lack of a detailed calibration of the efficiency of this assay, we cannot be certain that the EMA-positive cells in sample #142 were occult tumor cells. In order to estimate the frequency of these cells with reference to the original marrow harvest, we performed cell counts on representative cytospin fields, each of which was 1/10 the area of the entire cytospin preparation. The mean count was 351 ± 55 cells per field. Thus, there were two EMA-positive cells in a total of 3,510 cells on the cytospin. The only morphologically classifiable cells in the cytospin were histiocytes and other mononuclear cells presumed to derive from monocytes in addition to rare unidentifiable precursors in the original harvest. Three independent 600-cell differential counts performed on cytospins of the material obtained from the screens and placed into culture showed 69.4 ± 1.3% granulocytes (all types), 16.6 ± 1.8% nucleated erythroid cells, 11.1 ± 0.7% lymphocytes, 2.8 ± 0.1% monocytes, and a small proportion of other cells (megakaryocytes, plasma cells). Thus the presumed precursors of the histiocytes and mononuclear cells in the cultured sample most likely represent fewer than 2.8% of the cells of the original harvest. This suggests an enrichment factor of at least 36-fold, based on the loss of terminally differentiated cells and the selective enrichment of other cells by the culture process. We can estimate that this culture system is detecting EMA-positive cells at a frequency of 2 in a minimum of 125,000 cells of the original marrow harvest. If these EMA-positive cells are occult tumor cells, the efficiency of their detection by applying this culture system exceeds that of conventional histologic analysis, which has an estimated limit of 1 tumor cell in 1,000 to 10,000 nucleated bone marrow cells (11). The inclusion of this patient's marrow as a positive sample would raise the number of marrow harvests contaminated with occult tumor cells in breast cancer patients to over 40% of those examined.

Most of the occult breast cancer cells that were detected first became evident when we performed the assay for CFU-GEMM on the cultures. Morphologically, the adenocarcinoma cells differ significantly from the majority of hematopoietic cells and associated stroma that grow in this culture medium. Fresh or cultured normal marrow samples from allogeneic-transplant donors consistently failed to yield cells with epithelial morphology, nor do they contain EMA- or alpha-lactalbumin-positive cells. However, the suspected presence of "tumor" cells in the CFU-GEMM assay is not foolproof (e.g., #25, Table 1), which emphasizes the need for confirmatory cytology and immunocytochemistry.

Our observation of tumor cells in a high proportion of the cultures suggests these cells have a high plating efficiency or degree of autonomy, or

both. Additionally, the culture system may provide factors that support the growth of both hematopoietic progenitors and breast cancer cells. Two incidental observations suggest that growth factors may be an important component of this culture system. A significant number of the breast cancer cells were associated with hematopoietic colonies in the cultures, suggesting that the tumor cells may be producing colony-stimulating factors. A variety of tumors have been reported to produce such factors (12-14). In addition, using these techniques we have had greater difficulty establishing normal marrow harvests in long-term culture than in harvests from patients with cancer. Although the reasons for this are not clear, the production of growth factors by tumor cells, which are lacking in normal marrow, may play a role.

Additional studies are needed to determine the efficiency of this culture system in detecting occult breast cancer cells; to understand better and to improve the culture system; and to evaluate the clinical significance of occult tumor cells in autologous marrow harvests. Such culture techniques appear to have relevance and applicability in evaluating patients and in clinical decision making.

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Breast Cancer

E. Frei III and R. Bast, Chairmen

DR. K. ANTMAN: The planned protocol that we're actually beginning, hopefully within a month, is an induction of Adriamycin (doxorubicin) and methotrexate, with 5-fluorouracil (5-FU) if we can get it into the regimen—we're doing a phase I study of the induction regimen. The reasons for choosing that regimen were to avoid alkylating agents and to use a high dose of Adriamycin since the investigators at Mt. Sinai Hospital showed a very high complete response (CR) rate to single-agent Adriamycin at doses in excess of 90 mg/m^2. Patients who have a response, complete or partial or even a minimal response, will then receive transplants at this point, with cyclophosphamide and thio-TEPA. If we can, we will add carbinoplatinum to our cyclophosphamide thio-TEPA regimen as a phase I trial and, if that proves to be effective in patients with advanced disease, the three-drug regimen will be moved into the setting.

DR. G. SPITZER: Karen (Dr. Antman), how are you going to determine the relative effectiveness of this? What are the end points? How much better do the durations of disease-free survival have to be to determine whether a regimen is better?

DR. ANTMAN: At this point, these are all phase I studies. The idea is to see if this approach is feasible in patients who are young and otherwise have a 100% chance of dying. At the point when we find that it's feasible and also effective, then it will probably go into leukemia group B for appropriate randomization.

DR. E. FREI III: Gary Spitzer, in your breast cancer study of CVP in which you include induction and intensification, you had seven out of eight CRs at the end of intensification and in the second study, six out of eight. Are those figures correct? And the other question is: In the breast cancer you used just one transplant, I believe, or were there two?

DR. SPITZER: Routinely, it's two courses of intensification. There were two patients in CR when we gave the intensification. If I remember correctly, I thought there were 10 patients who were evaluable for response to the intensification. Five of these converted into CR so the CR rate at the end of it would have been about 7 of 12. As I mentioned earlier, a few of the patients with residual disease had minimal CAT scan abnormalities, so it was very hard to evaluate what the true CR rate was.

DR. FREI: How about tumor regression as a function of the first and second transplant courses?

DR. SPITZER: It appears to be a different rate of response than what Bill (Dr. W. Peters) has seen. A lot of these patients have pulmonary disease and mediastinal disease. I think (Dr.) Lenny Horwitz, who's helping me with the study, mentioned that he repeated an x ray on one of the patients recently transplanted, a lady whom I didn't have on the slide and was uncertain whether she'd responded at the end of the first course of chemotherapy; however, she was in CR at the end of the second course. The second intensification, we think, is very important.

I think that we're seeing a different pattern of response than you, Dr. Peters, and this may be because we're intensifying patients rather than using the megadose as the initial therapy.

DR. W. "BILL" PETERS: We've looked at the first derivative tumor volume with respect to time for the patients who have not been previously treated and patients who have received prior treatment. Initially, to my surprise, there is no difference in the rate of tumor volume regression between those two groups.

DR. SPITZER: Yes, but what you don't understand, Bill, is that the patients coming into this study are frequently responding already and while your patients in relapse may be regrowing, you're getting rid of the sensitive component with your high-dose therapy while we've already gotten rid of the potentially most sensitive component with the normal-dose therapy.

DR. PETERS: Where you've got a fairly precisely measurable disease, you do have an opportunity with two courses of the same intensification to analyze volume regression as a function of both as Souhami has done for cyclophosphamide in small cell lung cancer and where he saw evidence of a substantial decrease in tumor volume with the second course, suggesting that resistance occurs relatively early.

DR. SPITZER: I thought that Dr. Frei was skeptical about further reduction in the second course with cyclophosphamide.

DR. PETERS: No, when he added up the 11 or 12 cases, I think he saw with intensive cyclophosphamide something like an 80% regression on the median, first course, and 30% on the second course. So there was some, but it was substantially less.

DR. K. DICKE: I have a question for Bill and for Karen. What is the exact mortality of the regimens?

DR. ANTMAN: On the first regimen, cyclophosphamide, carbinoplatinum, BCNU (carmustine), our mortality was 15%. On the second regimen, the phase I study, there was one infectious death out of 23 patients—on the cyclophosphamide and thio-TEPA regimen.

DR. PETERS: We have the same results. Basically, you can predict the patients who are going to have a bad result. The patients who have extensive bulky disease at the time of treatment, who have poor performance status, those are the people who are likely to get into trouble. I think that as we move earlier in the disease our chance of getting into trouble will actually go down. I think that we will be unlikely to get below 10-15% in terms of serious toxicity and yet have a regimen that will produce a response rate that warrants testing in early disease, at least at this point.

DR. DICKE: Do you need bone marrow?

DR. PETERS: In the phase I trial, we looked at the time to hematologic reconstitution as a function of dose. Only at the first two dose escalations was there an increase in the duration of myelosuppression. After that it remained constant, suggesting that the time remained the same. Currently, we have an ongoing trial in patients who have limited bone disease in which we give 1 day of the 3-day regimen, that is to say cyclophosphamide at 1.8 g/m^2, cisplatin at 55 mg/m^2, and carmustine at 200 mg/m^2. I can guarantee you that every one of them has been in the hospital for at least 2 weeks and with each course, the myelosuppression is prolonged a little more.

DR. ANTMAN: My approach would be somewhat different. I believe that the idea is to evaluate the drugs in combination. We had two patients who required a second dose of marrow because they weren't re-engrafting quite as efficiently as we would have liked. And I suspect, therefore, that we do indeed need the marrow. In any case, the idea is to prevent deaths and I think that we can do this with marrow used as support. It's like a platelet transfusion, it's a supportive therapy.

DR. SPITZER: I don't think we can ignore, in this disease, the impressive frequency of bone marrow contamination and bone involvement. Bill, in view of the recent Adriamycin studies in adjuvant setting, do you think you are warranted at this moment to do a transplantation? Data that Drs. A. Buzdar and G. Hortobagyi have generated from this institution suggest that FAC (5-FU, Adriamycin [doxorubicin], cyclophosphamide) adjuvant chemotherapy in that group is markedly superior to CMF adjuvant chemotherapy, and there might be

a projected 40-50% disease-free survivorship in that group. You're going to have a lot of work cut out for you to prove the superiority of higher dose chemotherapy in that group.

DR. PETERS: Even if, in the 10-node adjuvant setting, the disease-free survival rate ends up being optimistically 30%, there's still 70% of the women who are being committed to dying of their disease. You will surely not make a major impact in the metastatic disease setting very soon and the only time to do it will be to do it early in the treatment settings. I think it's warranted.

DR. SPITZER: Except the problem is, Bill, that after we have the next set of adjuvant studies in this group, then maybe we will identify a subgroup within that group which has the 10-20% classic long-term, disease-free survivorship in whom it is appropriate to do transplant studies. Like in the inflammatory disease study, that subgroup is already identified as patients with residual disease after initial induction chemotherapy at the time of surgery. This is the subgroup with the bad outlook versus those who have a CR who have almost a 60-70% cure rate. So we should wait a little longer to identify the study group better.

DR. ANTMAN: One comment on bone marrow metastases in light of Dr. Spitzer's comment. There are three groups that I am aware of who are looking at cleaning up the marrow with breast cancer. There's the group at Jerusalem, the group in England at the Royal Marsden, and some groups in the United States. However, I think the important issue is to be able to get CRs in the patients first, in patients with known negative marrow, and then we can look at cleaning up the marrow.

DR. SPITZER: Yes, I agree, Karen. If we have a high response rate, CR rates of 70-80%, and still have continuing relapses, then I think we've found that marrow contamination is a problem.

V. Lung Cancer

Detection of Bone Marrow Metastases in Small Cell Lung Cancer by Tumor Stem Cell Assay and Monoclonal Antibodies

Michel Symann, Yves Humblet, Jean-Luc Canon, and Anne-Marie Lebacq-Verheyden

It is well known that small cell lung cancer (SCLC) has a high propensity to metastasize to the bone marrow and that such involvement is a prognostic factor (1). Depending on the series, incidence of positive bone marrow at the time of diagnosis is reported to be 17-29% (2-7).

In the setting of autologous bone marrow transplantation, the presence of tumor clonogenic cells in the graft should theoretically be avoided. This can be achieved by using appropriate screening tests to select patients or by purging in vitro marrow proven to be contaminated.

The objective of the present study was to investigate the use of growth of tumor colonies in soft agar and the use of three rat monoclonal antibodies (MAbs) in detecting metastases of SCLC in the bone marrow.

MATERIAL AND METHODS

Patient Population

Thirty-four patients at different stages of documented SCLC formed the basis of the study for the human tumor cloning system. Thirty-nine patients at the time of initial staging provided samples for the immunodetection. Staging was exhaustive and aggressive. Fifteen normal bone marrow samples were obtained from six healthy donors, one patient with a Ewing's sarcoma and four with neuroblastoma.

Collection of Bone Marrow for Culture and Immunodetection

Bone marrow aspirates were collected in a syringe containing 125 U/ml of Calparine (Choay, Paris, France). The samples were then diluted with an equal volume of calcium- and magnesium-free Hanks' medium. Mononuclear cells were concentrated by density centrifugation on Ficoll-Hypaque (1077 g/cm^3) (Pharmacia, Uppsala, Sweden) at 400 g on the interface during 30 minutes at 16° C. Next, the cell layer at the interface was collected, washed in Hanks' medium, and centrifuged at 400 g for 20 minutes. Finally, the pellet was counted using a Coulter Counter (Coulter Electronics 2 TD, England) and resuspended in calcium- and magnesium-free phosphate-buffered saline medium.

Human Tumor Cloning System

Cells were cultured according to Hamburger and Salmon (8), as described by Pollard *et al.* (9).

Anti-SCLC Rat Monoclonal Antibodies

The isolation and characterization of the MAbs anti-LCA1, LCA2, and LCA3 have been described elsewhere (10). The antibodies were biotinylated as described previously (11).

Indirect Immunofluorescence Assays

This assay was performed using each of the three MAbs separately. Cells from the SCLC cell line, 250,000, or 500,000 isolated marrow cells were incubated at 4°C during 30 minutes with 25 μl of biotinylated MAb diluted to 1:100. After two washings with phosphate-buffered saline-fetal bovine serum 2%-Azide 5%, 50 μl of fluorescent Avidine (Beckton Dickinson, California) diluted to 1:100 was added to the pellet. Following an incubation period of 60 minutes at 4°C, the cells were washed two more times. After having mounted cytocentrifuged cell smears in Tris-buffered glycerol (pH 9), the slides were examined with a reflected light fluorescent microscope equipped with a 40X objective (Olympus Optical Co., Japan). The entire smear was scanned and scored positive if at least one cell with bright surface fluorescence was detected. Positive controls using the NCI-H69 line were made with each test.

Two separate assays, with each MAb, were performed on each bone marrow sample to avoid technical errors.

RESULTS

A total of 67 bone marrow aspirations, performed on 34 patients, were also cultured in soft agar. Table 1 presents the results of this procedure. There were eight instances in which conventional morphology demonstrated SCLC in the bone marrow aspirate while no colony growth was observed. In seven instances of negative histology, the agar culture showed colony growth. Examination of the cells in the colonies by light microscopy in all instances revealed cells consistent with a nonhematopoietic origin.

Fifteen bone marrow samples originating from individuals free of SCLC were tested using anti-LCA1, LCA2, and LCA3. No fluorescent cells were observed. Indirect immunofluorescence staining of marrow aspirates by our three MAbs was included in the initial work-up of 39 patients. Sixteen samples were found to contain cells reactive with anti-LCA1, LCA2, or LCA3; of these, only five were also found to be involved by tumor on bone marrow smears or sections stained with May Grunwald-Giemsa or hematoxylin and eosin (Table 2).

In those patients, the results of antibody staining were compared with the extent of disease dissemination. Twenty-four patients were classified as having limited disease and 15 patients as having extensive disease. Table 3 summarizes the results of immunofluorescence testing of marrow aspirate according to the results of staging. Tumor cells stained by anti-LCA1, LCA2,

Table 1. Bone Marrow Involvement by SCLC: Comparison Between Conventional Morphology and Tumor Stem Cell Assay

		Tumor Stem Cell Assay		
		+	-	Total
Morphology	+	3	8	11
	-	7	49	56
Total		10	57	67

Table 2. Bone Marrow Involvement by SCLC: Comparison Between Conventional Morphology and Immunofluorescence

		Immunofluorescence		
		+	-	Total
Morphology	+	5	0	5
	-	11	23	34
Total		16	23	39

Table 3. Incidence of Tumor Cells Detected in the Marrow by Immunofluorescence as a Function of Disease Extension

Clinical Stage	Tumor Cells in the Marrow Detected by Immunofluorescence		
	Present	Absent	Total
Limited disease	7 (29%)	17	24
Extensive disease	9 (60%)	6	15
Total	16 (41%)	23	39

or LCA3 were found in 7 out of 24 patients with limited disease and in 9 out of 15 patients with extensive disease.

A remarkable heterogeneity in the tumor recognition was noted. Anti-LCA1 recognized tumor cells in 11 (68%) of 16 of the 16 marrows studied, and anti-LCA2 and LCA3 each recognized 7 (43%) out of the 16.

DISCUSSION

The goal of the present study was to investigate the human tumor stem cell assay and indirect immunofluorescence with anti-LCA1, LCA2, and LCA3 in the detection of SCLC metastases in the bone marrow.

In this study, we have not found a good correlation between morphological evidence of marrow involvement by SCLC and growth of tumor colonies in soft agar; the sensitivity ([true positives]/[true positives plus false negatives]) being 0.2. Nevertheless, there were seven samples of histologically negative marrow in which the tumor stem cell assay grew colonies. Even if this system has a low sensitivity, probably because of the low plating efficiency of the tumor cells, it has the advantage of documenting the presence of viable clonogenic cells in the bone marrow.

All patients with morphological evidence of marrow involvement had SCLC cells identified by anti-LCA1, LCA2, or LCA3 antibodies, the sensitivity of the indirect immunofluorescence being 1. Anti-LCA1, LCA2, and LCA3 were unreactive with 15 normal bone marrow samples but did react with 11 bone marrow samples negative by conventional morphological methods. The antigenic heterogeneity found on the cell surface using anti-LCA1, LCA2, and LCA3 highlights the advantage in using a panel of MAbs with different specificities. Bone marrow involvement was found in 24% of 24 patients who had otherwise limited disease. A positive pretreatment bone marrow is an unfavorable prognostic indicator both for survival and for duration of remission (1). The prognostic significance of bone marrow micrometastases detected by indirect immunofluorescence in patients with limited disease and morphologically negative bone marrow is not yet known. A prospective trial might address this question.

This experiment using human tumor stem cell assay and indirect

immunofluorescence indicates that detection of SCLC metastases unperceived by conventional methods can be achieved. In future studies, these methodologies will be applied to monitor autologous bone marrow grafts and efficacy of purging procedures.

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High-Dose Etoposide and Cisplatin With Autologous Bone Marrow Support as Treatment for Lung Cancer

Hillard M. Lazarus and Thomas R. Spitzer

Etoposide or VP-16-213, a semisynthetic congener of podophyllotoxin, is a drug important in the treatment of several human malignancies, including testicular and small cell lung cancer, leukemia, lymphoma, and Kaposi's sarcoma associated with the acquired immunodeficiency syndrome (AIDS) (1,2). Early trials using etoposide indicated that myelosuppression was the dose-limiting toxicity, although it was of modest degree and short duration (2-4). For ionizing radiation and a number of cytotoxic drugs including melphalan, thio-TEPA, cisplatin, 5-fluorouracil, and methotrexate, an escalation of drug dosage usually leads to an increased antitumor effect (5-13).

Given the reality of improved supportive care and the suggestion of a dose-response effect, several investigators undertook significant escalations in etoposide dose (with or without reinfusing autologous marrow) (14-17). Mucositis and toxicity to the marrow were severe, but antitumor responses were noted in several patients with advanced cancer. In addition, etoposide has exhibited synergistic activity with cisplatin and other antineoplastic agents in several human and animal tumor systems (1,18-21). Etoposide and cisplatin together are effective therapy for both small cell and non-small cell lung cancers (22-27). For these reasons we undertook a phase I trial in patients with

carcinoma of the lung to determine the maximum dose of etoposide that could be administered safely in conjunction with high-dose cisplatin and autologous bone marrow reinfusion.

MATERIALS AND METHODS

Fifteen lung cancer patients representing 18 treatment courses were evaluated. Three patients had small cell carcinoma, two of whom had not achieved a complete remission with conventional combination chemoradiation therapy and one who had relapsed after attaining remission. Six patients had surgically unresectable non-small cell cancer previously untreated, and six had active non-small cell disease after therapy. The median age for all patients was 59 years (range, 38-68 years). Twelve of these patients were men and three were women. No patient had evidence of active infection or tumorous involvement of the bone marrow. All patients had a Karnofsky performance status of at least 60%, an anticipated survival untreated greater than 8 weeks, measurable disease at physical examination or on external imaging, and no evidence of major organ system dysfunction not directly attributable to tumor. All patients had undergone a bone marrow harvest and cryopreservation procedure at least 4 weeks after completing any previous cytotoxic therapy. The marrow was frozen using previously described techniques and was stored in the liquid phase of a liquid nitrogen refrigerator until used (28). This protocol was approved by the Institutional Review Board for Human Subject Investigation of the University Hospitals of Cleveland, and all patients gave written informed consent.

At the start of therapy, patient evaluation included a complete history, physical examination, tumor measurement studies, complete blood count, serum tests of liver and renal function, 24-hour urinalysis for creatinine clearance, and audiometry. Patients were treated in single hospital rooms without specialized isolation procedures. Central venous catheters, parenteral alimentation, broad-spectrum antibiotics, and irradiated (30 Gy) blood-component support were used as indicated clinically. Persons with a history of "cold sores" or serum anti-herpes simplex antibody titers greater than 1:8 received intravenous acyclovir for 14 days (day T-1 through T+12). Time intervals were calculated from day 0, the day of marrow transplantation. The Eastern Cooperative Oncology Group (ECOG) criteria of response and toxicity were used to grade antitumor effect and organ system damage, respectively (except for hematologic dysfunction) (29). Frozen bone marrow was transported to the bedside in a liquid nitrogen-containing vessel for reinfusion 2 days after the final dose of chemotherapy. The marrow was thawed in a 37° C water bath and directly injected through a central venous catheter over 10 minutes without using blood filters.

The treatment schema is shown in Table 1. Patients received intravenous hydration with 0.9% saline beginning 12 hours before the first dose of

Table 1. Treatment Schema for High-Dose Etoposide, High-Dose Cisplatin, and Autologous Marrow Reinfusion

Agent	Day								
	-7	-6	-5	-4	-3	-2	-1	0	+1
Continuous saline infusion i.v.	x	x	x	x	x	x	x		
Cisplatin 40 mg/m²/day i.v. × 5 days		x	x	x	x	x			
Mannitol 25 g i.v.		x	x	x	x	x			
Etoposide[a] 600 mg/m²/day i.v. via syringe pump × 3 days		x	x	x					
Autologous bone marrow infusion								x	

[a] *Starting dose etoposide increased stepwise.*

cisplatin and continuing until 24 hours after the final dose. Cisplatin, 250 ml in 3% saline, was infused intravenously over 3 hours, followed immediately by 25 g mannitol. Cisplatin was administered at a dosage of 40 mg/m²/day for 5 consecutive days, or a total dosage of 200 mg/m² (days T-6 through T-2).

Etoposide was drawn into a syringe and infused directly into a central venous catheter using an infusion syringe pump (30). Etoposide (1800 mg/m²) was chosen as the initial dose. One third of the total dose, or 600 mg/m²/day, was administered each day for 3 consecutive days (days T-6 through T-4). The total dose of etoposide in mg/m², therefore, is the sum of the 3 consecutive daily doses. Subsequently escalated doses were 2400, 3000, and 3600 mg/m². Groups of at least four patients were entered at each dose level. Patients then were observed for signs of toxicity for a minimum of 4 weeks.

RESULTS

Evaluation of etoposide at four dose levels (1800, 2400, 3000, and 3600 mg/m²) has been completed; the maximum tolerated dose has not yet been reached (Table 2). Three of 15 patients died within 3 weeks of transplant, two owing to tumor progression and infection (despite normal recovery of WBC and polymorphonuclear cell counts) and one owing to infection alone. These three patients are inevaluable for antitumor response.

Toxicities due to high-dose etoposide and high-dose cisplatin therapy consisted of nausea and vomiting (in almost half the patients) but were mild to moderate and well controlled by antiemetics. A moderate to severe degree of alopecia occurred in most patients. Three patients had profound high-frequency hearing loss, which has persisted for several months, probably as a result of cisplatin-induced acoustic nerve damage. Fever, chills, metabolic

Table 2. Antitumor Results at Each Dose Level

No. of Courses	Etoposide Total Dose (mg/m^2)	Cisplatin Total Dose (mg/m^2)	Response				
			CR	PR	NR	TP	ED
6	1800	200		2	2		2
5	2400	200	1	2		1	1
4	3000	200		3	1		
3	3600	200	1		2		
Total 18			2	7	5	1	3

Abbreviations: CR, complete remission; PR, partial remission; NR, no response; TP, tumor progression; ED, early death.

acidosis, disorientation, peripheral neuropathy, and hypotension, toxicities noted by other investigators who have used these agents, were not observed.

Other important side effects of this combination of drugs included renal insufficiency, mucositis, diarrhea, and myelosuppression. Three patients experienced an elevation in serum creatinine level and a decrease in the 24-hour creatinine clearance rate. This problem occurred 2 to 3 weeks after the chemotherapy was completed, at a time when two of these three patients had been receiving antibacterial agents (including tobramycin) intravenously for fever and neutropenia. Peak and trough serum tobramycin concentrations were within the normal range, but synergistic nephrotoxicity with cisplatin could not be excluded. Ceftazidime was substituted for tobramycin and the serum creatinine rates decreased from 6.7 to 1.1 mg/dl and from 6.7 to 1.7 mg/dl, respectively. In subsequent patients ceftazidime was substituted for the aminoglycoside antibacterial agents. One patient, however, developed an increased serum creatinine rate (2.1 mg/dl) that improved to 1.3 mg/dl over time. Mucositis (grade 2) developed in only two patients. Diarrhea, ECOG grade 1 or 2, occurred in all seven patients who received 3000 or 3600 mg/m^2 etoposide.

Myelosuppression was prominent but of moderate duration in this study. One patient treated at the etoposide 1800 mg/m^2 dose did not become neutropenic or thrombocytopenic, while the peripheral blood counts of one patient in this dose group recovered within 10 days after transplant. For the entire patient population, peripheral blood neutrophil recovery (> 500/μl) occurred a median of 15 days (range, 10-23 days) after marrow reinfusion; platelet count in excess of 20,000/μl untransfused occurred 5-23 days (median, 15 days) after transplant.

Objective tumor regressions occurred in nine patients (Table 2). Two patients had complete remissions, one a woman with refractory small cell carcinoma (duration 5 months) and one a man with large cell lung cancer (duration 2+ months). Partial tumor responses were noted in seven other

patients (six with adenocarcinoma and one with large cell carcinoma); these responses lasted from 2 to 5 months.

DISCUSSION

The early results of this ongoing phase I trial are interesting, since we have shown that extraordinarily high doses of etoposide can be given safely, along with high-dose cisplatin, in association with autologous bone marrow reinfusion. Several groups showed that doses of etoposide can be augmented to 1500 mg/m^2 (single agent without bone marrow transplantation), but produce considerable myelosuppression and mucositis (16,17). Later, other investigators showed that further escalation was possible. Wolff and associates (14) administered up to 2700 mg/m^2 when autologous marrow was transplanted. Postmus and coworkers (15) infused patients with 3500 mg/m^2 over 3 days and reported the major toxicity was severe mucositis and that marrow injury was completely reversible. Adding other agents to etoposide, however, limited this approach. Sculier *et al.* (31) reported that patients with small cell lung cancer receiving late intensification with high-dose cyclophosphamide (200 mg/kg) and high-dose etoposide required autologous marrow reinfusion for etoposide doses 2000 mg/m^2 or greater. Their group did not continue to escalate the dose, since cardiac toxicity ensued, probably due to the effect of cyclophosphamide. In our study we have not yet reached the maximum tolerated dose of the combination of cisplatin and etoposide.

We have indirect evidence that marrow reinfusion is important to speed recovery of peripheral blood counts. Patients who received etoposide in lower doses (< 3000 mg/m^2) may not have needed marrow because of their more rapid recovery than that of those treated at the higher doses. In addition, cisplatin (200 mg/m^2) is associated with severe myeloid injury (32).

Toxicity with this regimen was quite acceptable. We did not observe the frequent renal and peripheral neurologic injury associated with cisplatin-containing regimens, probably because our therapy took place in only a single treatment rather than a series (33-37). We did, however, observe on audiograms the high-tone abnormalities that have been reported in patients receiving this type of cisplatin treatment.

Both in vitro and in vivo studies suggest a dose-response relationship for etoposide in several tumor types (38-41). More than half of the study patients evaluable for antitumor response (9 of 12) experienced a significant reduction in tumor size. Although only two of these responses were complete, the fact that a single intensive course of treatment was associated with a reduction in tumor size may be significant. In addition, etoposide in high doses has good central nervous system penetration that could be adapted for tumor prophylaxis or therapy (42). These favorable antitumor data as well as the acceptable toxicities of the regimen provide a strong

impetus to continue to explore the upper dose limit of etoposide. A confirmation of these encouraging results may be indicated through a phase II trial employing the final etoposide dose established in this phase I study, and perhaps using multiple courses of treatment in sequence with autologous marrow transplants.

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Lung Cancer

G. Spitzer and H. Lazarus, Chairmen

DR. W. "BILL" PETERS: As I recall from Steve Wolff's publications, the maximum tolerated dose (MTD) of VP-16-213 (etoposide) was about 2400 mg/m^2 in his studies. Is the difference you're seeing in the MTD related to the schedule or what?

DR. S. WOLFF: I was going to ask Hillard (Dr. Lazarus) how long the etoposide is infused by a syringe. Is it a rapid infusion or a prolonged, continuous infusion of etoposide?

DR. H. LAZARUS: The etoposide, as I indicated, is given over 4 hours. In response to Bill's question, as I recall you were at the microphone, Steve, your dose-limiting toxicity was 2400 because when you got to 2700 many of the patients had what was termed *unacceptable mucositis* which, in retrospect, may well have been owed to things such as herpes simplex or other types of infection. We have used acyclovir in the patients that are antibody positive and I think we may have resolved that problem. I don't really know that there is a ceiling for etoposide under those conditions. I think the data suggested we can continue to escalate the dose.

DR. PETERS: Have you looked at the pharmacy of the drug in the syringe? Does the drug stick to the walls or anything?

DR. LAZARUS: We've measured serum concentrations in several of these patients and they're very, very high. In fact, we sent some of the samples to Steve

to analyze. I think in a couple of them, the peak concentration was quite high. So I think that's a possibility, but fairly unlikely, I might add.

DR. WOLFF: I think that your data suggest that perhaps mucositis is a multifactorial process. I also think that when we did our study years ago we weren't quite aware of the prevalence of herpes simplex virus. I think that it is a possibility that we underestimated what the MTD dose is, especially since mucositis should probably never be an MTD unless it's a pan-mucositis, with the whole gastrointestinal system involved.

Gary, I just want to tell you what we're doing at Vanderbilt. Since you showed our slide I thought I would just tell you what our sequence is. We've developed a systematic approach to adding drugs. Our first drug was etoposide and we subsequently added cyclophosphamide and platinum. All the patients that you showed were extensive-stage patients, so what we've seen is with the combination of etoposide alone, etoposide plus Cytoxan, etoposide plus Cytoxan plus platinum, is that our latest complete response rate, which were all pathologically restaged, is now about 80% after two courses in extensive-stage small cell cancer patients. Our study, which is going on right now, is a randomization in limited-stage patients between two cycles of very high dose intense therapy. I also have to state that we have never used a bone marrow transplant in those patients so the doses that we're giving right now are without bone marrow transplant and those are 100/kg of Cytoxan, 1200/m^2 of etoposide, and 120/m^2 of platinum for two cycles as initial therapy and as a randomization for limited-stage patients between that therapy versus the same therapy, only at lower doses. We think that we're seeing some long-term disease-free survivors in extensive-stage patients and are quite anxious to move that therapy into a population that's going to benefit more from it. A fourth study that's currently going on in extensive-stage patients while they are in the hospital is the combination of induction therapy with intense weekly myelo-suppressive therapy. We're keeping the patients in the hospital for about 8 weeks straight, and what we're seeing right now is extremely rapid, complete responses 2 weeks after therapy.

DR. G. SPITZER: Steve, could I back you up a second? Tell us, in more detail, what the therapy with the extensive-disease patients is.

DR. WOLFF: As mentioned earlier, there is an 80% complete pathological response rate in extensive-stage patients. That therapy is now being tried upfront in limited-stage patients who have been randomized with conventional CEP. We're trying to show where there really is a meaningful dose-response relationship in a tumor group, limited-stage, that may potentially benefit from high-dose intensive therapy. And the next stage, which is the fourth, is to use that induction therapy with extensive-stage patients but add weekly marrow-toxic therapy for 8 weeks. I don't want to tell you of this study because it's just been started but in the first couple of patients, we've seen, at this time, all

complete responses within 2 weeks of beginning therapy in very extensive-disease patients.

DR. SPITZER: Steve, you've confused me on one point. The therapy in extensive disease is already myelotoxic. Are you then adding something on top of high-dose Cytoxan, etoposide, and platinum in extensive disease? Is that what you're saying? I think it's myelotoxic enough now, isn't it?

DR. WOLFF: Yes. The limitation for a high-dose intense therapy is that you have to wait 3-4 weeks before the next cycle of therapy. I think that you certainly get benefits from high-dose intense therapy but if you delay the intervals of therapy prohibitively long, I think you lose some of the effectiveness. We're essentially keeping patients in the hospital for about 8 weeks straight and giving them very intense therapy with weekly platinum, methotrexate, and vincristine, in the two induction therapies.

DR. SPITZER: So you've moved away from your original approach.

DR. WOLFF: No, no, we're adding to it. We're using the same induction therapy but adding weekly, further marrow-toxic therapy. We are really trying to clobber this extensive-stage disease. So far, the complete response rates are almost universal. So we think we're seeing a benefit, but it's too immature right now to make any comments except that in the previous study we are now seeing long-term, disease-free survivors in extensive-stage patients with brief therapy that we never saw before.

DR. SPITZER: Steve, the reason I showed the Vanderbilt data was to justify our addition of platinum to high-dose Cytoxan and etoposide. The addition of cisplatin at full dose, not necessarily escalated above outpatient setting, added significantly to the cytotoxic potential of the combination of Cytoxan and etoposide. However, I remember from the data on extensive-disease patients that there were some infectious deaths without marrow, particularly during the second course of therapy. The question of the necessity of marrow support will be important with this program. I think the high-dose Cytoxan, etoposide, and platinum combination will be used ultimately by a number of groups. So the marrow support question will be raised and I think it will be necessary, particularly if there is any prior chemotherapy.

DR. WOLFF: Right. I just should add one small caveat: in the extensive-stage patients that we're treating with a very heavily marrow-toxic therapy, this is a select group of patients. It's actually selected for large tumor bulk but excellent physiological performance data, because we certainly appreciate that many patients with small cell cancer are not very good performers. So this is a selected series as far as performance data. It's also selected to be negative as far as these patients have bulky tumor.

DR. SPITZER: I'd like to get some comments from Michel Symann and maybe Tom Frei. I hear that we're finally finding the combinations active in

small cell cancer just as the number of patients referred for this procedure is falling dramatically. We don't seem to be generating studies on defined groups of patients adequately to prove our points, but instead Hillard, who may have an interesting program, is investigating primarily relapsed patients. I'd like to hear where a number of people in this area are proceeding in a defined way, so that we may be able to come back aggressively into the field of small cell lung cancer and define a role for high-dose therapy with marrow support in it.

DR. E. FREI: Well, we think that with your observations, if you can get patients into complete remission by some kind of an induction regimen, that's the optimal time to intensify them, maybe times two. That to me remains, along with Dr. Symann's comparative experience, compelling evidence that you can do something. I think Steve Wolff's program for extensive disease certainly sounds like the way to go, but for a selected group of patients. Why do you think patients are not being referred? Certainly nobody else has a better approach to therapy that I'm aware of.

DR. SPITZER: There has been a lot of the negative studies of small groups of patients in extensive disease with minimally intensive therapy, which have recently been published with an associated number of confusing editorials. Somehow we need to try even more strongly to define studies which may be more convincing.

DR. LAZARUS: I might ask Dr. Symann about the monoclonal antibody studies for the detection of tumor in the bone marrow. There have been a number of investigators, including the National Cancer Institute group and Ruff and Pert, who've shown that small cell lung cancer is perhaps more analogous to a hematologic malignancy in that it shares antigens with cells like macrophages and monocytes. In their hands and in other people's hands, the number of surface antigens that it shared, for example, with squamous and adeno, was less, and I just wondered about the choice. If you couldn't increase your sensitivity by using other antibodies, then the others that you had shown that were, I guess, LCA1, LCA2, and LCA3 were either derived from or reacted with squamous cell.

DR. M. SYMANN: The antibodies I was referring to are not useful for diagnostic purposes because they cross-react with squamous as well as with oat cell carcinoma of the lung. Nevertheless, they do not recognize normal hematopoietic cells. For instance, I didn't show the results, but in a batch of 50 normal bone marrow samples we were completely unable to show any positive cells. Probably, three is not enough, anyway, looking at the heterogeneity of antigenic expression. The more we search, the more we find. Probably if we could increase the number we could detect more positive bone marrows. Now, with respect to the findings we may derive from that, when we looked at the bone marrow we collected for autologous graft purposes, even if the sample was negative on the small samples, like a 2- or 3-ml aspirate, before bone marrow

collection for grafting—when we went back to examine samples from the 1 liter we did collect, we were again able to find positive cells. So I think that in this kind of disease, we have probably more than 80% of patients with some positive cells in the bone marrow.

DR. A. ELIAS: I want to agree with Gary concerning the lack of referrals for small cell lung cancer. At the Dana-Farber Cancer Institute we are attempting an induction-phase protocol followed by high-dose combined alkylating agents for consolidation in small cell lung cancer. I would say that the likelihood is small that most practitioners are impressed by the short-term favorable responses in small cell lung cancer. I think that's one reason why we're not seeing patients, because there are plenty of patients out there who are suitable.

DR. SPITZER: Yes, I think basically if the data coming out of Vanderbilt are correct and our preliminary data on the Cytoxan, etoposide, and platinum in small cell lung cancer are true, we're going to have to possibly pool our resources to try to get a definitive study done in limited-disease oat cell. Michel, I just wanted to ask you one question. What is the difference in these antibodies that you're reporting and the panel that was reported by Sam Bernall of Dana-Farber Cancer Institute on the detection of small cell lung cancer and relative frequency of upgrading the extent in these studies versus yours?

DR. SYMANN: Referring to the Dana-Farber study, because we used three monoclonal antibodies, a number of limited-disease patients became extensive disease because bone marrow was involved. All monoclonal antibodies were not as selective as the SM1 from Dana-Farber Cancer Institute because this one was able to discriminate between squamous and oat cell; ours were not. Just thinking about your concern about the decrease of your referral, in Europe we have a different problem. Lung cancer is usually seen first by pulmonary specialists and the problem is those people don't like to send their patients to oncologists because they feel they are losing them. So, the struggle is just at the beginning of the disease.

DR. FREI: Dr. Lazarus, as far as I understand it, you had six patients with previously treated unresectable non-small cell lung cancer, right? Did you see any partial or complete response in these patients?

DR. LAZARUS: Yes, there were several responses in patients that were previously treated.

DR. FREI: Previously refractory to standard regimen?

DR. LAZARUS: Yes, that's right. I'll have to get the data for you later. I think there were two patients.

VI. Melanoma/Sarcoma/Carcinoma

Treatment of Advanced Melanoma With High-Dose Chemotherapy and Autologous Bone Marrow Transplantation

R. H. Herzig, S. N. Wolff, J. W. Fay, C. F. LeMaistre, D. Frei-Lahr, J. N. Lowder, G. L. Phillips, and G. P. Herzig

The treatment of metastatic malignant melanoma is quite difficult because of the resistance to chemotherapy. A large number of agents have been studied, with DTIC (dacarbazine) most extensively evaluated. The response rate (complete and partial) in patients receiving dacarbazine alone or in combination is about 20% (1,2). We have explored the use of autologous bone marrow to permit dose intensification of alkylating agents with the anticipation of improving the response in patients with metastatic malignant melanoma. The results of our phase I and II studies include the single-agent studies of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (or carmustine), melphalan, and triethylenethiophosphoramide (thio-TEPA); also included is the study of the combination of carmustine and melphalan.

MATERIALS AND METHODS

Patients

Patients eligible for treatment had metastatic melanoma and met the requirements of participation in our phase I and II trials of intensive

chemotherapy and autologous marrow rescue. Informed consent, approved at each institution, was obtained before beginning treatment.

Marrow Processing

Before high-dose therapy was administered, marrow was collected and cryopreserved by standard methods (3). Histology was normal and the marrow was collected a minimum of 4 weeks after previous chemotherapy. It was then cryopreserved in dimethyl sulfoxide and kept at −196° C in the liquid phase of liquid nitrogen. Three to four days after completing chemotherapy, the marrow was rapidly reinfused intravenously.

Chemotherapy

Dose escalation of the chemotherapy was accomplished by a modified Fibonacci scheme. During the single-agent studies, the total dose was administered over 3 days and given intravenously over 2 hours (carmustine, thio-TEPA) or by rapid intravenous bolus administration (melphalan). When the combination of carmustine and melphalan was given, the starting dose was 50% of the maximally tolerated dose determined in the respective phase I single-agent study. Each drug was escalated singly, holding the other drug dose constant. The melphalan was administered for the first 3 days followed by carmustine on the subsequent 3 days.

Evaluation of Response

Responses were defined using standard criteria. Complete response (CR) was the complete disappearance of all measurable disease for more than 1 month; partial response (PR) represented a more than 50% reduction of measurable disease; any response less than partial was considered no response (NR). Duration of response was calculated from the day of marrow infusion. The results of our phase I and II studies have been previously reported (4-8) and have been updated. For statistical comparisons, the confidence interval method described by Simon, Fisher's exact test, or chi-square method were used (9).

RESULTS

The results of treating advanced melanoma in patients with maximally tolerated doses of carmustine, melphalan, or thio-TEPA are presented in Table 1. These doses were determined to be the maximally tolerated doses in the phase I trials. In the case of thio-TEPA, there was a dose-response relation noted at 900 mg/m^2, with a significantly higher response in patients receiving more than 990 mg/m^2 compared with patients who received 180-720 mg/m^2. The overall response rates for melphalan and thio-TEPA were better than for carmustine. The lower response rate for carmustine can be

Table 1. Three Drugs and Autologous Bone Marrow Transplantation for Advanced Melanoma

Drug[a]	No. of Patients	Response		
		CR (%)	PR (%)	Total (%)
Carmustine				
Prior Rx	15	0	2 (13)	2 (13)
No prior Rx	16	4 (25)	8 (50)	12 (75)
Total	31	4 (13)	10 (32)	14 (45)
Melphalan				
Prior Rx	16	3 (19)	7 (44)	10 (63)
No prior Rx	10	3 (30)	5 (50)	8 (80)
Total	26	6 (23)	12 (46)	18 (69)
Thio-TEPA				
Prior Rx	12	1 (8)	6 (50)	7 (58)
No prior Rx	15	3 (20)	7 (47)	10 (67)
Total	27	4 (15)	13 (48)	17 (63)

[a] *Carmustine, 1200 mg/m²; melphalan, 180–225 mg/m²; thio-TEPA, 900–1575 mg/m².*

Note: Prior Rx is for patients who received prior therapy before autografting; no prior Rx is for patients who received no therapy before autografting.

accounted for by the significantly poorer response in patients who had received previous therapy (13% for carmustine compared to 63% and 58% for melphalan and thio-TEPA, respectively, $P < .02$). In patients who had not received previous therapy before autografting, there were excellent responses with all three agents. The effect of the extent of metastases in which patients with visceral metastases are compared with patients with skin and/or lymph node disease (see Table 2).

The response rates for high-dose carmustine (45%), melphalan (69%), and thio-TEPA (63%) are compared with the responses seen with each of these agents used in conventional doses for advanced melanoma (Table 3). In each instance, the response is significantly greater for the high dose with autografting ($P < .05$ by confidence interval). The median duration of response was similar for all three agents: carmustine, 6 months (range, 2-46+ months); melphalan, 4 months (range, 2-14 months); thio-TEPA, 4 months (range, 2-15+ months). However, twice as many patients treated with carmustine had unmaintained responses greater than 1 year compared with patients who received melphalan (15% versus 8%). Patients who received thio-TEPA have been most recently treated, with seven of 17 patients still alive and only one more than 1 year.

Since there were no overlapping toxicities between the high-dose melphalan and carmustine studies, and since both agents demonstrated

Table 2. Effect of Extent of Metastatic Disease on the Outcome of Drugs With Autologous Bone Marrow Transplantation for Patients With Advanced Melanoma

Drug and Extent of Metastases[a]	No. of Patients	Response		
		CR (%)	PR (%)	Total (%)
Carmustine				
Skin ± lymph node	5	1 (20)	2 (40)	3 (60)
Visceral	26	3 (12)	8 (31)	11 (42)
Melphalan				
Skin ± lymph node	2	1 (50)	0	1 (50)
Visceral	24	5 (21)	12 (50)	17 (71)
Thio-TEPA				
Skin ± lymph node	7	4 (57)	2 (29)	6 (86)
Visceral	20	0	11 (55)	11 (55)

Abbreviations: CR, complete response; PR, partial response.

[a]*Skin ± lymph node: skin and/or lymph node metastases only; visceral: visceral organ involvement with or without skin and lymph node involvement.*

Table 3. Conventional-Dose versus High-Dose Therapy for Advanced Melanoma

Drug	No. of Patients	Response Rate (%)	95% Confidence Interval
Carmustine[a]	110	15	10–23
Carmustine/ABMT[b]	31	45	29–62
Melphalan[a]	24	17	7–36
Melphalan/ABMT[b]	26	69	50–83
Thio-TEPA[a]	55	16	9–28
Thio-TEPA/ABMT[b]	27	63	44–78

Abbreviations: ABMT, autologous bone marrow transplantation; thio-TEPA, triethylenethiophosphoramide.

[a]*Conventional dose.*

[b]*High-dose with autologous bone marrow transplantation.*

significant antitumor responses (with a somewhat better response rate with melphalan and a possibly improved duration of response with carmustine), we performed a phase I dose-escalation study of both agents in combination for patients with metastatic melanoma (7). We began at 50% of the maximal doses from the previous studies: melphalan (90 mg/m^2) and carmustine (600 mg/m^2). Four dose escalations (five treatment levels) were accomplished: melphalan (135 mg/m^2) and carmustine (600 mg/m^2); melphalan (135 mg/m^2) and carmustine (900 mg/m^2); melphalan (180 mg/m^2) and carmustine (900 mg/m^2); melphalan (180 mg/m^2) and carmustine (1200

mg/m^2). The pattern of marrow recovery (granulocytes >500/μl and platelets >20,000/μl untransfused) were similar to each agent alone. Increased toxic reactions (e.g., pulmonary [noninfectious diffuse interstitial pneumonitis and adult respiratory distress syndrome] and gastrointestinal [diarrhea]) were significantly greater at the highest level (100% of both agents) compared to the previous level (100% melphalan [180 mg/m^2], 75% carmustine [900 mg/m^2]) or to any lower level. The pulmonary toxicity of melphalan (180 μg/m^2) and carmustine (1200 mg/m^2) was significantly greater than that observed with carmustine alone at 1200 mg/m^2, thus indicating the potential for additive toxicity.

The response rate showed a trend for improved responses with increasing doses, but too few patients were entered at each level to be statistically significant (Table 4). The extent of disease (skin and/or lymph node involvement versus visceral organ involvement) was not different than that seen with each agent alone: 4 out of 10 patients (40%) with skin and/or lymph node but with no CRs, and 30 out of 48 (62%) with visceral organ involvement (including all six CRs). The response rate was not statistically different from melphalan alone, but was marginally better than carmustine alone (P = .08, Fisher's exact test). At the maximally tolerated dose (180 mg/m^2 of melphalan and 900 mg/m^2 of carmustine), the response rate was identical to thio-TEPA. The duration of response, however, resembled carmustine with a median duration of 5 months (range, 2-30+ months), with 15% greater than 1 year.

SUMMARY

The results of these studies suggest that autologous bone marrow transplantation will permit moderate increases in doses sufficient to obtain responses where conventional doses fail. Although increased response rates

Table 4. High-Dose Melphalan and Carmustine Combination and Autologous Bone Marrow Transplantation for Patients With Advanced Melanoma

Melphalan/Carmustine (mg/m^2)	No. of Patients	Response		
		CR (%)	PR (%)	Total (%)
90/600	9	1 (11)	3 (33)	4 (44)
135/600	7	2 (29)	2 (29)	4 (59)
135/900	5	1 (20)	3 (60)	4 (80)
180/900	27	2 (7)	14 (52)	16 (59)
180/1200	10	0	6 (60)	6 (60)
Total	58	6 (10)	28 (48)	34 (59)

Abbreviations: CR, complete response; PR, partial response.

with either single agents or in combination were observed, the duration of the responses were generally of short duration (only about 10% > 1 year). Possible solutions to improve the duration of response include multiple courses of high-dose therapy and/or other combinations of agents. Multiple courses of therapy have been tried in few patients without increased toxicity. No conclusions can be drawn with the small number of patients treated. The results of other combinations have not been shown to be superior (10; K. Antman, personal communication).

A more practical approach would be to extend this type of therapy to patients with less extensive disease, but with a poor prognosis for cure. For example, adjuvant therapy for patients with stage II melanoma might be such a group of patients. While conventional therapy has not been shown to be of benefit in the adjuvant setting, it also has not shown significant benefit in patients with advanced disease. One of the prerequisites of adjuvant therapy is a demonstrated effectiveness in the patients with advanced disease. Since high-dose regimens appear to be effective in advanced disease, certain high-risk stage II patients might particularly benefit from adjuvant high-dose therapy and autografting.

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Melanoma/Sarcoma/Carcinoma

R. Benjamin and R. Herzig, Chairmen

Dr. K. Dicke: Since the toxicities might be overlapping, why would you choose melphalan and thio-TEPA as opposed to BCNU?

Dr. J. Miser: Well, the main reason for choosing it would be that we know that melphalan and thio-TEPA clearly have a lot of activity. With BCNU it's not as certain because so few patients have been treated. I think you could reasonably ask it with either of those. If we get into trouble with a combination in terms of not being able to use reasonably large doses of the two, then I think that a combination of either of those with BCNU would make sense. There is some information that you already saw for melanoma, we know what the maximum tolerated dose of melphalan and BCNU is in combination and I think there's good reason to think that BCNU and thio-TEPA would be tolerated as well. I think those are reasonable combinations.

Dr. G. Spitzer: I wonder if any of the speakers, particularly Geoff (Dr. G. Herzig), would comment on the rate of tumor regression. We've heard of this in the past for breast cancer.

Dr. G. Herzig: In the case of colon cancer, many of the responses are relatively slow, that is, it's not usual to see a response. I think the earliest might be at about a month posttreatment. Some of them continue to show signs of regression for another month after that.

Dr. Dicke: I have a question for Geoff. Since the prognosis of colon

cancer is so bad, why don't you use a combination of BCNU and melphalan, which has been worked out already, for instance, in Duke-C disease? The reason is that in this minimal residual disease setting, the prognosis is poor.

DR. G. HERZIG: I think it's a reasonable study to perform. However, we have to convince the surgeons, first of all, that that's a reasonable study to do because they're the ones who see those patients. Maybe if we can get some publicity for the responses, we'll be able to convince some of them that this is the thing to do.

DR. R. PINKERTON: I'd like to ask Dr. Miser a question. We, too, have rather disappointing experiences with high-dose melphalan and single-fraction total body irradiation (TBI) in both stage IV rhabdomyosarcoma and high-risk Ewing's sarcoma. I just wondered what sort of ideas for future combination strategies he had in mind in view of the apparent inefficacy of the combination of high-dose melphalan and TBI in these diseases.

DR. MISER: You noticed I skirted that issue. We got remission with melphalan but of short duration; also, you don't get durable response rates in relapsed patients. I think that relates to some of the other factors that I talked about—the amount of disease and the resistance of the disease to radiation and chemotherapy. It's possible that there will be a better long-term response rate with high-dose melphalan and TBI in first remission. That really hasn't been looked at in a big population. But, again, I'm not highly optimistic about it. I think it is actually time now to look at some other therapies in Ewing's sarcoma, high-dose chemotherapy that is active in the disease, maybe VP-16-213 (etoposide). I think high-dose etoposide with transplant might be effective. That's one area that is going to require some phase I and phase II studies. The second may be ifosfamide. Ifosfamide is a very active drug; therefore, ifosfamide and etoposide may be a very active drug combination in those diseases. High-dose ifosfamide has not been looked at. There are other toxicities, however, of high-dose ifosfamide to the central nervous system and renal disease that may intervene. I think those are two possible strategies.

DR. SPITZER: A question for Dr. Miser. Would you comment on the relapse pattern in your patients? You didn't mention that and that may be of major importance.

DR. MISER: I'm sorry, I didn't mention that. In fact, the number of patients with bone marrow disease at diagnosis was approximately 15. The relapse pattern, in fact, was not that of the pattern you might expect, whatever that might be, of reinfused bone marrow. We did not see widespread metastatic disease in the lung, and we did not see widespread bone marrow relapse. They were generally isolated relapses in one or two sites. The local relapse rate was very, very low. I mean with local relapse, a relapse which occurs at places where there is metastatic disease or at the primary site of the disease. Those were not the primary problems. The primary problems were

relapses in bulk sites and other sites, generally solitary. I don't feel we reinfused a tumor, at least we don't have evidence of that.

DR. E. FREI: Roger (Dr. R. Herzig), this is a question for you. When one uses agents in combination, there are obviously many variables to deal with. Very often, for cells in culture the cells have to see both drugs together, and not necessarily in sequence. And I noted that when you put BCNU and phenylalanine mustard together you gave the first for 3 days and the second for the next 3 days. So, in fact, neither the host, probably, and certainly not the tumor, saw the two drugs at the same time. I don't have a good answer for this myself. I realize there are a lot of practicalities in those strategies. But I wonder if when you do that you have truly tested the potential for synergism between the two. Why did you do that, for example, rather than put them together concurrently?

DR. R. HERZIG: Well, partly because we were embarking on our first trial in putting them together and were worried about synergistic toxicity as well as potential synergistic antitumor effect, particularly with liver. So we spaced them out, giving them sequentially one after the other, or changing the order.

DR. DICKE: Dr. Miser, on what kind of data do you base that etoposide is an effective drug?

DR. MISER: If you look at etoposide in spindle cell sarcomas, the response rate is very low. If you look at etoposide as a single agent in small round cell sarcomas, the response rate across the board is about 20-30%. If you look at it in combination with ifosfamide, the response rate and also the relapse rate in Ewing's sarcoma approach 100%. If you look at it in combination with ifosfamide in rhabdomyosarcoma, it approaches 80%, and it's on the basis of standard doses, 500 mg/m^2 of etoposide per course and 9 g/m^2 of ifosfamide per course. I think there may be some advantage to using those two high-dose drugs together.

DR. DICKE: I think that there might be a dose response for etoposide, at least in Ewing's sarcoma.

DR. R. HERZIG: I think that's as much my opinion as it is based on documented fact and literature, but I would hold to that opinion, yes.

DR. DICKE: But, besides the combination of chemotherapy, which has been designed by Norman Jaffe, who else has demonstrated a dose response, and why are the responses still so poor with high-dose chemotherapy?

DR. R. HERZIG: The standard response rate of the intergroup rhabdomyosarcoma trial and the intergroup Ewing's sarcoma trial with patients with metastatic disease is approximately 50-60%. If you look at the Boston Ewing's trial in which they used high-dose vincristine, Adriamycin, and Cytoxan like mine, and if you look at my trial, our response rate in Ewing's

sarcoma is 100%, the response rate in rhabdo is about 90%. Although we didn't do it in a randomized fashion, I think there is in fact a dose-response curve in induction.

DR. DICKE: Right, but your long-term survival rate is only 20%.

DR. R. HERZIG: The long-term survival rate of the Ewing's sarcoma patients is about 50%. Now, if you look at metastatic patients with Ewing's sarcoma in the published trials, their survival rate is 30%, but if you look at those patients, a lot of them had isolated metastatic disease in the lungs, which yields reasonable response rates and reasonable durations of response. If you look at patients with bone and bone marrow disease, they have almost uniformly died. The majority of patients in our trial had bone and bone marrow disease, and they are doing better than in previous trials, but I don't know that it's worth jumping to.

DR. DICKE: Do you think it is already time to do a randomized trial between a high-dose regimen versus normal-dose chemotherapy in one of the sarcomas?

DR. R. HERZIG: No, I guess that was my final sentence. I don't think we ought to use this strategy without a little more development. Is 50% good enough in that group before trying to come up with a better regimen? If you had a high-dose regimen to randomize, what high-dose regimen would it be? I'd like a better one before we did the randomized trial.

VII. Brain Cancer

Intensive Monochemotherapy With Carmustine and Autologous Bone Marrow Transplantation for Malignant Glioma

Gordon L. Phillips, Joseph W. Fay, Geoffrey P. Herzig, Roger H. Herzig, Hillard M. Lazarus, and Steven N. Wolff

Despite multimodal therapy, patients with high-grade malignant gliomas remain a therapeutic challenge. The median survival of patients with such tumors is only about 12 months, with very few long-term survivors (1). Despite the localized nature of these tumors, advanced techniques of surgery and radiotherapy have not been effective in their eradication (2,3). Nor has chemotherapy been very successful, probably because of the intrinsic heterogeneity of gliomas, the pharmacologic features of the central nervous system, and poor host tolerance of the most active agents, the chloroethyl nitrosoureas, especially carmustine (BCNU) (4,5). Nevertheless, it is likely that future therapeutic advances must be derived from drugs, as major progress with local therapies seems improbable.

Bone marrow rescue allows the use of augmented-dose chemotherapy (6). While probably important in the treatment of many cancers, such therapy may be particularly important in the malignant gliomas, for which the dose of chloroethyl nitrosoureas is initially limited by myelosuppression. This fact, plus the virtual absence of marrow involvement in the gliomas, has led to several

studies in which marrow rescue was used to permit augmented chemotherapy with carmustine (7). A major criticism of these studies is the exposure of the patient to systemic toxicity for the treatment of a localized tumor.

In 1978, we began a phase I study of escalating-dose carmustine and autologous bone marrow transplantation (ABMT) in patients with refractory cancer (8). During this study, we observed striking antitumor effects in some patients with progressive glioblastoma. We therefore initiated a phase II study of the maximum dose of carmustine we then believed tolerable with bone marrow transplantation (1200 mg/m^2) in patients with progressive malignant glioma. Subsequently, we evaluated this therapy with slightly lower carmustine doses, after further evaluating the 1200 mg/m^2 dose as adjuvant therapy in 18 patients before disease progression.

METHODS

The patients' characteristics are listed in Table 1. Altogether, 45 patients were treated; 27 had progressive disease, and the 18 who did not were treated in

Table 1. Patient Characteristics

Characteristic	Progressive Disease Group	Adjuvant Therapy Group
Number	27	18
Age (years)		
Median	39	34
Range	8–68	23–67
Histology		
Glioblastoma	20	15
Anaplastic astrocytoma	7	3
Previous therapy		
Surgery	27	18
Radiotherapy	27	18
Chemotherapy	3	2
Interval between diagnosis and carmustine (months)		
Median	7	3
Range	0–60	3–12
Performance status		
0–1	13	18
2–4	14	0
Carmustine dose (mg/m^2)		
900	—	2
1050	6	14
1200	19	2
1350	2	—

an adjuvant fashion. All patients had received maximal local therapy, but few had been given conventional chemotherapy. Marrow was harvested and cryopreserved shortly before therapy in all patients. Forty-three patients received 900-1200 mg/m^2 of carmustine in divided doses over 3 days; one patient also underwent ABMT at that time. Two patients each received 600 and 750 mg/m^2 of carmustine (total, 1350 mg/m^2) with two transplantations (the total marrow was obtained at one pretherapy harvest) early in the phase I study. A minimum of 1×10^8 nucleated cells per kg of body weight was frozen, then thawed and infused 3-7 days after the carmustine therapy. Patients were followed up with neurologic examinations and CT scans at 3-month intervals.

The results were analyzed as of 31 December 1986.

RESULTS

Antitumor Effects

Our initial study of the progressive disease group included some patients who were treated during the phase I study. Twelve of these 27 patients (44%) responded. Four patients survived 12 months or more after treatment, including two who had progression-free intervals of more than 48 months, one of whom is healthy 102 months later. The median survival was 3 months (range, 1-102+ months). The corrected progression rate was 95%.

All but two of the patients treated in an adjuvant fashion received carmustine within 3 months of surgery. In general, slightly lower doses of carmustine were used after cases of pulmonary toxicity were noted at the 1200 mg/m^2 dose. Four patients are alive, all more than 24 months after carmustine therapy. Two remain free of progression at 63 and 80 months. The median survival in this group was 18 months (range, 4-80+ months). The corrected progression rate was 86%.

Figure 1 details overall survival in both groups.

Toxicity

Although hematologic toxicity was manageable, nonhematologic toxicity was severe. Nine patients died of pulmonary (n = 6), hepatic (n = 2), or central nervous system (n = 1) toxicity, an overall toxic death rate of 20%. We were unable to identify factors including carmustine dose, that predicted toxic death. (Because of the fairly narrow dose range, this was not particularly surprising.) In addition, one of the adjuvant therapy patients who is progression-free at 80+ months has progressive neurologic deterioration of unknown etiology, as did one of the long-term survivors in the progressive disease group some years before her death (she eventually died of glioma).

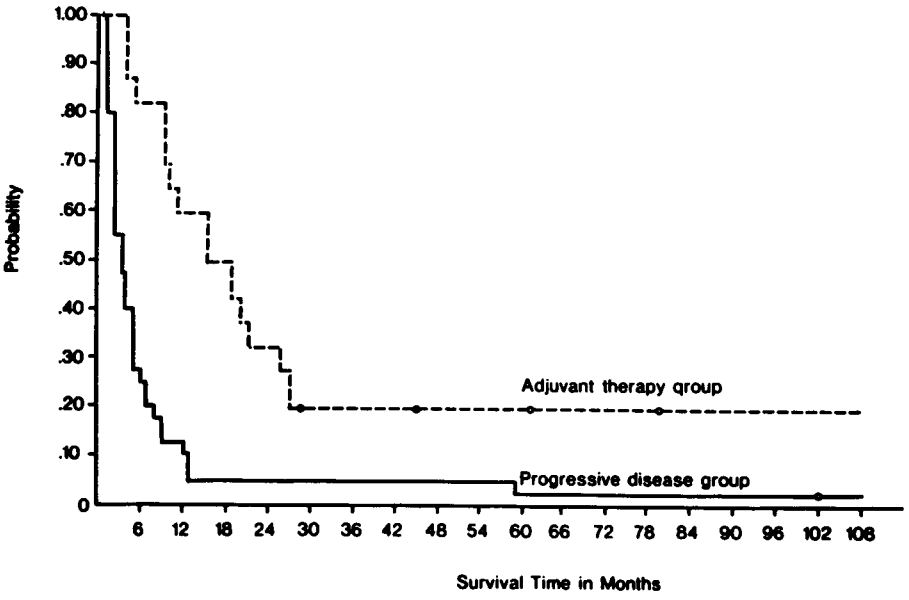

Figure 1. *Survival of patients in adjuvant therapy and progressive disease groups. Open circles indicate living, progression-free patients; closed circles indicate living patients with evidence of progression.*

DISCUSSION

The responses noted with high-dose carmustine and ABMT, particularly among the long survivors, are unexpected with conventional therapy. Moreover, although these results are modest, they are similar to those obtained in early studies using allogeneic bone marrow transplantation for patients with refractory hematologic malignancy (9). Nevertheless, our results are disappointing in two ways. First, even patients treated before progression had a high progression rate; second, toxicity was excessive.

Concerning future studies, several approaches are possible. Patients may be treated even earlier than those in our adjuvant therapy study, perhaps in a "neoadjuvant" condition. Also, it is probably naive to expect single-agent therapy, regardless of dose, to reliably cure clinically apparent malignancy (10), and the addition of other agents known to penetrate the central nervous system (e.g., AZQ [aziridinylbenzoquinone], thio-TEPA, VP-16-213) is indicated.

Efforts to decrease the toxicity of high-dose carmustine with ABMT are essential. Since it is difficult to avoid the conclusion that the dose rates of carmustine must be reduced to be lower than those we used, it is likely that earlier therapy with carmustine plus other agents will be required to improve results.

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Pilot Study of High-Dose Carmustine, Transplantation, Radiotherapy, and Surgery for Patients With Glioma

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High-grade gliomas of the brain (anaplastic astrocytoma and glioblastoma multiforme) are the most frequently occurring primary CNS tumors. Despite conventional treatment including surgical resection, postoperative irradiation, and adjuvant chemotherapy, the prognosis has remained very poor (1). Postoperative irradiation had increased overall survival rates significantly (2). Attempts to improve these results by using adjuvant mono- or polychemotherapy improved neither response rate nor survival (3-5). The lack of activity of chemotherapy in such tumors is explained not only by their low chemosensitivity but also by low penetration of the drugs into the CNS. One way to overcome the problem of the so-called blood-brain barrier is to use the dose-effect relationship and especially high doses of chloroethyl nitrosoureas, such as carmustine (BCNU), which has been proved to be efficient at conventional dosage (3).

Since 1980 (6), many reports have been published on escalation of dose for gliomas (7-10). The objective of our pilot study is to evaluate the feasibility, efficiency, and toxicity of a therapeutic program for patients with

high-grade gliomas, combining 1) cytoreductive surgery, 2) early postoperative chemotherapy, 3) early intensification with high-dose carmustine followed by autologous bone marrow transplantation (ABMT), and 4) whole-brain irradiation followed by a local boost to the tumor bed.

As is shown in Table 1, this whole protocol was scheduled over a period of 2 months with the objectives of reducing hospitalization time for these patients with poor prognoses and beginning radiation therapy after the conventional period of postoperative delay.

PATIENTS AND METHODS

Between March and September 1986, 15 patients entered the study (Table 2). Diagnoses were established by arteriographic and, for two patients, by CT scan criteria. Among the 13 others, 9 patients had a grade 4 astrocytoma or glioblastoma multiforme and 4 patients had a grade 3 astrocytoma. Thirteen patients were treated during the initial phase of the disease, and two patients were treated at relapse.

Initial surgical procedures were biopsy for four patients, partial resection for three patients, complete resection for five patients, and no biopsy for three patients (two patients already mentioned and one patient in relapse). Furthermore, four patients underwent a second surgical procedure after high-dose carmustine therapy (three complete resections and one partial resection).

Table 1. Treatment Protocols

Time	Procedure	Dosage
Strategy 1		
Day 0	Surgery	
Day 3	Teniposide (VM-26)	150 mg/m^2
	Carmustine	100 mg/m^2
Days 3–10	Procarbazine	100 mg/m^2
Day 21	Marrow harvesting high-dose carmustine	800 mg/m^2
Day 24	Marrow infusion	
Days 45–63	Radiotherapy	45 Gy
Strategy 2		
Day 0	Biopsy	
Days 3–10	Marrow harvesting high-dose carmustine	800 mg/m^2
Days 6–13	Marrow infusion	
Day 30	Surgery if indicated	
Day 33	Teniposide	150 mg/m^2
	Carmustine	100 mg/m^2
	Procarbazine	100 mg/m^2
Days 45–63	Radiotherapy	45 Gy

Table 2. Patients' Characteristics

Number		15
Age in years	Median	50.9
	Range	27–64
Gender	Women	10
	Men	5
Performance status		
World Health Organization scale	Grade 1	9
	Grade 2	5
	Grade 3	1
Histologic tumor type	Astrocytoma 3	4
	Astrocytoma 4 or glioblastoma	9

Depending on the feasibility or infeasibility of surgery, two treatment strategies were used (Table 1).

Strategy 1: Surgery Followed by Chemotherapy

Cytoreductive surgery was performed on six patients on day 0. The first course of chemotherapy was administered on day 3 after histologic confirmation. The drugs (Table 1) were administered in the following fashion: teniposide, diluted in 500 ml of 5% dextrose, over an 8-hour infusion; carmustine, in a 5-minute intravenous short infusion through a peripheral venous access; procarbazine, per os.

The autologous bone marrow harvesting was scheduled between days 21 and 24, as previously reported (11), with 1×10^8 mononuclear cells/kg harvested, and a buffy coat performed to reduce the volume to 100–150 ml. Then the buffy coat was mixed with 4% albumin in a 600-ml Travenol bag (Travenol Laboratories, Norfolk, England) and stored at approximately 8° C. On the afternoon of marrow harvest, the high-dose chemotherapy (800 mg/m^2 of carmustine) was administered diluted in 100 ml of 5% dextrose in a 2-hour infusion using a peripheral venous access. The marrow was centrifuged at 1000 *g* for 25 minutes, resuspended in 200 ml of saline with 4% albumin, and reinfused at body temperature 72 hours after carmustine administration. For radiation therapy, scheduled to begin on or about day 45, the total delivered dose was 45 Gy in 19 days, using the 18-MeV energy of a linear accelerator: 24 Gy in 8 fractions to the whole subtentorial brain, followed by a localized boost of 21 Gy in 7 fractions to the tumor or tumor bed. Every radiotherapy field was scheduled according to the dosimetry CT scan planning system.

Strategy 2: Chemotherapy Followed by Surgery

If tumorectomy was not possible at diagnosis (as was true for seven patients) or at relapse, a biopsy examination was performed. After that, patients obtained high-dose carmustine, followed by an operation if possible. Patients then received the "conventional" chemotherapy (teniposide, carmustine, procarbazine) and finally underwent irradiation.

Patients were treated in single rooms, without isolation procedures. The first four patients were hospitalized during the whole post-ABMT period, but supervision was quickly organized on an outpatient basis with a twice-weekly blood count, hepatic biologic assay, physical staging, and chest x ray.

Evaluation of the response of patients with gliomas remains difficult because the only way to monitor these patients is by CT scan. In this study, the overall survival rate, which provided a rapid answer because of these patients' poor prognosis, was chosen as an evaluation criterion. Measuring the patients' quality of life was also a major objective of the pilot study.

RESULTS

Toxicity

Carmustine and ABMT Toxicity

The toxicity effects of the whole protocol, especially those of high-dose carmustine (Table 3), show different aspects and degrees. The immediate tolerance after a high dose of carmustine with grade 2 nausea and vomiting, was moderate. This is partially because of the short, 6-hour delay between bone marrow harvesting under general anesthesia and the carmustine infusion.

Hematologic Toxicity

Hematologic toxicity was mild. Three patients had a severe neutrocytopenia (< 1000 WBC/mm^3 and < 500 polyneutrophils/mm^3), which lasted fewer than 10 days in all three cases, but two patients had fever. In 12 patients (80%) no neutrocytopenia was observed. The platelet count evolution was similar: only two patients (13%) had a severe thrombocytopenia that required transfusions. The nadir of WBCs appeared at day 21, and for platelets at day 17. Consequently, almost all the patients, except the first ones, were followed as outpatients.

Nonhematologic Toxicity

Almost every patient presented with biological hepatic abnormalities on the cholestasis tests, but none had a clinical hepatitis or jaundice. Hepatic steatosis has, however, been found in the one autopsy performed.

Three patients had leg phlebitis, and one patient had a pulmonary embolism 15 days after high-dose carmustine.

Two patients had nondocumented fevers. One patient experienced

Table 3. Toxic Effects

Characteristics	No. of Patients	%	Grading
Nausea, vomiting	15	100	2
Hematologic toxicity	15	100	
White blood cells	2	13.5	1
	6	40	2
	4	26.5	3
	3	20 <1000 WBC, <500 PN/mm^3	4 <10 days
Platelets	1	6.5	0
	7	46.5	2
	5	33.5	3
	2	13.5 <25,000/mm^3	4 <10 days
Phlebitis	3	20	
Infection < 30 days	4	26.5	
> 30 days	2	13.5	
Interstitial pneumonitis	4	26.5 (3 regressive, 1 lethal)	
Dermatitis	2	13.5	
Cerebral hemorrhage	1	6.5 (1 lethal)	
Extradural hematoma	1	6.5 (1 lethal)	

orchitis and one a urinary infection before day 30. *Staphylococcus epidermidis* sepsis appeared in one patient after day 30, and localized herpes zoster in another.

Pulmonary complications were of big concern in these patients, since four cases of interstitial pneumonitis were observed; two were life-threatening, requiring intubation and assisted ventilation, and one was documented as putatively the result of a *Candida albicans* infection. All cases were regressive, but the patient with the most severe one, probably because of *Candida*, secondarily relapsed with *Pneumocystis carinii* and died. The etiology of the three other cases of interstitial pneumonitis was not proved, because dyspnea, cough, and fever without an infectious agent were observed and no biopsy was performed. These findings are, however, in accordance with those already published concerning pulmonary fibrosis caused by carmustine. Finally, two patients presented 6 weeks after ABMT with severe toxemia and extensive cutaneous edema, erythema, and desquamation. One case was severe and associated with highly elevated eosinophilia (240/mm^3). These symptoms disappeared progressively when the patient's anticonvulsant treatment with phenytoin was discontinued. Skin biopsy did

not give any specific information, and no criterion for a hypereosinophilic syndrome was found.

Other Toxicities

Irradiation was done as scheduled for 11 patients (2 patients in relapse had had anterior irradiation, and 2 patients could not tolerate the scheduled radiotherapy course). The patients' treatment tolerance was very good, and the classical cerebral edema often present at the beginning of treatment could be controlled readily with appropriate medications. However, one patient died 3 days after the end of the radiotherapy course in a sudden coma; the autopsy showed a cerebral hemorrhage, when platelet count was normal; the remaining part of the tumor was completely necrotic and no cerebral edema was found. This type of complication is well known and classical in cerebral irradiation.

No major problems were recorded in the majority of patients after surgery, but this probably reflects a bias in selection of patients, who entered this protocol only when their performance status was good. One patient, however, became comatose and died of an immediate postoperative extradural hematoma.

Altogether, three deaths (20%) of toxic reactions at 2, 3, and 6 months after completion of the whole treatment protocol were recorded. Only one (7%) was directly consecutive to the high dose of carmustine.

Response

As of November 15, 1986, the median follow-up has been only 4 months, and only the feasibility of the protocol is reported here in detail.

All patients, except three who died of toxic effects, had at least the same performance status before and after treatment (1-1), and four patients initially scored as having grade 2 status became grade 1 after treatment.

Histologic assays of the four patients treated with surgery after high-dose carmustine, showed remaining neoplastic cells. Even if complete remission was not fully achieved after the high-dose chemotherapy, however, additional tumor reduction as a result of spontaneous necrosis has been observed. In the patient who died of cerebrospinal hemorrhage, no residual tumor cells were noticed at autopsy.

The 15 patients' survival is plotted in Figure 1. In addition to the three patients who died, one patient has been in relapse, as shown by CT scan changes, since day 90. The other patients are alive, disease-free, and have a stable performance status.

DISCUSSION AND CONCLUSION

The objective of this pilot study was to evaluate the feasibility, efficiency, and tolerance of a new protocol combining surgery, conventional poly-

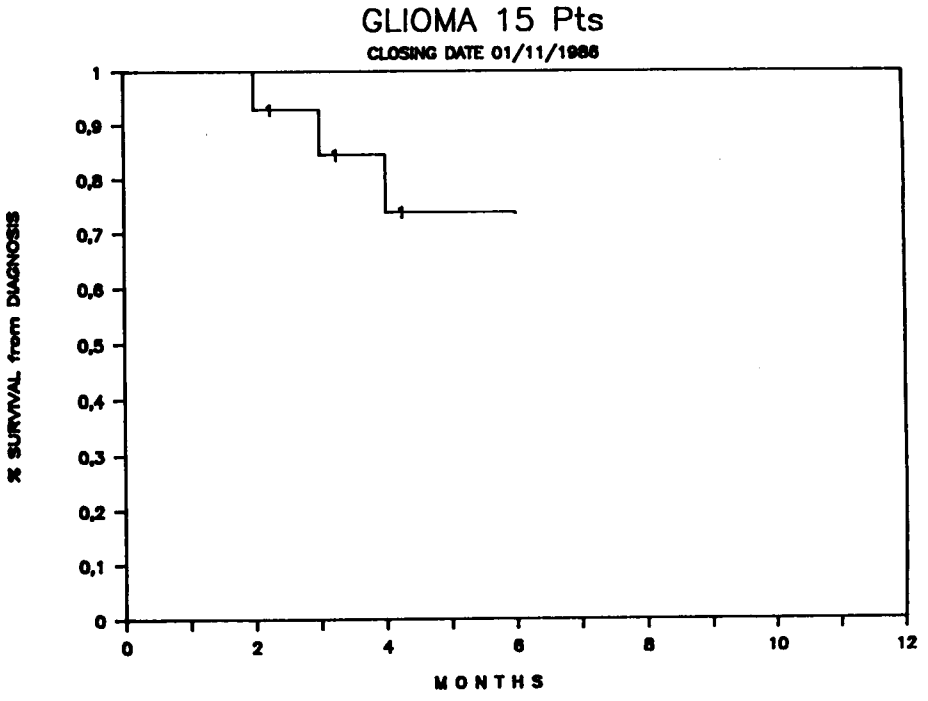

Figure 1. *Kaplan-Meier survival plot of 15 patients with glioma.*

chemotherapy, high doses of carmustine followed by ABMT, and radiation. This treatment protocol appears to be feasible in practice, although its efficiency cannot yet be evaluated; the only brain autopsy done showed a complete necrosis of the remaining tumor.

A toxic death rate of 20% was observed, comparable to that of other ABMT programs. Although the poor prognosis of this disease allows such a risk, the risk is acceptable only if previously reported improvement of results can be confirmed. At the moment, our preliminary results seem comparable to those reported in the literature, since 70% of the patients are alive and well, after a median follow-up time of 4 months.

Finally, the hospitalization time never exceeded 2 months, and the patients' quality of life is acceptable. The majority of patients were ambulatory during a large part of the treatment course, except for the patient who died of interstitial pneumonitis of carmustine origin and was hospitalized for more than 3 months. Patients can complete the program within 2 months.

The dose-effect relationship in gliomas may be explored in the future without major problems.

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High-Dose Chemotherapy With Autologous Marrow Transplantation for Gliomas of the Central Nervous System

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Standard treatment for primary tumors of the central nervous system (CNS) is partial surgical resection followed by maximal-dose radiation. Recently, the addition of chemotherapy to this approach has been evaluated. However, despite this multimodality effort, survival gains have been insignificant (1). Virtually all patients with primary glial tumors of the CNS succumb to that disease. High-grade gliomas (glioblastoma multiforme) are particularly virulent; median patient survival is less than 1 year and few, if any, are long-term survivors. The inability of chemotherapy to add to radiation and surgery is due to the intrinsic drug resistance of these tumors, inadequate CNS penetration of the drug, and poor patient tolerance.

One of the mechanisms for improving cytotoxic therapy is to intensify dose and frequency of drug administration (2). Bone marrow transplantation, by assuring reconstitution of hematopoiesis, has allowed the escalation of myelotoxic agents to the limits of extramedullary toxicity. Since increased CNS penetration is anticipated from these novel high-dose regimens, tumors

of the CNS were a high priority for evaluation. This report details our experience treating CNS tumors with high-dose chemotherapy and autologous bone marrow transplantation (ABMT).

MATERIALS AND METHODS

Institutions

Patients were treated at the following medical centers: Baylor University Medical Center, Dallas, TX; Case Western Reserve University, Cleveland, OH; Cleveland Clinic Foundation, Cleveland, OH; The University of Texas Health Science Center at San Antonio, San Antonio, TX; Vanderbilt University, Nashville, TN; and Washington University, St. Louis, MO. Prior to treatment, patients gave their informed consent as approved by the individual institutional review board.

Bone Marrow Harvest, Cryopreservation, and Reinfusion

Bone marrow collection, processing, cryopreservation, storage, and reinfusion were performed as previously reported (3). The minimum required cell dose per transplant was greater than 1.0×10^8/kg of body mass.

Evaluation Definitions

Tumor response was evaluated by the criteria of Wilson *et al.* (4). A response required improvement evident on a clinical neurologic examination concomitant with a completed tomography scan of the head demonstrating less tumor enhancement and mass effect while the patient was on a stable or decreasing dose of corticosteroids. Patients who died of therapy-related toxicity were analyzed as having progressive tumor at the date of death. Survival and response durations were calculated from the date of bone marrow transplantation for phase I and II studies and from the date of diagnosis for phase III studies.

Statistical Analysis

Actuarial survival rate was calculated by the product-limit method of Kaplan and Meier (5). Confidence-limit intervals were calculated as described by Simon (6).

Chemotherapy

Three agents, BCNU (carmustine), VP-16-213 (etoposide), and thio-TEPA, were evaluated in phase I and II studies. Each was administered as three equal daily infusions followed in 3 or 4 days by ABMT. The combination of carmustine and etoposide was administered as a 6-day course, 3 days of carmustine followed by 3 days of etoposide. Carmustine was also evaluated adjuvantly in a phase III study. The results of these studies are shown in Tables 1 and 2.

Table 1. Summary of High-Dose Studies

Phase	Drug	Total Dose (mg/m²)	Dose/day (mg/m²)	Schedule[a]
I	Thio-TEPA	180–1575	60–525	Days −6,−5,−4
I	Carmustine and etoposide	1050 1200–2400	350 400–800	Days −8,−7,−6 Days −5,−4,−3
II	Carmustine	1050–1350	350–450	Days −5,−4,−3
II	Etoposide	2400	800	Days −5,−4,−3
III	Carmustine	900–1050	300–350	Days −5,−4,−3

[a] *Autologous bone marrow transplantation was performed on day 0.*

Table 2. Summary of Phase I and II Responses

Study	n	Response			Median Survival Time (mo)
		No.	%	95% CI[a]	
Carmustine	27	12	44	28–63	5
Etoposide	16	3	19	5–30	4
Carmustine and etoposide	4	0	0	0–50	3
Thio-TEPA	4	1	25	5–70	2+

[a] *95% confidence intervals.*

RESULTS

Thio-TEPA

We have recently completed phase I evaluation of high-dose thio-TEPA and are now conducting a phase II study (7). The initial total dose administered was 180 mg/m² (60 mg/m²/day for 3 days) and the highest dose has been 1575 mg/m² (525 mg/m²/day for 3 days). To date, only four patients with tumors of the CNS have been treated on the phase II study; one has responded. Toxicities of thio-TEPA have been predominantly myelosuppression, mucositis, and dermatologic reaction; a novel CNS toxicity has been noted at the higher dose levels.

Carmustine

Twenty-seven patients with progressive malignant gliomas after maximal radiation therapy were treated with high-dose carmustine at a dose of 1050-1350 mg/m² (350-450 mg/m²/day for 3 days). Responses were noted in 12 patients (44%) (8). The median survival time of all patients was 5 months; two were alive and apparently disease-free at 60 and 84 months after transplantation. Toxicities in this study were predominantly nonhematologic, including hepatic, pulmonary, and CNS. The overall rate of fatal nonhematologic toxicity was 17%.

Etoposide

Sixteen patients have been treated on a phase II study with high-dose etoposide administered at a dose of 2400 mg/m^2 (800 mg/m^2/day for 3 days). All patients had progressive gliomas after prior maximal radiation therapy, and half had been exposed previously to carmustine. Three responses provided an overall response rate of 19% (9). The median survival time of the entire group was 4 months; the three responders lived 9, 10, and 54+ months. Toxicity of this treatment was predominantly myelosuppression; nonhematologic toxicity was modest.

Carmustine and Etoposide

In this study, high-dose carmustine at the maximal dose of 1050 mg/m^2 (350 mg/m^2/day for 3 days) was combined with high-dose etoposide at two dose levels, 1200 mg/m^2 and 2400 mg/m^2 (400 and 800 mg/m^2/day for 3 days) (10). Three patients were treated at the first level and one patient at the second. No responses were noted, and the study was stopped because of the severe hepatic toxicity that occurred in two patients. The median survival time of all patients was 3 months from transplantation.

Adjuvant Carmustine

High-dose carmustine at a dose of 900-1050 mg/m^2 (300-350 mg/m^2/day for 3 days) was administered to 18 patients with glioblastoma multiforme or anaplastic astrocytoma shortly after they completed standard radiation therapy (11). Toxicity of this study was predominantly pulmonary and included four fatal episodes. The actuarial probability of survival of the entire group was 19% at 27 months after diagnosis. At present, four patients are alive but two have recurrent and progressive tumor. Two patients are apparently long-term disease-free survivors at 60 and 77 months from diagnosis, the former patient is well but the latter is severely neurologically compromised owing to severe encephalomyelopathy (12). The actuarial probability of survival for all patients is shown in Figure 1.

DISCUSSION

Primary tumors of the CNS are a formidable challenge due to their inherent drug resistance and the pharmacologic sanctuary of the blood-brain barrier. High-dose chemotherapy can overcome inadequate CNS penetration and, if a dose-response relationship exists, can overcome relative drug resistance. For these reasons, treatment of CNS tumors with high-dose chemotherapy and ABMT has been actively studied. In this report, we summarize our collaborative systematic program of evaluating high-dose therapy for tumors of the CNS in phase I, II, and III studies.

Carmustine avidly crossed the blood-brain barrier and is the most active agent for treating CNS tumors. Using the criteria of Wilson *et al.*, the

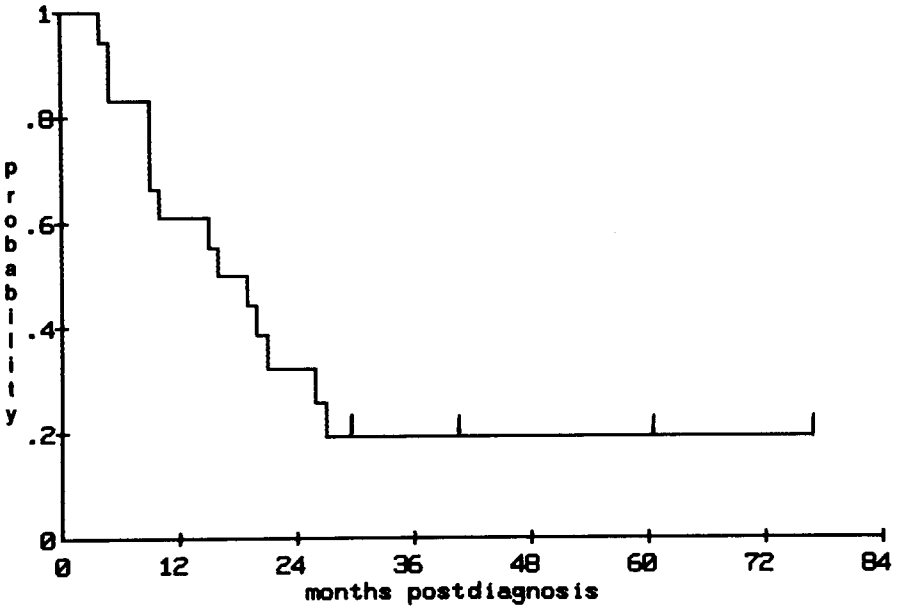

Figure 1. *The actuarial probability of survival for all patients adjuvantly treated with high-dose carmustine and ABMT after irradiation for high-grade glioma of the central nervous system. Tick marks indicate patients alive. The probability of survival is 19% at 27 months after diagnosis.*

response rate for standard-dose carmustine is 13-33% (13). High-dose carmustine was the first agent studied with autologous bone marrow support, and it demonstrated a response rate of 46%. Two of the 27 patients treated for progressive high-grade gliomas are long-term survivors, an observation not characteristic of standard chemotherapy (14). Therefore, because of the high response rate and the existence of long-term survivors treated for progressive tumors, we concluded that high-dose carmustine appeared to have augmented cytotoxic activity when compared to the historical data of standard-dose carmustine. However, the toxicity of high-dose carmustine was substantial, carrying a 17% incidence of fatal pulmonary, hepatic, or CNS toxicity.

Once we recognized the increased antitumor activity of high-dose carmustine in patients with progressive glioma, the next step of evaluation was to administer the drug before the tumor progressed. Accordingly, we administered high-dose carmustine at a dose of 900-1050 mg/m^2 to patients with high-grade gliomas shortly after they completed standard radiation therapy. We postulated that the augmented activity of high-dose carmustine would be most meaningful if administered when the tumor burden was small before drug-resistant cells developed. Standard-dose carmustine has been extensively studied in this context, producing a 2-year survival rate of less than

20% (15). Our study, therefore, was historically controlled and was designed to be able to detect a 40% 2-year survival rate. The dose chosen for administration was less than the maximal dose to avoid a substantial degree of pulmonary toxicity. Eighteen patients were treated with early promising results. However, pulmonary toxicity and late relapses resulted in a survival rate that was not statistically different from that of standard therapy. As in the phase II carmustine study, nonmyeloid toxicity was severe, including four episodes of fatal pulmonary toxicity and two additional patients who had severe toxicity that responded to corticosteroid therapy. Although high-dose carmustine had a high response rate in patients with progressive gliomas, the magnitude of tumor reduction was insufficient to influence survival when the drug was administered adjuvantly. However, even though high-dose carmustine did not produce a benefit in the population as a whole, two patients are long-term disease-free survivors more than 5 years after diagnosis, although one patient has developed severe encephalomyelopathy.

The next agent studied was etoposide at a dose of 2400 mg/m^2. This agent in standard-dose evaluation had demonstrated modest activity against CNS tumors (16). Sixteen patients were treated, and only three (19%) responded, results similar to those of standard-dose therapy. Toxicity of this therapy was predominantly myelosuppression; mucositis was modest. Two patients developed fatal infectious complications during the cytopenic period, but no fatal extramedullary toxicity was noted.

For most tumors, meaningful survival benefit is achieved only when drugs are administered as synergistic combination chemotherapy. Since high-dose etoposide is dose-limited by mucositis and does not cause pulmonary, hepatic, or CNS toxicity, it seemed an ideal agent to combine with carmustine, which does not cause mucositis and is dose-limited by hepatic and pulmonary toxicity. Four patients were treated with the combination of high-dose carmustine and high-dose etoposide. Unfortunately, severe hepatic toxicity developed in two patients before any patient responded. Although etoposide at the maximal tolerated dose does not cause severe hepatic toxicity, large cumulative doses had been associated with that toxicity (17). This combination produced synergistic toxicity without synergistic antitumor activity, a possibility of high-dose combination chemotherapy.

One of the requirements of cytotoxic agents anticipated to have activity against CNS tumors is adequate penetration of the blood-brain barrier. Thio-TEPA, one of the earliest alkylating agents developed, fulfills this requirement; it produces drug concentrations in the cerebrospinal fluid almost equal to that in the serum. Standard-dose thio-TEPA had not been adequately evaluated against CNS tumors because of imprecise response criteria and the lack of availability of CT scanning to measure exact tumor response (18). Presently, only four patients have been treated with high-dose thio-TEPA with one response noted.

Other studies are now beginning, including an evaluation of high-dose

etoposide with the synergistic agent cisplatin. Pending the completion of the phase II thio-TEPA study, this agent will be studied in combination chemotherapy. Although high-dose carmustine is the most active agent, its use is limited by the high frequency of fatal extramedullary toxicity. If this agent is to be used in high-dose studies, a substantial reduction from the maximal dose should be considered.

In summary, CNS tumors have been treated with high doses of a variety of drugs. Unfortunately, although high response rates have been observed, survival has not yet been prolonged meaningfully. However, new combinations are being investigated to create synergistic drug combinations that, it is hoped, can be used as adjuvant therapy for high-grade gliomas.

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Brain Cancer

S. Wolff and G. Herzig, Chairmen

Dr. H. Vriesendorp: Brain tumors are a local problem, not a systemic disease. Autologous bone marrow transplantation (ABMT) so far has had a lot of toxic effects and not a lot of useful therapeutic results. Is any consideration being given to approaching the problem in a local way?

Dr. S. Wolff: I think those directions are being explored, but not by people undergoing ABMT obviously.

Dr. K. Dicke: When you increase a systemic dose of VP-16-213 (etoposide), do you get higher levels of etoposide in the CNS?

Dr. Wolff: We have looked at CSF levels. We have also had the opportunity in one patient who had an Ommaya reservoir inside a cystic neoplasm to sample etoposide levels. There are higher levels compared to what you would achieve with normal-dose etoposide in those patients, either in the CSF or in the cystic internal fluid of a high-grade neoplasm. So I think you do get higher levels into the CNS with high-dose systemic therapy. One of the reasons that we've done these studies with systemic therapy is that, although brain tumors are localized processes, they're really not very localized. They intercollate throughout an extensive part of the brain. Many of the infusional studies cannot be done because high-grade tumors classically cross the midline, and I think that one of the things that we're really accepting now is that this is a fairly extensive neoplasm at the time of diagnosis.

Although, certainly, with infusional studies you would certainly avoid most of the systemic toxicities.

DR. DICKE: I have one other question. How dose responsive do you think the glioblastoma might be?

DR. G. PHILLIPS: That's a very hard question to answer. I think there are some in vitro models growing these cells which show dose responses. Those models are very interesting because this tumor at surgery is very heterogeneous, both biochemically and with regard to chemosensitivity. The only clinical data that we have is the BCNU (carmustine) data for progressive patients without prior chemotherapy but not treated immediately after radiation therapy. Historically, the response rate in critically evaluated studies ranges from 12 to 30%, so around 20% would be an acceptable median response to normal-dose carmustine. In our 27 patients, we had a 44% response rate, which might be an indication of an increased response rate.

DR. WOLFF: By and large, the question just hasn't been addressed in clinical studies. We might have a chance with thio-TEPA. At least there's a comparable group of patients who were treated with what was thought to be high-dose, something around 60/m^2 at the maximum, and we may be able to, retrospectively at least, compare these results with what may be 50 times that dose of thio-TEPA in a similar group of patients and get an answer for thio-TEPA, or at least a suggestion.

DR. G. MESSERSCHMIDT: I just wanted to update some of our studies in glioma, mainly because it seems to contrast with what's been presented this morning. We've now treated 31 patients and in our protocol these patients have all received a transplant within 3 weeks of their surgical diagnosis, so that the delay from surgery has been minimal. We've used 1500 mg/m^2 over 3 days before the transplant. Pulmonary toxicity has not been significant. Of interest, though, four people did develop infiltrates. Two were idiopathic and resolved on their own; two others had surgical biopsies in which results were shown to be pneumocystic. Both those patients died. Since then, we've treated these patients with Septra prophylactically and we have not had another case of pulmonary infiltrates. Also, we've followed CAT scans on all these patients every 3 months for well over 3 years. Some of these tumors do not begin to regress on CAT scan for up to 12-18 months following the transplant. So, trying to determine response early is often very difficult. Of the 31 patients, only three have progressed and all three of those have now died, so progressive disease on our present protocol is very minimal. I forgot to mention that when they recover from the transplant they are given 60 Gy of whole-brain radiation.

DR. PHILLIPS: These are patients with new diagnoses of grades III and IV glioblastoma?

DR. MESSERSCHMIDT: Yes.

DR. PHILLIPS: And they all receive treatment immediately after surgery?

DR. MESSERSCHMIDT: Yes, immediately after surgery.

DR. PHILLIPS: So it's difficult to measure tumor regression, presumably because they've mostly been completely resected.

DR. MESSERSCHMIDT: They all have CAT scans immediately after surgery before the transplant and it's very easy to see the tumor on that CAT scan. But what you see over the next 12-18 months is a decrease in size, a decrease in the amount of contrast enhancement, and eventually, calcification in the area of the tumor. We have had one patient die of lupus nephritis 1 year after the transplant. She also had the same sort of CAT scan calcification, and an autopsy showed no viable or visible tumor there. The long-term survival seems to indicate that the patients, even though they do not have totally normal CAT scans in all cases, do not have viable tumors. So we're very encouraged by this study.

DR. PHILLIPS: What is the 18-month overall survival now?

DR. MESSERSCHMIDT: Eighteen months, with everyone included. Let me point out that everyone referred to us received transplants including two people who were comatose. The overall survival rate is 50%; that includes the patient with pneumocystis and four early deaths.

DR. PHILLIPS: I would like to add just one fact. When we had three fatalities at 1050 BCNU/m^2, we further dropped the adjuvant dose to 900/m^2. The first patient to be treated at 900/m^2 died of fatal pulmonary toxicity.

DR. G. SPITZER: Does the North American Bone Marrow Transplant Study Group have phase I and II data on the combination of etoposide and total body irradiation (TBI) for tumors other than glioblastoma?

DR. WOLFF: We've used that combination of TBI and etoposide with cyclophosphamide.

DR. SPITZER: What dose of etoposide, and can you comment on the toxicity?

DR. WOLFF: It appears that the maximum dose of etoposide given as a single infusion is going to be 1800/m^2, with liver toxicity at 2250 when given with 150/kg of cyclophosphamide and 10-12 Gy of TBI.

VIII. New Regimens

Intensification Regimens for Autologous Bone Marrow Transplantation

Emil Frei III, Carol A. Cucchi, and Beverly Teicher

Intensification chemotherapy and total body radiotherapy have been curative for some patients with hematologic malignancies; accordingly, they are under increasing study for these diseases and also, more recently, for solid tumors. The construct of intensification regimens has been largely empirical and provides a major challenge to progress in autologous bone marrow transplantation. In this chapter we discuss the development and study of preclinical models designed to provide information that will help us to construct such intensification regimens to treat human cancers.

IN VIVO MODEL

Quantitative models of dose intensification into the range required for marrow transplantation have been limited. A relevant method is presented in Figure 1. Inbred mice bearing a measurable transplanted tumor are treated with a single injection of a given drug with a dose ranging upward from the LD_{10}. Since toxicity and death from most antitumor agents are delayed, excision of the tumor at 24 hours for quantitative study is possible. Important aspects of the physiologic pharmacology have been completed by 24 hours, and intracellular events will continue to occur whether the tumor is left in place or excised for quantitation. The excised tumor may be analyzed by in

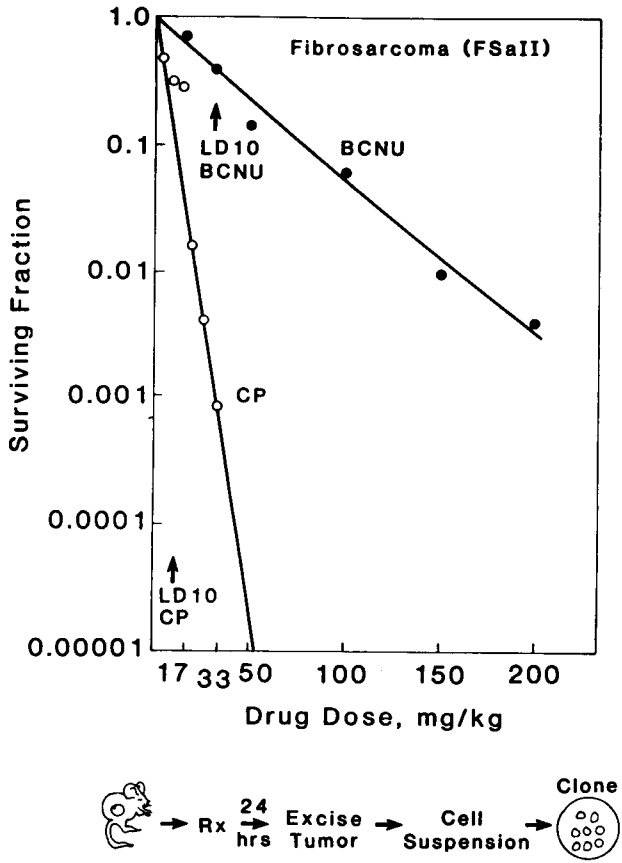

Figure 1. *In vivo model of high-dose intensification regimen. Abbreviations: LD_{10}, lethal dose; CP, cisplatin.*

vitro cloning or by quantitative assay in inbred strains (1;Teicher *et al.*, unpublished data).

Figure 1 presents data with respect to the action of cisplatin and BCNU (carmustine) in a transplantable fibrosarcoma. The LD_{10} of cisplatin produces a less than 1 log cell kill; that is, enough to result in transient tumor regression. With increasing doses, up to 3- to 5-fold the LD_{10}, an exponential increase in log kill is achieved. The slope for carmustine is less steep, but it still maintains the log linear relationship between cell kill and dose.

Using this model, we have demonstrated in three different tumor systems that the alkylating agents maintain a linear log relationship between dose and cell kill down through 4-5 logs (the limit of the assay). For nonalkylating agents, and particularly for cell cycle-sensitive agents, the killing curves are curvilinear, producing marginal increments in cell kill at higher doses. This system has demonstrated clearly that alkylating agents have properties

appropriate to the transplant arena. We are now using this system to analyze a number of variables, particularly those relating to effect of tumor size on fractional kill, the effect of different drugs and different tumors, and the effect of schedule and combination therapy on cell kill.

CROSS-RESISTANCE AMONG ALKYLATING AGENTS IN COMBINATION CHEMOTHERAPY

Schabel and colleagues, some 7 years ago, demonstrated for L1210 mouse leukemia that induction of resistance to a given alkylating agent usually did not confer cross-resistance to the other alkylating agents (Table 1) (2). This observation, which was counterdogma at the time, was of major significance, since it indicated that the alkylating agents, perhaps the most important class of antitumor agent, were not "one" radiomimetic agent with minor differences with respect to toxicity, route of administration, etc., but rather were fundamentally different one from the other. This difference, expressed in terms of lack of cross-resistance, could provide a major basis for combination alkylating agent therapy. Indeed, Schabel and colleagues went on to demonstrate that various combinations of alkylating agents were commonly synergistic (2,3). Since such agents have steep dose-response curves as already indicated, have myelosuppression as the dose-limiting toxicity, and often have differing nonmyelosuppressive dose-limiting toxicities, combining them intensively represented an attractive approach to transplant therapy. However, before proceeding it was necessary to demonstrate their properties, particularly the lack of cross-resistance, in human tumors.

Accordingly, alkylating agent-resistant lines of human tumors were produced by prolonged progressive selection pressure (Fig 2). Using a comparable protocol against a variety of human tumor cell lines, high levels (4-5 logs) of resistance to methotrexate and resistance in excess of 600-fold to Adriamycin can be achieved (4).

For nonalkylating agents, it is a generalization that, with appropriate selection pressure, high levels of resistance can be achieved readily. In

Table 1. Cross-Resistance Among Alkylating Agents (L1210 Mouse Leukemia)

	L1210	L1210/CPA	L1210/PAM	L1210/BCNU	L1210/Platinum
CPA	7[a]	0-1	5	7	5
PAM	6	4	0	7	5
BCNU	5	5	5	0-1	3
Platinum	5	5	5	5	0-1

Abbreviations: CPA, cyclophosphamide; PAM, phenylalanine mustard; BCNU, carmustine; platinum, cisplatin.

[a]*Tumor cell kill (in logs) achieved in L1210 in vivo with LD_{10} dose of drug.*

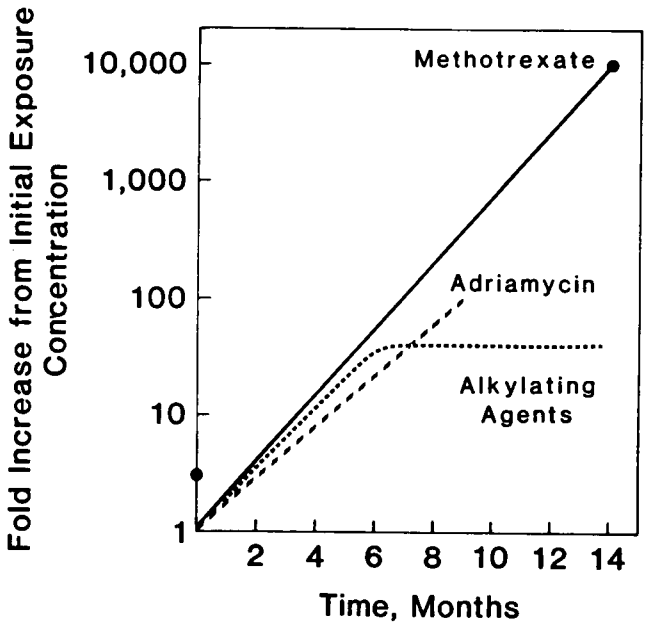

Figure 2. *Schematic of the development of drug resistance using selection pressure.*

contrast, for radiotherapy specific resistance has been extremely difficult to produce. Transient resistance at low levels (2- to 3-fold) has been achieved by Courtenay and has been reported in lines with increased glutathione transferase and glutathione (5).

The alkylating agents more closely resemble radiotherapy in this regard. Thus we found it extremely difficult and time-consuming to develop resistance to differing alkylating agents in different cell lines. While up to 20- to 30-fold the initial IC_{50} could be achieved, back mutations were frequent, and cloned lines were generally resistant only 5- to 10-fold (Table 2). This property is in itself important, indicating that resistance (a major obstacle to cure) is much less prominent for the alkylating agents than for other agents and that resistance is relative and can be overcome, at least in part, by a 5- to 10-fold increase in dose, something readily accomplishable in the marrow transplant setting.

The issue of cross-resistance to alkylating agents on the part of human cell lines is presented in Tables 2 and 3. Although low levels of alkylating-agent cross-resistance were common, major cross-resistance was distinctly uncommon, consistent with Schabel's L1210 data (6,7). In this era of multi-drug resistance, it was also important to determine whether alkylating-agent resistance imparted resistance to nonalkylating agents (Table 3) (6). Collateral sensitivity to Adriamycin and perhaps to vincristine was consistently observed for different cell lines and different alkylating agents, and one

Table 2. Resistance Ratios[a] to Various Alkylating Agents

Cell Line	Agent									
	HN_2[b]	MEL[b]	BCNU[b]	4-HC[b]	Busulfan[c]	thio-TEPA[c]	CDDP[b]	HO_2-IFA[b]	MitoC[b]	MitoC[d]
Raji/BCNU	1.9	4.0	5.3	1.6	—	—	4.0	—	1.0	—
Raji/HN_2	6.6	1.4	1.9	2.8	1.2	1.6	2.3	0.9	1.4	2.0
Raji/CP	3.2	1.3	2.6	1.0	0.9	1.5	7.3	1.2	0.8	1.5
SCC-25/CP	1.8	5.0	2.0	2.8	1.0	1.7	12.0	1.6	1.0	1.2
MCF7/CP	1.6	1.9	0.42	3.0	—	2.3	4.3	—	—	—

[a] *Resistance ratio = IC_{50} of resistant line/IC_{50} of parental line. The IC_{50}s (micromolar) for the Raji parental line were: HN_2, 2.6; MEL, 20; BCNU, 30; 4-HC, 18; busulfan, 6.6; thio-TEPA, 2.4; CDDP, 30; HO_2-IFA, 23.3; MitoC[b], 10.2; and MitoC[d], 0.042. The IC_{50}s (micromolar) for the SCC-25 parent line were: HN_2, 11; MEL, 8; BCNU, 200; 4-HC, 25; busulfan, 15.5; thio-TEPA, 2.4; CDDP, 17; HO_2-IFA, 25; MitoC[b], 3.3; and MitoC[d], 0.004.*

[b] *Drug exposure for 30 min.*

[c] *Drug exposure for 24 hr.*

[d] *Drug exposure continuous.*

Abbreviations: HN_2, nitrogen mustard; MEL, melphalan; BCNU, carmustine; 4-HC, 4-hydroperoxycyclophosphamide; CPA, cyclophosphamide; CDDP, cisplatin; HO_2-IFA, hydroperoxy alpha-interferon; MitoC, mitomycin C.

Table 3. Resistance Ratios[a] to Various Antineoplastic Agents

Cell Line	Agent					
	ADR[b]	ADR[c]	VCR[b]	FU[d]	MTX[b]	MTX[c]
Raji/BCNU	0.3	—	0.7	1.0	1.4	—
Raji/HN_2	0.3	0.5	0.7	3.4	1.2	2.5
Raji/CP	0.2	0.5	0.2	1.9	1.6	1.5
SCC-25/CP	0.6	0.4	0.9	0.6	23.8	7.3

[a]*Resistance ratio = IC_{50} of resistant line/IC_{50} of parental line. The IC_{50}s (micromolar) for the Raji parental line were: ADR[b], 0.66; ADR[c], 0.04; VCR[b], 0.11; FU[d], 44; MTX[b], 41; and MTX[c], 0.022. The IC_{50}s (micromolar) for the SCC-25 parental line were: ADR[b], 2.8; ADR[c], 0.013; VCR[b], 0.21; FU[d], 52; MTX[b], 34; and MTX[c], 0.006.*

[b]*Drug exposure for 30 min.*

[c]*Drug exposure continuous.*

[d]*Drug exposure for 24 hr.*

Abbreviations: ADR, Adriamycin; VCR, vincristine; FU, 5-fluorouracil; MTX, methotrexate; HN_2, nitrogen mustard; CP, cyclophosphamide.

clone showed substantial cross-resistance to methotrexate. As a generalization, however, alkylating-agent resistance did not cross to other antitumor agents.

Developing studies with respect to the biochemistry of alkylating-agent resistance indicate that a variety of mechanisms of resistance may be involved, some of which are specific for a given alkylating agent (Table 4). Resistance is often multifactorial. Teicher *et al.* have found, for example, that resistance to cisplatin of a squamous cell carcinoma of the head and neck cell line was due to: a decrease in plasma membrane transport, an increase in protein sulfhydryls in the cytosol (and some cross-resistance to cadmium), an increase in glutathione reductase, and possibly an increase in DNA repair (8). Some of these mechanisms should be unique to cisplatin, whereas others

Table 4. Documented Mechanisms of Alkylating Agent Resistance

Drug	Resistance Mechanism
Nitrogen mustard (HN_2)	Transport reduction Glutathione reductase increase DNA repair increase
Melphalan	Transport reduction Glutathione increase
Nitrosoureas	DNA repair increase
Cyclophosphamide	Aldehyde dehydrogenase increase
Cisplatin	Transport reduction Glutathione reductase increase Protein-sulfhydryl increase

should cross to other alkylating agents. Thus low-level or partial cross-resistance should be common, which in fact is the case, based on the aforementioned studies.

ANALYSIS OF IN VITRO MULTILOG KILLING CURVES

In general, sensitivity and cross-resistance are measured by determining the IC_{50} over a 1-log dose range. This provides information that may be relevant to remission induction in the clinic but could be misleading with respect to multilog cell kill, a property essential to cure. Figure 3 presents multilog cell-kill curves for three alkylating agents and five representative human solid tumor cell lines. Note that phenylalanine mustard (PAM) and cisplatin maintain linearity with respect to log kill in some tumors, while in others they become less effective with increasing dose. The latter presumably represents heterogeneity with respect to drug resistance (9). Skipper and colleagues have determined that this property may be crucial to the effects of initial treatment. Thus they have found on the basis of preclinical in vivo studies that where 10^{-2} tumor cells are resistant, tumor regression will not occur; if 10^{-4} are resistant, moderate response may be achieved; if 10^{-6} are resistant, a major antitumor effect occurs (10). On this basis, we divided the curves in Figure 3 accordingly, with 4+ representing a straight-line effect, and 1 to 2+ a plateau at the level of 1-2 logs. These data are presented semiquan-

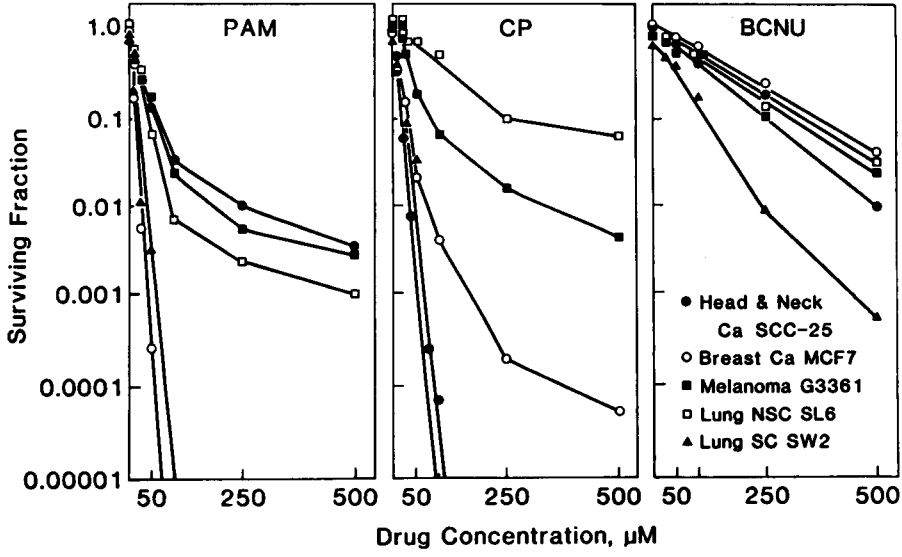

Figure 3. *Relative effects of three alkylating agents, PAM (phenylalanine mustard), cyclophosphamide (CP), and BCNU (carmustine), on cell lines of head and neck, breast, melanoma, lung non-small cell, and lung small cell cancers.*

Table 5. Relative Effects of Three Alkylating Agents on Five Human Tumors (in logs)

Cell Lines	PAM	C-Plat	BCNU
Head and Neck SCC25	1-2+	4+	1+
Breast MCF7	4+	2+	1+
Melanoma G3361	1-2+	1-2+	1+
Lung A/SC SL6	1-2+	1+	1+
Lung SC SW2	4+	4+	2+

Abbreviations: PAM, phenylalanine mustard; C-Plat, cisplatin; BCNU, carmustine; SC, small cell.

titatively in Table 5. A significant correlation is observed between the curve and the known antitumor effect of the agent against the tumor in question. This supports the position that these in vitro experimental models have clinical relevance, and particularly it supports the position that multilog analyses (9) provide important and supplementary information not always evident from IC_{50} studies.

Using this system, we have studied combinations of chemotherapeutic agents. We were particularly interested in the combination of cyclophosphamide and thio-TEPA against the MCF7 breast cancer line because of compelling clinical factors. We found that relatively low concentrations of thio-TEPA provided striking synergy (up to 1,000-fold) with cyclophosphamide, particularly high concentrations of cyclophosphamide (9). This phenomenon has been reproducible both in vitro and in vivo and is under study with respect to the intimate time-schedule relationships and with respect to molecular pharmacology.

In summary, we believe the models described provide a valid approach to the construct of clinical intensification regimens. Based on the history of the development of curative cancer chemotherapy, including that with relationship to transplantation, and based on developing knowledge of drug resistance, it seems likely that combinations of agents employed intensively provide a major opportunity for advancing treatment. At the same time, this approach represents a major research challenge.

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High-Dose Busulfan and Cyclophosphamide in Advanced Childhood Cancers: A Phase II Study of 30 Patients

O. Hartmann, F. Beaujean, J. L. Pico, E. Benhamou, C. Kalifa, C. Patte, F. Flamant, and J. Lemerle

High-dose chemotherapy followed by bone marrow transplantation (BMT) appears presently to be a promising therapy for patients with poor-prognosis, chemosensitive malignancies. In patients with leukemia or solid tumors, several studies have demonstrated that results are much better when patients treated have only minimal residual disease (complete remission or partial remission) rather than progressive or bulky residual disease (1,2). However, even under the best of conditions (i.e., patients are in complete remission), the relapse rate remains high. The need for more efficient high-dose regimens therefore remains great in order to decrease the posttransplantation relapse rate. Taking into account the very impressive results of the high-dose busulfan and cyclophosphamide (BU + CY) combination in acute myeloblastic leukemia (3), we designed a phase II study using this combination in children with relapsed or refractory nonleukemic malignancies. In this chapter we report our results with 30 patients.

PATIENTS AND METHODS

Patients' Characteristics

Thirty patients entered this study. Their median age at diagnosis was 8 years (range, 1.5-20 years), and their sex ratio was two males to one female. Eleven children had neuroblastoma, eight non-Hodgkin's lymphoma, six Ewing's sarcoma, and five rhabdomyosarcoma.

Before entering this phase II study, all patients had been extensively treated. They had received 3-10 different drugs (median, 6 drugs), for a median duration of 11 months (range, 3-50 months). Surgical excision of the primary had been attempted in 10 of 11 with neuroblastoma, of 8 with non-Hodgkin's lymphoma, 1 of 6 with Ewing's sarcoma, and 4 of 5 with rhabdomyosarcoma. Radiation therapy had been previously administered to three of six Ewing's sarcoma primary tumors and to primary and metastatic lymph nodes of one of the patients with rhabdomyosarcoma. Nine of the 30 patients had also previously received high-dose cyclophosphamide with autologous bone marrow transplantation (ABMT); conditioning regimens included a combination of melphalan (180 mg/m^2) with VM-26 (teniposide), BCNU (carmustine), and, for some patients, procarbazine. Despite the use of such aggressive therapies, at the time of entering this phase II study, 14 patients had primarily refractory disease, 14 were in their first progressive relapse, and 2 were in the second progressive relapse (see Table 1).

Bone Marrow Procedures

Twenty-eight patients had an ABMT, and two had a human leukocyte antigen-matched allogeneic BMT. The technique of bone marrow harvesting has been described elsewhere (6). Samples of harvested bone marrow were cytologically proved to be free of residual involvement. All harvested bone marrow was cryopreserved in dimethyl sulfoxide as previously described (7). Twelve out of 28 bone marrow harvests were purged by Asta Z 100 μmol/ml (Beaujean *et al.*, unpublished data). This purging technique was used for the majority of the harvests taken from patients who had bone marrow involve-

Table 1. Patients' Status Before Busulfan and Cyclophosphamide

Diagnosis	Primarily Resistant Disease	First Progressive Relapse	Second Progressive Relapse
Neuroblastoma	6	5	
Non-Hodgkin's lymphoma	4	4	
Rhabdomyosarcoma	2	2	1
Ewing's sarcoma	2	3	1
Total	14	14	2

ment at diagnosis and with non-Hodgkin's lymphoma for some whose marrow was cytologically normal, but was harvested during a progressive phase of their disease.

Drug Administration

On day -8, busulfan (4 mg/kg/day) was administered orally in four divided doses for 4 consecutive days. Beginning on day -4, cyclophosphamide (50 mg/kg/day) was infused intravenously over 60 minutes on 4 consecutive days. On day 0, thawed bone marrow was infused intravenously. During the 8 days of chemotherapy, hydration was maintained with 3 l/m^2/day 5% dextrose, and mesna was administered to decrease the risk of cyclophosphamide-associated cystitis.

Supportive Care

All patients were treated under simple reverse isolation barrier conditions. They received standardized parenteral nutrition and irradiated blood products when needed. Prophylactic oral nonabsorbable antibiotics were not used. When their temperature rose above 38° C, patients received broad-spectrum antibiotics.

Evaluation of Response

Response was judged on serial evaluations of a) catecholamine metabolites in urinary excretion, for patients with neuroblastoma, b) normalization of bone lesions on radioisotopic bone scan, c) clearing of bone marrow involvement, and d) changes of three-dimensional measurements of primary on ultrasound tomography and computed tomography (CT) scan. In three patients, two laparotomies, one before and one after the BMT, were performed by the same surgeon.

Complete response (CR) was defined as complete disappearance of any sign of disease for at least 4 weeks. Partial response (PR) required a greater than 50% reduction of all measurable sites of disease for at least 4 weeks. Objective effect (OE) was defined as a less than 50% and greater than 20% reduction of all measurable sites for at least 4 weeks. All other situations were judged as failure (F).

Evaluation of Toxicity

The intensity of gut toxicity was established by means described elsewhere (8). The criteria for evaluation of infectious complications were duration of fever over 38° C and the occurrence of bacteremia or of documented infections.

RESULTS

Table 2 presents the responses of all 30 patients.

Table 2. Response After Bulsulfan and Cyclophosphamide

Diagnosis	Response					
	CR	PR	OE	F	NE	CR + PR (%)
Neuroblastoma	2	1	5	3		27
Non-Hodgkin's lymphoma	6	0	0	2		75
Rhabdomyosarcoma	1	1	1	2		40
Ewing's sarcoma	1	3	1	0	1	66
Total	10	5	7	7	1	52

Abbreviations: CR, complete remission; PR, partial remission; OE, objective effect; F, failure; NE, nonevaluable.

Tumor Response

One patient died of treatment-related toxicity on day 13 post-BMT. He is not *actually* evaluable for response, since, to be evaluated, the duration of response should be at least 4 weeks. Among the 29 evaluable patients, 10 achieved a CR, 5 a PR, 7 an OE, and 7 F.

Of the 11 patients with neuroblastoma, 2 achieved a CR, 1 a PR, 5 an OE, and 3 F. The CRs were achieved by two patients with residual bone marrow involvement refractory to conventional chemotherapy. Two patients were treated for unresectable stage III abdominal disease. The effects, as measured by ultrasound tomography and CT scan, were only OE. However, the possibilities of local excision were considerably improved after high-dose chemotherapy and BMT: second laparotomies, performed in both patients after BMT, led to a greater than 75% excision. Histologic examination of the tumor tissue showed partially matured neuroblastoma.

Six of the eight patients with non-Hodgkin's lymphoma achieved CR. Six of these lymphomas were of the Burkitt type and two of the lymphoblastic type. It is noteworthy that in one of these patients central nervous system involvement completely disappeared under this high-dose chemotherapy regimen.

Of the six patients with metastatic Ewing's sarcoma, one died early of toxicity. Histologic postmortem examination of the primary and the bone metastases showed no detectable residual tumor. Among the five remaining patients, one achieved a CR, three a PR, and one an OE.

Of the five patients with rhabdomyosarcoma, one achieved CR but subsequently died of complications, 46 days post-BMT. Histologic post-mortem examination did not show any detectable residual disease. The four remaining patients achieved one PR, one OE, and two Fs.

Duration of Response

In these patients with very advanced disease, duration of response was usually short. It lasted 1-23+ months, for a median of 6 months.

Survival

Seventeen patients died of progression or relapse and seven died of toxic or infectious complications. Four of these deaths occurred early during the chemotherapy-induced aplastic phase. They were related to streptococcal septicemia with acute respiratory distress syndrome in one patient, aspergillosis in two patients, and interstitial pneumonitis in one patient. Three deaths occurred several months after BMT and were related to cytomegaloviral infection in two patients and to late pneumococcal septicemia in one other (who had been splenectomized).

The six living patients include three with non-Hodgkin's lymphoma in complete remission 17+, 20+, and 23+ months post-BMT, and three with Ewing's sarcoma alive with disease at 5+, 7+, and 15+ months post-BMT.

Toxicity

All patients experienced profound myelosuppression. The median duration of granulocytopenia less than $0.5 \times 10^9/l$ was 19 days (range, 7-53 days). Leukocytopenia less than $1 \times 10^9/l$ and thrombocytopenia less than $50 \times 10^9/l$ lasted for a median duration of 20 (range, 7-33 days) and 31.5 (range, 17-103 days) days, respectively. No difference in duration of these levels could be demonstrated between the recipients of nonpurged versus purged marrow.

Mild desquamation of the skin occurred in most patients; all had, at least, a marked melanoderma. Gut toxicity was generally mild. Moderate or severe vomiting was observed in 10 of 30 patients. Diarrhea was absent in 10 patients, mild in 11 patients, and moderate or severe in 9 patients. Mucositis was moderate in nine patients and severe in one.

Three patients developed severe but nonfatal hemorrhagic cystitis that resolved without sequelae.

Hepatic toxicity was marked by an elevation of serum glutamic oxaloacetic transaminase in six patients. The values observed were between three and four times the normal values. No clinical or biologic signs suggested subclinical veno-occlusive disease.

One instance of veno-occlusive disease occurred in a patient with pulmonary aspergillosis and multiple visceral failure. This patient developed a resultant hepatorenal syndrome causing severe renal impairment.

Three instances of generalized seizure were observed during busulfan administration. They occurred respectively at days 3, 3, and 4 of busulfan therapy, rapidly resolved with appropriate treatment, and did not recur. Prophylactic phenytoin treatment was not used in these patients.

No cardiac toxicity directly attributable to the drugs was evident.

Eight patients developed pulmonary complications. Seven were attributable to infection. One patient died of interstitial pneumonitis. Postmortem histologic examination of the lungs failed to show any sign of infection. This idiopathic interstitial pneumonitis was possibly related to drug toxicity.

Infectious Complications

All patients experienced fever for a median duration of 11 days (range, 3-32 days). Six of the 30 patients developed documented bacteremias: three with nonhemolytic *Streptococcus,* two with *Staphylococcus epidermidis,* and one with *Pseudomonas aeruginosa.* One of these septicemias was rapidly complicated by a lethal acute respiratory distress syndrome. The other five patients responded to appropriate antibiotic therapy and recovered from this septicemic episode.

Seven pulmonary complications were related to infection. Two occurred during a septicemic episode (*Streptococcus; P. aeruginosa*). Two were related to aspergillosis and were lethal, one was due to *Pneumocystis carinii* and resolved, two developed in patients with polyvisceral cytomegalovirus infections and were lethal. These last two patients were still leukocytopenic and thrombocytopenic when they developed cytomegalovirus infection.

Three cases of herpes simplex; two of localized herpes zoster, and two of varicella were observed during the first 6 months following BMT. All patients recovered rapidly under acyclovir therapy.

DISCUSSION

The response rate to low-dose busulfan in solid tumors is low (9). Significant responses have been described using higher intermittent doses in lymphomas (10), leukemias (11), and solid tumors (12,13). This high-dose busulfan combined with high-dose cyclophosphamide at the Johns Hopkins Center demonstrated high antileukemic properties in acute myeloblastic (3) and other leukemias and lymphomas (14), which were confirmed in our study.

Although patients entering this study were heterogeneous in terms of diagnosis, age, and previous therapy, they were evaluable for tumor response and toxicity. All had measurable disease prior to the high-dose chemotherapy. Twenty-one of them had progressive disease refractory to any kind of conventional therapy, and nine were in progressive relapse after previous exposure to high-dose chemotherapy and ABMT. Under busulfan and cyclophosphamide therapy, the response rate (15/29: CR + PR = 50%) in evaluable patients is particularly high for a phase II study on refractory disease.

For neuroblastoma, high-dose melphalan followed by BMT has previously been used as a phase II therapy. Although objective responses have been observed (15-17), no measurable effects were obtained on refractory disease in our experience (18). August *et al.* (19) described encouraging results when patients with neuroblastoma were conditioned by high-dose polychemotherapy, total body irradiation, and bone marrow reconstitution. In the present study of patients with refractory disease, only 3 of 11 patients receiving the busulfan and cyclophosphamide combination failed completely. Surgical excision of a previously unresectable primary was rendered possible

by this high-dose regimen, and 3 of 11 (27%) achieved CR plus PR. Taking into account the particularly poor prognosis of this group of patients with neuroblastoma, these measurable effects appear particularly promising, and this high-dose conditioning regimen should be tested in patients with less advanced disease.

For non-Hodgkin's lymphoma, several studies of high-dose chemotherapy followed by BMT have been published. Under BACT (BCNU [carmustine], ara-C [cytarabine], cyclophosphamide, thioguanine) (20) or modified BACT (1,21) therapy, the response rate in refractory patients was low and invariably of short duration. Lu *et al.* (14) showed encouraging results with busulfan and cyclophosphamide in three patients with non-Hodgkin's lymphoma, but details of disease status prior to the chemotherapy are not provided. Our series of eight patients with non-Hodgkin's lymphoma treated with busulfan and cyclophosphamide is inadequate for drawing any statistically significant conclusion; but our results—six of eight CRs, three of whom remain in continuous CR at 17+, 20+, 23+ months post-BMT—are encouraging and should be confirmed in a larger group of patients.

For metastatic Ewing's sarcoma and rhabdomyosarcoma, scattered studies with high-dose melphalan (16,22) have produced some CRs or PRs. Our results are too preliminary and the number of patients too small to permit any comparison, but it is noteworthy that four of five such patients responded after a relapse following our high-dose combined melphalan therapy.

In this study of previously heavily treated patients, the incidence and intensity of the complications we encountered were, as expected, high, but similar to those described by Lu *et al.* (14) using the same procedure. The same sort of generalized seizures observed in three of our patients have already been described in another study of patients under busulfan therapy (23). Although lethal viral interstitial pneumonitis is apparently rare after syngeneic BMT (24), the incidence of pneumopathy is particularly high in this study of patients treated with ABMT and was related in most patients to documented opportunistic infections. The understandable presence of infection in such previously heavily treated patients does not preclude, as an adjuvant factor, a direct toxic effect of the high-dose chemotherapy on the lungs (25). However, we must emphasize that four of these seven deaths occurred in patients who had relapsed after a previous course of high-dose chemotherapy and ABMT.

CONCLUSION

In this phase II study, high-dose busulfan and cyclophosphamide produced a high response rate on advanced pediatric malignancies. The toxicity of this regimen was high but acceptable in these previously heavily treated patients. The use of this conditioning regimen in patients with a less advanced stage of the disease should improve results and carry a lower incidence of complications; it deserves further investigation.

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The Intensive Use of Carmustine With Autologous Bone Marrow Transplantation

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In 1978, our group began the first in a series of clinical studies designed to evaluate escalating-dose monochemotherapy accompanied by autologous bone marrow support (1). We initially chose to evaluate BCNU (carmustine) because it appeared to be a suitable agent for this type of study: 1) Carmustine is active in vitro against resting-phase cells. 2) It is active against a moderately wide spectrum of malignancies and has activity against some tumors that are notoriously unresponsive to other agents. 3) Carmustine's major acute toxic effect is myelosuppression, making it ideal not only for autologous bone marrow transplantation (ABMT) studies but potentially also for use in intensive-treatment combinations. Moreover, at the time we initiated this study, the cumulative dose-limiting toxic effects on nonhematologic organs such as the lung, and to a lesser degree the kidney, were not widely known.

We completed the phase I trial in 1980, determining that the maximum carmustine dose associated with acceptable toxicity was 1200 mg/m^2. Pulmonary toxicity was believed to be the dose-limiting nonhematologic toxicity, the risk of severe or fatal interstitial pneumonitis being 10%. We have observed serious carmustine-related pneumonitis at doses as low as 900 mg/m^2 but are unable to provide the incidence of toxicity at these doses.

Pulmonary toxicity seems to be greater when carmustine is given in a brief, intensive schedule with bone marrow transplantation, compared with the same dose in a conventional schedule (2). Severe hepatotoxicity and neurotoxicity appeared at higher doses.

During the phase I study and subsequent phase II trial, impressive antitumor responses occurred in several types of tumors, including melanoma, glioblastoma, small cell carcinoma of the lung, and metastatic tumors of the central nervous system.

Unfortunately, attempts to exploit this single-agent activity by combining carmustine with other agents have not been overly successful. For instance, Herzig (3) reported on the use of carmustine at doses of 600-1200 mg/m^2 and melphalan at 90-180 mg/m^2 in treating metastatic melanoma. The complete and overall response rates of 10% and 59%, respectively, were not superior to those observed with either single-agent carmustine (4) or melphalan (5). Moreover, pulmonary toxicity seemed to increase with the higher doses of this regimen. Peters *et al.* (6) experienced difficulty with their intensive combination-chemotherapy regimens that include carmustine at 600-750 mg/m^2. The subsequent deletion of carmustine, cisplatin, cyclophosphamide plus melphalan, and carmustine, cyclophosphamide, cisplatin plus etoposide produced altered, often diminished, patterns of renal and hepatic toxicity, respectively (Peters *et al.*, personal communication).

We are currently attempting to give total carmustine doses of 1200-1800 mg/m^2 in two or three "fractionated" courses over several months. This will be successful only if damage is not invariably cumulative.

In summary, carmustine monochemotherapy with ABMT is not likely to find a major place in antineoplastic therapy; the doses required to provide a measure of tumor control are probably too toxic. Although there are problems with the combinations noted above, carmustine probably is still useful, especially for treating tumors for which carmustine is active at a conventional dose. Examples include the malignant gliomas, Hodgkin's disease, and perhaps certain other tumors. In any case, it is unlikely that a carmustine dose of more than 600-750 mg/m^2 can be given without incurring an at least 10% incidence of severe interstitial pneumonia.

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A Phase II Study of a New Cytoreductive Conditioning Regimen With Autologous Bone Marrow Transplantation for Lymphomas: The BEAM Protocol

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Since the initial report by Appelbaum *et al.* (1) of high-dose chemotherapy followed by autologous bone marrow transplantation (ABMT) in patients with non-Hodgkin's lymphomas (NHL), this procedure has been applied extensively. Most conditioning regimens were derived from the initial BACT combination (BCNU [carmustine], aracytine [cytarabine], cyclophosphamide, thioguanine). The carmustine dosage was increased in the Institut Gustave-Roussy's BACT modification (2) or replaced by CCNU (lomustine) in the TACC regimen (thioguanine, aracytine [cytarabine], CCNU, cyclophosphamide) (3). At present there is no convincing evidence of the superiority of any single conditioning regimen for any subtype of lymphoma (4). In particular, no statistically significant differences were identified until now between patients treated with chemotherapy or with chemoradiotherapy conditioning regimens, although legitimate doubt remains about the ablative potential of regimens lacking an irradiation component. The current

approach is to combine a chemotherapy regimen with involved-field radiotherapy (see Armitage "Bone Marrow Transplantation in Relapsed Diffuse Large Cell Lymphoma," and T. Philip *et al.* "Autologous Bone Marrow Transplantation in Burkitt's Lymphoma: 50 Cases in the Lyons Protocol," both in this volume).

The overall toxicity of the chemotherapy conditioning regimen until now was in the range of 15% to 20% (4). In 1983, in an attempt to decrease the conditioning regimen's toxicity without decreasing the response rate we set up a new regimen, the BEAM protocol (BCNU, etoposide, aracytine, melphalan) derived from the BACT regimen (5-7). Carmustine and cytarabine, proved to be efficient in NHL, were kept, whereas 6-thioguanine was replaced by etoposide (5). Cyclophosphamide, found to be responsible for fatal congestive heart failure, was deleted and replaced by melphalan as the alkylating agent (6). For the BEAM regimen, in order to replace 6-thioguanine, we decided to use etoposide (VP-16-213) because of a 40% response rate in phase II studies of patients with NHL (7). With the introduction of melphalan, no cardiotoxicity was observed, and a good response rate has been reported in acute lymphocytic leukemia (ALL) (8) and NHL. This report is a review of 52 patients treated with this protocol during the period of 1983-1986, with particular reference to response rate and toxicity.

PATIENTS AND METHODS

Between January 1984 and January 1986, 52 BEAM conditioning regimens were administered in a multicentric study to patients with NHL or Hodgkin's disease. Institutions that entered patients in the study were: Centre Leon Berard (Lyons, France, 26 patients), University College Hospital (London, England, 11 patients), Centre Hospitalier de Tours (France, 7 patients), Hopital Saint-Antoine (Paris, France, 4 patients), and Institut Paoli Calmettes (Marseilles, France, 3 patients).

Patients were children and adults ranging in age from 1 to 65 years with a median age of 30.4 years. Thirteen patients were younger than 16 years (range, 1-15 years; median, 9.5 years), and 39 were adults (range, 18-65 years; median, 37.4 years). Thirty-eight patients were male. As is shown in Table 1, of 45 patients with NHL, 23 had high-grade, 18 had intermediate-grade, and 4 had low-grade NHL. Seven patients had Hodgkin's disease.

All patients with NHL had previously received a doxorubicin-containing regimen. Twelve patients were in first complete remission (CR), five in first partial remission (PR), and two were in primary refractory condition after first-line therapy. Twenty-six patients were in relapse; among these, 19 were still sensitive to a second-line chemotherapy (so-called sensitive relapse [SR]), and 7 were resistant to this salvage chemotherapy (resistant relapse [RR]). All patients with Hodgkin's disease were in relapse (two in RR and five in SR), but only four of them had received a doxorubicin-containing regimen.

Table 1. Histologic Characteristics of Patients' Non-Hodgkin's Lymphomas

Non-Hodgkin's Lymphoma	No. of Patients
High-grade (23)	
Burkitt	13
Lymphoblastic	4
Immunoblastic	6
Intermediate-grade (18)	
Follicular large cells	1
Diffuse mixed	9
Diffuse large cells	6
Diffuse small cells	2
Low-grade (4)	
Follicular mixed	1
Follicular small cells	3

Patients or parents were fully informed of the protocol risks and gave informed consent in the presence of at least three collaborators.

Bone marrow harvesting and freezing procedures were performed as previously reported (9,10); 24 marrows were purged, either with monoclonal antibodies (11) or with mafosfamide. In 28 cases, the marrow was unpurged. Bone marrow was harvested from 15 patients in first CR, at relapse in 28, and during first PR in 7 patients; 2 patients had disease progression with bone marrow involvement when the marrow was taken.

Patients were isolated in a sterile room but not always in a laminar air flow unit. They all received standardized gut decontamination, except for the patients in London, and bacteriological survey and transfusion policies were used as previously described by the participating centers.

The BEAM regimen (Table 2) consisted of carmustine given over 30 minutes, diluted in 100 cc of dextrose; etoposide, 100 mg/m^2 administered intravenously every 12 hours over 30 minutes, diluted in 200 ml of dextrose; cytarabine, 100 mg/m^2 intravenously every 12 hours over 30 minutes in 200 ml dextrose; and melphalan as intravenous bolus over 5 minutes. Forced diuresis was instituted at least on the last 3 days with a 3 l/m^2 dextrose hydration. On day 7, or at least 24 hours after melphalan and 48 hours after the last etoposide administration, autologous bone marrow was reinfused.

RESULTS

Response Rate

Details of response according to status before ABMT are shown in Table 3. For the 26 patients whose response was evaluable, the overall response rate was 19 of 26 patients (73%), with responses from 13 of 19 NHL patients (68%), and 6 of 7 Hodgkin's disease patients (85.5%). In the group of seven NHL patients with resistant relapse or progressive disease, however, only two

Table 2. BEAM Protocol

BEAM	Day 1	2	3	4	5	6	7
BCNU (carmustine) 300 mg/m²	x						
Etoposide 200 mg/m²/day		x	x	x	x		
Aracytine (cytarabine) 200 mg/m²/day		x	x	x	x		
Melphalan 140 mg/m²						x	
ABMT infusion							x

Abbreviation: ABMT, autologous bone marrow transplantation.

Table 3. BEAM Response Rate of Patients With Non-Hodgkin's Lymphoma (NHL) and Hodgkin's Disease (HD)

Patients' Status	No. of Patients	CCR	CR	PR	NR	PD	NE
NHL Patients							
Resistant relapse	7		2	1	2	1	1
and PD	2					2	
Sensitive relapse							
in PR	8		5				3
in CR	11	11					
first PR	5		5				
first CR	12	11				1	
HD Patients							
in RR	2		1	1			
in SR	5		4			1	

Abbreviations: BEAM, BCNU (carmustine), etoposide, aracytine (cytarabine), melphalan; CCR, continuous complete remission; CR, complete response; PR, partial response: NR, no response; PD, progressive disease; NE, not evaluable; RR, resistant relapse; SR, sensitive relapse.

achieved CR or even PR. Among the group of 10 NHL patients sensitive to conventional chemotherapy, or after relapse, or in PR, all achieved a CR. Among Hodgkin's disease patients, the response rate was high, with five of seven CR and only one disease progression.

Toxicity

Reduction of toxicity was a major objective in this study. The result may be summarized as follows (Table 4):

Immediate tolerance was very good, with only grade 1 nausea and vomiting after carmustine and high-dose melphalan, and with good tolerance of etoposide and cytarabine.

Table 4. Toxicity of BEAM Regimen

Mucositis: mild in 80% of patients	
Aplasia: No. of days	
WBC < 1000/mm^3: median, 17.7 (range, 7–31)	
Neutrophils < 500/mm^3: median, 19.7 (range, 10–33)	
Neutrophils < 200/mm^3: median, 13.7 (range, 4–24)	
Platelets < 50,000/mm^3: median, 26.5 (5 patients: > 45 days)	
Prolonged fever: 15	
Interstitial pneumonitis: 7 (3 fatal)	
Miscellaneous toxicities:	
1 cystitis, 1 bowel obstruction, 1 hepatic injury, 1 sudden death,	
2 renal failures (1 fatal)	
1 lung edema (1 fatal)	
General toxicity of BEAM:	
morbidity 28 patients	54%
mortality 6 patients	11.5%
5 in progression or in relapse	18.1%
1 in CR	5.8%

Abbreviations: BEAM, BCNU (carmustine), etoposide, aracytine (cytarabine), melphalan; CR, complete response.

During aplasia, when prolonged fevers were observed in 15 patients, seven of these fevers were not characterized, four were in relation to a gram-positive sepsis (3 *Staphylococcus epidermidis* and 1 *Streptococcus D*) and two in relation to a gram-negative sepsis (1 *Pseudomonas aeruginosa* and 1 *Proteus morgani*). One fungal infection with *Torulopsis glabrata* and one documented herpes infection were also recorded.

Seven patients experienced an interstitial pneumonitis. Two were not characterized, two were related to a proved cytomegalovirus infection, one to a proved *Candida albicans,* one to aspergillosis, and one to *Mycoplasma pneumoniae.* Three of the seven (1 cytomegalovirus-related, 1 aspergillosis-related, and 1 undocumented infection) were fatal.

Miscellaneous toxic effects were observed in seven patients. The two patients who had renal failure had received a cisplatin-containing regimen as second-line chemotherapy 4 weeks before the BEAM. In the patient who died of renal failure, the vancomycin level was very high after only 2 days of treatment and probably contributed to the fatal outcome. The fatal, acute lung edema observed in another patient was probably of infectious origin.

The sudden death, on day 38 without preceding or accompanying symptoms, of a 44-year-old patient as he was watching TV at home must also be recorded. We have no explanation for this death in remission (the family refused necropsy).

In summary, as the data in Table 4 show, morbidity was observed in 28 of 52 patients and toxic death in 6 patients. There was no difference in the

morbidity rate of the patients treated during relapse (18 of 28, 64.2%) and patients treated as first line (10 of 17, 59%). In contrast, a difference was observed, although not a significant one, in the toxic death rate: 5 of 28 (18.1%) patients treated during relapse died of toxic effects, compared with the deaths of 1 of 17 (5.8%) patients who received first-line treatment.

DISCUSSION

BEAM showed an effective response rate for Hodgkin's disease in this preliminary study, with six responses in seven patients, and five complete responses. These results, even if preliminary, indicated that this regimen should be investigated in relation to relapse of Hodgkin's disease in a larger group of patients.

In patients with non-Hodgkin's lymphoma, the overall response rate was 73%, with 65% of patients showing a complete response. A high response rate among patients in sensitive relapse and first PR was related to a poor response rate for patients in resistant relapse and disease progression (Table 5). Despite the small number of patients in this group, these results suggested that the BEAM regimen is not indicated for progressive disease. However, the BEAM regimen was devised for "good-risk" patients in an attempt to reduce toxicity, a goal that was achieved. Indeed, tolerance and toxicity with this regimen seemed better than those of other conditioning regimens used in NHL (4). An important improvement with BEAM was that the generally observed mucositis was very mild.

Miscellaneous complications remained of concern, however, and the interstitial pneumonitis and lung edema observed were an indirect toxic effect of carmustine and melphalan. The renal failures were cisplatin-related and could be avoided if one removed cisplatin from the salvage chemotherapy preceding high-dose melphalan.

The sudden death of a patient remains unexplained but may be related to

Table 5. Morbidity and Mortality of BEAM Regimen

Patient Condition	No. of Patients	Morbidity		Mortality	
		No.	%	No.	%
Resistant relapse, progressive disease	9	4	44.4	1	11.1
Sensitive relapse	19	14	73.6	4	21
Total	28	18	64	5	17.8
First partial response	5	3	60	0	0
First complete response	12	7	58	1	8.3
Total	17	10	59	1	5.8

Abbreviation: BEAM, BCNU (carmustine), etoposide, aracytine (cytarabine), melphalan.

Table 6. Toxic Death Rate: Comparison of BACT and BEAM

Authors	Ref.	Regimen	No. of Patients	Toxic Deaths	
				Number	Percentage
Appelbaum *et al.*	1,6	BACT	22	4	18.2
Philip *et al.*	9,10	BACT +/- TBI	17	4	23.5
Hartmann *et al.*	2	BACT-IGR	16	3	18.7
This report		BEAM	52	6	11.5

Abbreviations: BACT, carmustine, cytarabine, cyclophosphamide, thioguanine; BEAM, BCNU (carmustine), etoposide, aracytine (cytarabine), melphalan; TBI, total body irradiation; IGR, Institut Gustave-Roussy.

the case of a patient described by Hartmann *et al.* (2), a patient who also died suddenly, 14 days after BACT without other symptoms or explanations.

A comparison of the toxic death rate observed with BACT with that of BEAM (Table 6) emphasizes the fact that the toxic cost of the BEAM regimen is low. Furthermore, this toxic cost is only 5.8% for patients treated in first PR or CR.

In conclusion, we recommend the BEAM regimen because there is a good response rate in NHL patients not in CR after induction chemotherapy and in patients in relapse who are still sensitive to a rescue protocol. The toxicity of this regimen is very low (in both mild mucositis and mortality); however, more aggressive conditioning regimens should be explored for patients in resistant relapses and for primary refractory patients even if high morbidity and mortality rates are expected.

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Total Body Irradiation, High-Dose Cytarabine, and High-Dose Melphalan: A Phase II Study

J. Y. Cahn, E. Plouvier, M. Flesch, G. Souillet, P. Bordigoni, P. Lutz, P. Montcuquet, and P. Herve

The combination of total body irradiation (TBI) and cyclophosphamide followed by autologous or allogeneic bone marrow transplantation has become the preferred treatment for acute childhood lymphoblastic leukemia in second complete remission (CR2) (1-4). Recurrent leukemia remains, however, a common cause of failure in children who received transplants during CR2 after an early relapse while on therapy (2-4). We therefore began a pilot study of the high risk of relapse of acute lymphoblastic leukemia (ALL) patients, using a more intensive conditioning regimen. The efficacy of high-dose cytarabine (HD ara-C) and high-dose melphalan (HDM) for refractory leukemias (5-8) led us to explore their association, in conjunction with fractionated TBI, as a preparative regimen for allogeneic or autologous bone marrow transplantation.

In addition to the antileukemic effect of this combination, we tested its antihost-versus-graft efficiency in a group of patients who underwent familial human leukocyte antigen (HLA) nonidentical transplantation. We summarize our initial experience, including the toxicity and response rate in a group of children and adults younger than 30 years.

PATIENTS AND METHODS

Twenty-four patients (these included 19 children and 5 young adults, from 15 to 28 years old; median age was 10.5 years [range, 4-28 years]) underwent bone marrow transplantation, using a preparative regimen consisting of fractionated TBI (12 Gy, 6 fractions in 3 days) from day −11 to day −9 followed by 8 doses of HD ara-C, 3 g/m² intravenously over 3 hours every 12 hours for 8 doses (day −7 to day −4). Melphalan was administered over half an hour (140 mg/m²) under hyperhydration on day −2 (RALL 86 protocol).

Bone marrow was infused intravenously on day 0 (Table 1). All patients were isolated in a protective environment and all blood products were irradiated with 45 Gy.

Among ALL patients, 12 were treated in CR2 after early relapse on therapy, 4 in CR3 or later, and 4 during relapse.

One adult patient was treated for acute myeloblastic leukemia (AML) in second relapse, and three patients were treated for chronic myeloid leukemia (CML; one chronic, one accelerated, one acute).

Eight patients received an autologous bone marrow transplantation (ABMT) after in vitro treatment of the marrow with chemotherapy or monoclonal antibodies and complement. Nine patients received an HLA-identical allogeneic bone marrow transplantation (BMT) unmanipulated in seven of nine cases, using methotrexate for graft-versus-host disease prophylaxis until the thirtieth day, and in two cases marrow depleted of T cells (CD 2, 5, 7 and C') (9). Seven patients (three with chronic, four with acute leukemia) received transplants from familial nonidentical donors using T-cell depletion of the graft and additional immunosuppression with rabbit antithymocyte globulins (Institut Merieux, France) (manuscript in preparation). Twenty-one of the 24 patients had previously been heavily pretreated.

Table 1. RALL 86 Conditioning Regimen

Regimen	Days −12	−11	−10	−9	−8	−7	−6	−5	−4	−3	−2	−1	0
TBI 12 Gy, 6 fractions		x	x	x									
HD ara-C 3 g/m² q 12 hr						x	x	x	x				
Melphalan 140 mg/m²											x		
Transplantation													x

RESULTS

The Regimen's Toxicity

Since all 24 patients completed the preparative RALL 86 regimen, its toxicity was evaluable according to Herzig's score in all of them. The patients' toxic reactions were: reactions of the eye—10 patients, 41.7%, no severe toxicity; gastrointestinal—11 patients, 45.8%, 4 severe; CNS—4 patients, 16.7%, 3 severe including 1 lethal; skin—10 patients, 41.7%, with dry desquamation; liver—9 patients, 33.3%, 3 severe including 1 lethal; documented infections—9 patients, 37.5%, 2 lethal.

Among noninfectious toxic reactions, two were lethal in two children with ALL in CR2 (1 allogeneic BMT and 1 ABMT): lethal encephalopathy occurred a few days after transplantation in a child who had received radiotherapy (18 Gy) to the CNS 3 weeks before transplantation, and the second child died of veno-occlusive disease of the liver 35 days after transplantation. Two infection-related toxic reactions were lethal, one in a patient who developed pseudomonas cellulitis the day before transplantation, and the other in a patient who died of cytomegalovirus pneumonitis 72 days after transplantation. Both infection-related deaths occurred in patients who had received mismatched marrow.

The global mortality of patients in the pilot study was 16.6%. Engraftment was stable in all 22 evaluable patients, especially in the mismatched group who had no severe graft-versus-host disease, graft rejection, or failure.

Antileukemic Response

Seven patients (4 with ALL, 1 AML, and 2 CML) whose antileukemic response was evaluable obtained complete remissions. We need a longer follow-up to evaluate the duration of response in this group, as well as in the other patients in remission.

DISCUSSION

Different approaches to improve antileukemic treatments are being investigated as pretransplantation regimens in high-risk patients who have relapsed. The combined use of fractioned TBI, HD ara-C, and HDM has an acceptable toxicity in young patients. Only one death occurred in the ABMT group. The regimen's antileukemic activity needs to be confirmed in a larger series of patients and with a longer follow-up. Because of the encouraging results of this study, we are now evaluating the feasibility of using this protocol with lower doses of ara-C (12 g/m^2) in older patients.

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New Regimens

K. Antman and M. Symann, Chairmen

DR. W. PETERS: Dr. Frei, according to what you said about the emergence of an isosensitivity for Adriamycin in those cell lines resistant to alkylating agents, could you extrapolate from that to define the best schedule in, for instance, breast cancer to apply Adriamycin? Should we plan to put Adriamycin in frontline therapy?

DR. E. FREI: Well, that's an important question and I forgot to mention the other way in my talk. Alkylating agent-resistant lines are collaterally sensitive to Adriamycin. The issue is whether Adriamycin-resistant lines react to the alkylating agents. Although we haven't done definitive studies in that area, it would appear that they are neither cross-resistant nor collaterally sensitive. If they were collaterally sensitive, then the sequence of Adriamycin to alkylating agents would make sense. Whether the studies are such that the alkylating agents should be given first, intensively, followed by Adriamycin, I think that is a very difficult clinical strategy and I really am not prepared to support that yet. What we've decided to do is give Adriamycin first because it is a very good remission-inducing agent, probably the best in breast cancer, that will lower the tumor burden. There is no cross-resistance to the alkylating agents and hopefully that will be a setting for maximizing the response.

DR. G. SPITZER: Tom (Dr. Frei), have you studied the pattern of cross-resistance in an alkylating agent-resistant line to VP-16-213 (etoposide), and what is the dose response to etoposide in all these lines?

DR. FREI: Gary, we haven't done it.

DR. M. BERAN: Dr. Frei, we know that when cells become Adriamycin resistant, they frequently display much slower growth kinetics. What about the growth kinetics of alkylating agent-resistant cells?

DR. FREI: That's a very good question. Very often, I would say that about 80% of the time that we have an alkylating agent-resistant line, it grows a little bit slower—the doubling time may be 30 hours as compared with 24 or 26—than the parent line. It's hard to believe that it would happen in vivo because if that were to happen, you would never select it for growth. I mean the more rapidly growing cells would almost always predominate. So I'm not quite sure what that means. Occasionally, we get a line that has cytogenetic changes that has substantially slower growth kinetics. When that happens, you've obviously got to be careful in interpreting your IC_{50} data.

DR. G. DINI: I was really impressed by the results proposed by Dr. Hartmann in neuroblastoma patients treated with two ablative regimens. How many patients were treated? What was the time interval between the two transplants?

DR. O. HARTMANN: In this study, the time interval is variable.

DR. DINI: The bone marrow was never purged?

DR. HARTMANN: Yes, about half of the patients had bone marrow purged by Asta Z. The hematologic recovery was very close to that with nonpurged bone marrow transplantation.

DR. K. ANTMAN: I think we'll end this session here. Thank you.

IX. Supportive Therapy

Herpesvirus Infections After Autologous Bone Marrow Transplantation

John R. Wingard, William H. Burns, and Rein Saral

Herpesvirus infections impose substantial morbidity and mortality after allogeneic bone marrow transplantation. Detailed studies of herpesvirus infections after autologous bone marrow transplantation (ABMT) have not been published as yet. We have found reactivated herpes simplex virus (HSV) infections in patients who have received allogeneic marrow and in intensively treated patients with acute leukemia to be comparable in severity and timing (1,2). Reactivation occurs in two thirds of HSV seropositive patients during the third week after initiating cytotoxic therapy. The prophylactic use of intravenously administered acyclovir completely suppresses reactivation of the virus during administration of the drug. Because of the comparability of patients who undergo ABMT to these other two patient populations, we routinely use prophylactic acyclovir after ABMT to maximize antiviral efficacy and to minimize emergence of resistant virus. This strategy employed at our institution was reviewed previously at the first of these symposia (3).

We have reviewed our experience with cytomegalovirus (CMV) infection in 143 consecutive patients who received ABMT over a 9-year period. Twenty-seven patients (19%) developed positive CMV cultures (Table 1). Seven patients (5%) developed CMV viremia. No patient developed CMV retinitis, but three (2%) developed CMV pneumonitis.

The incidence of CMV pneumonitis (2%) after ABMT was substantially

Table 1. CMV Infections in Patients Receiving Autologous and Allogeneic Bone Marrow Transplants Over a 9-Year Period at the Johns Hopkins Oncology Center

Infection	Type of Transplant: Allogeneic n = 386	Type of Transplant: Autologous n = 143	Significance (*P* value)
Positive culture	119 (31%)	27 (19%)	.008
during first 50 days	58 (15%)	23 (16%)	NS
Viremia	49 (13%)	7 (5%)	.02
during first 50 days	23 (6%)	5 (4%)	NS
Retinitis	1 (0.3%)	0 (0%)	NS
Pneumonitis	45 (12%)	3 (2%)	.0002

Abbreviation: CMV, cytomegalovirus.

lower than that after allogeneic transplantation (12%). The rates of positive CMV cultures and CMV viremia were lower in autologous transplant recipients than in allogeneic transplant recipients. The median onset of positive cultures and viremia were 48 and 49 days, respectively. At our institution, patients who have received ABMT are routinely followed in our bone marrow transplantation clinic for only 50 days, rather than the 100 days that are routine after allogeneic transplantation, because of fewer postengraftment complications after ABMT. Thus, we examined the infection rate in the first 50 days only, to exclude a bias in surveillance. During the first 50 days, the infection and viremia rates were the same in the two types of transplantation.

Despite a high rate of CMV infection in these patients, CMV retinitis was a rare event, occurring in only one patient (an allogeneic marrow recipient) (4). This is in marked contrast to the experience with severe CMV infections in patients with the acquired immune deficiency syndrome, where CMV retinitis is much more frequent.

A comparable proportion of patients receiving both types of transplants (67% allogeneic and 70% autologous) were seropositive for CMV prior to transplantation (Table 2). Of those who were seronegative prior to transplant, 49% of the autologous marrow recipients seroconverted and 46% of the allogeneic marrow recipients seroconverted after transplant. This indicates that primary infection with CMV was common, probably because of the use of contaminated blood products and, in some cases of allogeneic transplantation, to virus contamination of the marrow. Although the risk for developing positive CMV cultures appeared to be less after ABMT than after allogeneic transplantation, irrespective of the pretransplant serology (Table 2), during the first 50 days the infection rate was similar in patients receiving either type. Both allogeneic and autologous marrow recipients with positive sera were

Table 2. Comparison of CMV Infections in Patients Receiving Autologous and Allogeneic Bone Marrow Transplantation

	Type of Transplant				
	Allogeneic		Autologous		
Infectious Indication	No.	(%)	No.	(%)	
Patients with positive pre-transplant serology/patients tested	232/347	(67%)	94/134	(70%)	P = NS
Patients with negative pre-transplant serology who seroconverted	49/106	(46%)	18/37	(49%)	P = NS
Patients with positive cultures/ Patients with:					
Positive pre-BMT serology	97/232	(42%)	24/94	(26%)	P = .009
(during first 50 days)	46/232	(20%)	20/94	(21%)	P = NS
Negative pre-BMT serology	15/115	(13%)	2/40	(5%)	P = .008
(during first 50 days)	7/115	(6%)	2/40	(5%)	P = NS
Unknown pre-BMT serology	7/39	(18%)	1/9	(11%)	P = .53
(during first 50 days)	5/39	(13%)	1/9	(11%)	P = NS
Patients with viremia/Patients with positive cultures	49/119	(41%)	7/27	(26%)	P = .21
(during first 50 days)	23/58	(40%)	5/23	(22%)	P = .20

Abbreviation: BMT, bone marrow transplantation.

more likely to have positive cultures than those with negative sera (P = .001 and P = .01, respectively).

These data support the conclusion that rates of reactivation of latent CMV and new infection do not explain the difference in rates of CMV pneumonitis. A plausible explanation for the difference in risk for CMV pneumonitis is the difference in immunodeficiency after autologous compared to allogeneic transplantation. This is supported by the finding that the frequency of CMV pneumonia in allogeneic marrow recipients without acute graft-versus-host disease (GVHD) (5%) was not significantly greater than the rate of CMV pneumonia after ABMT (2%) (Table 3). In contrast, patients with acute GVHD after allogeneic transplantation had a significantly higher rate of CMV pneumonia than either autologous or allogeneic marrow recipients without acute GVHD. This is presumably due to the immunodeficiency imposed by the GVHD itself as well as the immunosuppressive therapy used to treat GVHD.

Several new approaches to serious CMV infection have been studied in recent years. Cytomegalovirus immune plasma and immunoglobulin have been studied in several patient populations after allogeneic marrow transplantation (5-9). In general, such treatments have been beneficial in the

Table 3. The Contribution of Graft-versus-Host Disease to the Risk for CMV Pneumonitis After Bone Marrow Transplantation

Type of Transplant	Acute GVHD	Proportion With CMV Pneumonitis
Autologous	—	3 of 143 (2%)[a]
Allogeneic	No	9 of 168 (5%)[a]
Allogeneic	Yes	36 of 218 (16%)[a,b]

Abbreviations: CMV, cytomegalovirus; GVHD, graft-versus-host disease.
[a] P = .000003.
[b] P = .001.

prevention of CMV infection or CMV pneumonia, although not universally so (9): One preliminary report suggested a beneficial effect of CMV immunoglobulin in treating CMV pneumonia (10) and another did not (11). Furthermore, the benefit has occurred largely in patients who are seronegative prior to transplantation. An alternative strategy is the screening of blood products for evidence of contamination by CMV. Studies performed in a variety of patient populations including those with bone marrow transplantation (9, our unpublished observations) have demonstrated the ability to prevent CMV infection when only CMV seronegative blood products are used. An added benefit may be a more rapid recovery of platelets and reduced need for platelet transfusion support (12).

Although gancyclovir (DHPG) (9 [1-3-dihydroxyl-2-propoxymethyl] guanine) has been shown to have potent anti-CMV activity in vitro and has shown beneficial effects in the treatment of CMV retinitis in patients with the acquired immune deficiency syndrome (13,14), it has had very little beneficial effect in bone marrow recipients with CMV pneumonitis (15). Acyclovir given prophylactically in standard doses has not been effective in preventing CMV pneumonia after marrow transplantation, but a preliminary study that employed twice the standard dose (500 mg/m^2 every 8 hours administered from day −5 to day 25) has recently been reported to reduce CMV infection and pneumonia in patients who received allogeneic bone marrow (16). Although this was not a randomized trial, preliminary analysis of the various risk factors for CMV pneumonia did not reveal any other variable that might have accounted for this beneficial effect. Further analysis of this data is under way.

The frequency of reactivation of varicella zoster virus (VZV) and the severity of infection has not been studied in detail after ABMT. It has been our impression that, as with VZV infection after allogeneic transplantation, reactivation is common, occurs several months after transplantation, and frequently disseminates. Because VZV is less susceptible to acyclovir than HSV (17), higher blood levels are needed to inhibit the virus. In general, because of poor absorption of acyclovir after oral administration, adequate blood levels of the drug are not achieved for prolonged periods. Accordingly, administering oral acyclovir to patients with high risk of dissemination should be discouraged. In

contrast, intravenous acyclovir is very effective in abrogating the progession of VZV infection (18). A new drug, 2-amino-9-[(2-hydroxyethoxy)methyl] purine (descyclovir, BW515U), is an analogue of acyclovir that is metabolized by xanthine oxidase to acyclovir. Excellent absorption after oral administration occurs and high blood levels of acyclovir are achieved (19). Randomized trials comparing descyclovir with placebo in the treatment of dermatomal zoster in immunocompromised patients are currently under way and, if successful, should lead to an effective ambulatory therapy of VZV infection.

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Autologous Blood Stem Cell Transplantation: A New Treatment Concept for Patients With Malignant Lymphohematopoietic Disorders

Martin Korbling and Hans Martin

The usual source of hematopoietic stem cells for transplantation is the bone marrow. However, evidence in rodents, canines, and nonhuman primates indicates that stem cells with marrow-repopulating ability also circulate in the peripheral blood (1-4). Using the circulating blood as the primary source of stem cells with which to repopulate an aplastic bone marrow is a concept that reflects the physiological pattern within which fetal hematopoiesis develops. The fetal bone marrow becomes a site of hematopoiesis after pluripotent hematopoietic stem cells migrate into its stromal matrix via the circulating blood. Thus transfusing blood-derived stem cells in adults may be considered as repeating the prenatal seeding of hematopoiesis into the bone marrow (5,6).

In humans the repopulating ability of circulating stem cells is less well established. Goldman *et al.* (7) were the first who showed that blood-derived hematopoietic stem cells can reestablish hematopoietic function after myeloablative treatment for blast crisis in chronic myelogenous leukemia (CML). However, in these patients most stem cells collected and eventually transfused originate from a Philadelphia chromosome (Ph^1)-positive tumor cell clone. Their repopulating capability does not necessarily mean that

"normal" stem cells act the same way. In 1980, we in the Oncology Center of the Johns Hopkins Hospital in Baltimore collected blood stem cells during a transient chemotherapeutically induced Ph^1-negative phase. The retransfusion of those "normal" stem cells into the patient after myeloablative treatment resulted in complete hematopoietic reconstitution without reappearance of the Ph^1-positive cell clone, but the follow-up was too short to evaluate the reconstitutive potential of those transfused stem cells on a long-term basis (8).

There are reports of autologous blood stem cell transplantation (ABSCT) performed as an alternative to bone marrow transplantation in patients with malignant lymphohematopoietic disorders other than CML or with solid tumors (9-20; see also Reiffers *et al.* "Autologous Transplantation of Circulating Stem Cells in Acute Nonlymphocytic Leukemia," this volume). These provide clear evidence that hematopoietic engraftment can be achieved with human stem cells collected from the circulating blood rather than from the marrow site. In this chapter we describe the reconstitutive potential of blood-derived hematopoietic stem cells in patients with malignant lymphohematopoietic disorders and discuss ways of mobilizing stem cells into the peripheral blood in order to optimize the stem cell-collection procedure.

METHODS

Stem Cell Harvest

Peripheral blood stem cells were collected by "stem cell apheresis," using a continuous-flow blood-cell separator (Fenwal CS-3000, Fenwal Laboratories, Deerfield, IL). The total blood volume processed per run was 10 liters at a flow rate of 50-70 ml/minute. Centrifuge speed was 1000-1600 rpm. Anticoagulants added were 5000 U of heparin as a bolus at the start of apheresis, anticoagulant-citrate-dextrose-A (ACD-A) by continuous infusion (500 ml per run), and 5000 U of heparin injected into the cell-collection bag at the completion of apheresis. The interval between stem cell harvests was between 1 and 3 days (21). To optimize the stem cell yield, stem cell aphereses were performed in most cases after transient myelosuppression, during the subsequent expansion of the circulating blood stem cell pool.

To collect a mononuclear cell product with low RBC contamination we employed procedure number 1 (platelets) of the Fenwal system, using the 35 Collection Chamber and the Granulo-Separation Chamber. The basic computer program was modified dependent on the blood flow rate (L-68 was changed to 0750), *not* on the patient's hematocrit, and the secondary spin procedure that removes the platelets from the collected mononuclear cells was omitted to minimize possible cell loss.

Cryopreservation of Apheresis-Derived Stem Cells

The 200-ml cell suspension collected per run was concentrated to 100 ml and mixed with the same volume of Spinner-minimum essential medium supplemented with 20% dimethyl sulfoxide. The final 200-ml cell suspension was distributed into two 100-ml polyolefine bags (DELMED, Canton, MA) and frozen to −100°C in a computerized freezer (CRYOSON BV-6, Cryoson Deutschland GmbH, Schöllkrippen, West Germany). The frozen cells were stored in the liquid phase of nitrogen until use.

Thawing and Transplanting Apheresis-Derived Stem Cells

The frozen bags were thawed by immersing them into a 40°C water bath. The cell suspension was immediately injected into the patient using a central line. Postthaw washing and spinning of cells was avoided to minimize the risk of cell clumping and stem cell loss. The total volume of cell suspension injected into the patient was in the range of 1500 ml and was given over 2 hours. Forced diuresis was started after infusing 500 ml. The transfusion of the remaining free hemoglobin transiently impaired renal function (creatinine was raised to 2.0-2.5 mg/dl for 24-48 hours). In our experience this procedure produced no major risk to the patient, nor did the infusion of a cooled 6°C-8°C cell suspension into the right atrium of the heart cause any cardiac arrhythmia.

Pretransplant Conditioning Regimens

Regimen 1

Total body irradiation (TBI) using a linear accelerator, superfractionated over 4 days at 120 cGy single dose up to a total of 1320-1440 cGy (lungs, 900 cGy). Three single doses were administered per day at 8 A.M., noon, and 4 P.M.. Following TBI, cyclophosphamide (50 mg/kg) was given on each of 4 consecutive days (total dose 200 mg/kg) (patients 1, 3, and 6).

Regimen 2

TBI, superfractionated over 3 days up to a total of 1200 cGy, followed by a single dose of melphalan (120 mg/m^2) (patient 4).

Regimen 3

Cyclophosphamide (1.5 g/m^2 × 4), carmustine (300 mg/m^2), and etoposide (125 mg/m^2 × 3) (CBV regimen [22]) (patient 2).

Regimen 4

Mitoxantrone (12 mg/m^2 × 2 days), carmustine (300 mg/m^2), and etoposide (125 mg/m^2 × 3 days) (patient 5).

Regimen 5

Melphalan (200 mg/m^2) (patient 7).

ABSCT was performed 48 hours after completion of chemotherapy in regimens 1 and 3 and 24 hours after chemotherapy in regimens 2, 4, and 5.

In Vitro Stem Cell Assay

To determine the concentration of hematopoietic progenitor cells in each harvested cell suspension and in the peripheral blood before and after ABSCT, we used the human multilineage in vitro assay in methylcellulose—the human pluripotent stem cell (CFU-GEMM) assay—previously described by Fauser and Messner (23) and modified according to Ash *et al.* (24).

RESULTS

Blood Stem Cell Collection

Stem Cell Yield in Seven Patients Pretreated and Subsequently Infused With Transplanted Blood Stem Cells

Patients had received various chemotherapeutic regimens before stem cell apheresis was started.

In patient 1, who had non-Hodgkin's lymphoma, seven aphereses were performed from 2 to 4 weeks after the conclusion of chemotherapy with cyclophosphamide, vincristine (Oncovin), methotrexate, and prednisone (COMP).

Patient 2, who had recurrent Hodgkin's disease, was heavily pretreated with radiation and polychemotherapy (6 × cyclophosphamide, Oncovin, prednisone, procarabazine [COPP]; 1 × cyclophosphamide, hydroxydaunomycin [Adriamycin], Oncovin, prednisone [CHOP]; 6 × Adriamycin, bleomycin, vinblastine, dacarbazine [ABVD]) and, after his third relapse, received three cycles of hydroxydaunomycin (Adriamycin), Oncovin (vincristine), ara-C (cytarabine), prednisone, and bleomycin (HOAP-Bleo). Aphereses were performed early in the fourth remission (four runs 3-4 weeks after the first cycle of HOAP-Bleo and five runs 2-3 weeks after cyclophosphamide [15 mg/kg × 4 days] administered 4 weeks after the third cycle of HOAP-Bleo).

Patient 3, who had end-stage acute myelogenous leukemia (AML), was pretreated with two courses of 6-thioguanine, ara-C (cytarabine), and daunorubicin (TAD), two courses of amsacrine, two courses of mitoxantrone/VP-16-213 (etoposide), and one course of high-dose ara-C. Stem cells were collected by seven aphereses early in the third complete remission (CR), 3-5 weeks after the completion of the last cytotoxic treatment (second course of mitoxantrone/etoposide).

Patient 4 had sarcoma (Askin tumor). Treatment consisted of four cycles of vincristine, Adriamycin, ifosfamide, and actinomycin D (VAIA). Eight

leukaphereses were performed 4-7 weeks after the end of the fourth block of VAIA.

Patient 5, who had recurrent Hodgkin's disease, was pretreated with radiation and polychemotherapy—six courses of COPP and five of ABVD. A total of 14 aphereses were performed during the third remission, six runs 2-5 weeks after a course of cyclophosphamide (15 mg/kg × 4 days) and another eight runs 4 months later without intermittent chemotherapy.

Patient 6, who had newly diagnosed AML, was treated with two courses of TAD within 4 weeks. Ten aphereses were performed early in the first remission, 2-7 weeks after completion of the second TAD cycle.

Patient 7 had rhabdomyosarcoma and was pretreated with polychemotherapy—two courses of VAIA and two courses of cisplatin (Platinex), ifosfamide, Adriamycin, and vincristine (PIAV). A total of 10 leukaphereses was performed, six runs 2-5 weeks after a course of cyclophosphamide (15 mg/kg × 4 days) and another four runs 4 months later without further chemotherapy.

The number of leukaphereses and the cell yield per run is shown in Table 1 for each of the seven patients. The highest total stem cell number was reached in patient 1: 15.1×10^6 granulocyte-macrophage colony-forming units (CFU-GM); the lowest was in patient 4 (a 9-year-old boy weighing 25 kg): 0.25×10^6 CFU-GM.

Stem Cell Yield in Heavily Pretreated Patients After Cyclophosphamide Treatment

In nine heavily pretreated patients, aphereses were performed after a course of cyclophosphamide (15 mg/kg × 4 days). Five of them had end-stage Hodgkin's disease and were pretreated at least with cyclophosphamide, mechlorethamine, Oncovin, procarbazine, and prednisone (C-MOPP) and ABVD. One patient with rhabdomyosarcoma received prior polychemotherapy (2 × VAIA and 2 × PIAV). These patients underwent a total of 37 aphereses. The average harvest per run was 3.1×10^4 CFU-GM. Three patients with non-Hodgkin's lymphoma who had been pretreated with standard multiple chemotherapy underwent a total of 16 apheresis runs. The average number of cells harvested per run in those patients was 10.4×10^4 CFU-GM.

Figure 1 diagrams the stem cell yield per run following treatment with cyclophosphamide (15 mg/kg × 4 days). Stem cell apheresis was started 2-3 weeks after cyclophosphamide treatment. Usually six to eight runs were performed over a period of 2-3 weeks. As one can see, in this group of heavily pretreated patients, prior cytotoxic treatment with cyclophosphamide did not result in a subsequent CFU-GM overshooting as occurred in patients in early remission (25-32). The most probable explanation is an exhaustion of the mobilizable progenitor-cell pool by heavy and prolonged cytotoxic treatment.

Table 1. Cell Yield by Leukapheresis in Seven Patients Subsequently Transplanted With Autologous Blood Stem Cells

Apheresis Run No.	Mononuclear Cells $\times 10^9$							Granulocyte-Macrophage Colony-Forming Units $\times 10^3$						
	Pt. 1	Pt. 2	Pt. 3	Pt. 4	Pt. 5	Pt. 6	Pt. 7	Pt. 1	Pt. 2	Pt. 3	Pt. 4	Pt. 5	Pt. 6	Pt. 7
1	6.8	4.2	2.1	2.1	2.1	12.3	2.4	2000	620	130	6	10.5	272	4.8
2	4.5	4.8	1.5	1.6	3.8	4.8	2.7	900	300	62	63	—	114	5.4
3	6.4	4.1	2.4	2.0	2.45	3.0	2.4	ND	650	230	108	—	48	36
4	5.0	2.6	1.27	1.7	2.5	5.0	2.1	2600	340	100	10	45	20	17.8
5	15.0	3.9	1.2	3.5	0.75	2.3	2.1	3900	370	91	10	39	34	40
6	7.5	3.1	2.1	2.7	4.5	2.8	3.0	3800	1070	50	10	194	3	45
7	10.0	1.5	4.4	2.0	8.5	5.6	6.2	1900	180	490	32	68	66	37
8		5.6		0.6	4.8	4.9	3.8		530		14	9.6	68	12
9		6.3			6	4.8	2.7		210			6	34	11
10					0.95	5.9	2.9					3.8		37
11					2.5							5		
12					3.7							11		
13					4.3							39		
14					1.1							3		
Total per kg of body weight	0.77	0.53	0.20	0.65	0.75	0.58	0.5	210	60	16	10	6.7	7.5	4

Abbreviation: ND, not done.

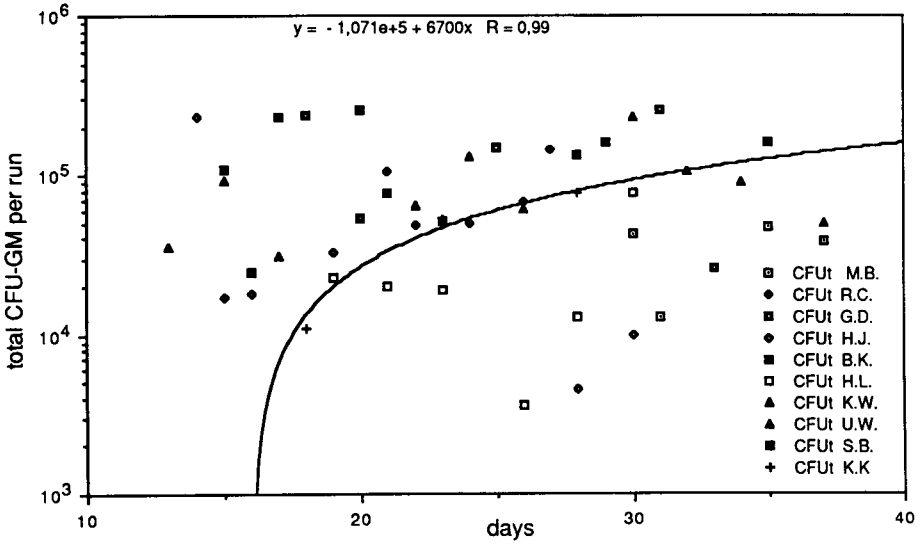

Figure 1. *Granulocyte-macrophage colony-forming units (CFU-GM) per apheresis run in heavily pretreated patients after a course of cyclophosphamide (15 mg/kg body weight x 4 days). The abscissa represents days after the beginning of the cyclophosphamide treatment.*

Reconstitution of Leukocytes and Platelets After ABSCT

Blood cell reconstitution after ABSCT occurred very rapidly in patients 1 and 2; it reached 1000 leukocytes/μl on day 9 (patient 1) and day 10 (patient 2), 500 granulocytes/μl on days 12 and 10, respectively, and 50,000 platelets/μl on day 10 for both (Figs 2 and 3). Normal blood counts were reached within 2 weeks. Both patients show complete and permanent trilineage engraftment at 21+ months (patient 1) and 10+ months (patient 2) posttransplantation.

Patients 3 to 7 received 0.4-1.6 x 10^4 transplanted CFU-GM/kg body weight (b.w.), a stem cell dose believed to be at the lower limit for safe engraftment. Compared to patients 1 and 2, leukocyte and platelet reconstitution were variably delayed. Patient 3 reached 1000 leukocytes and 500 neutrophils on days 14 and 16, respectively, after ABSCT (Figs 2 and 3). On day 120 his bone marrow was still slightly hypocellular and his WBC was about 1800, with 1000 neutrophils. Patient 4 reached 1000 leukocytes and 500 neutrophils on day 14. On day 70 he had 2300 WBCs. Patient 3 (day 120) and patient 4 (day 70) had not yet reached 50,000 platelets without platelet support.

Patients 5 and 6 reached 1000 leukocytes on days 20 and 10, respectively, and 50,000 platelets on days 18 and 15, respectively. The cell count on day 28 was 3300 and 4600 leukocytes and 405,000 and 50,000

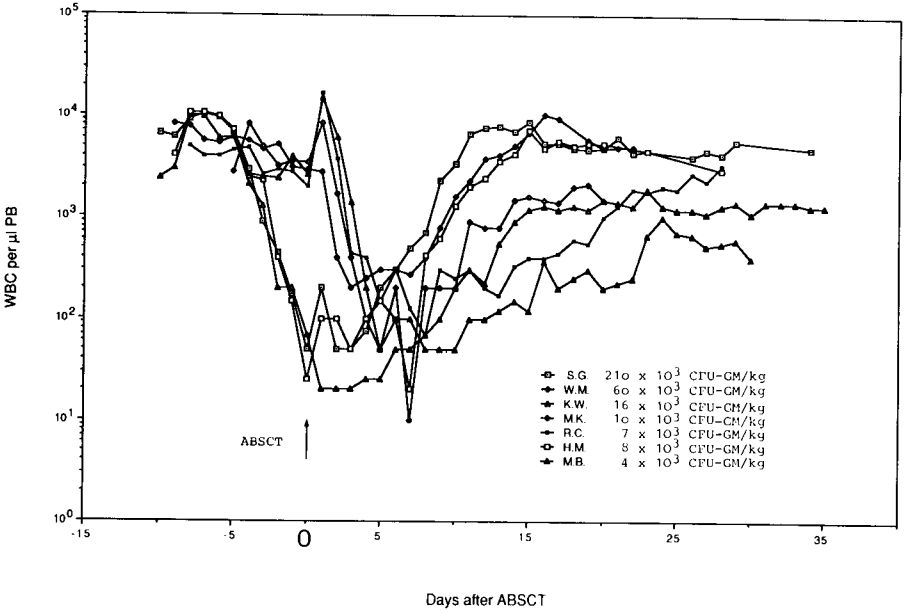

Figure 2. *Early reconstitution of WBC after autologous blood stem cell transplantation (ABSCT).*

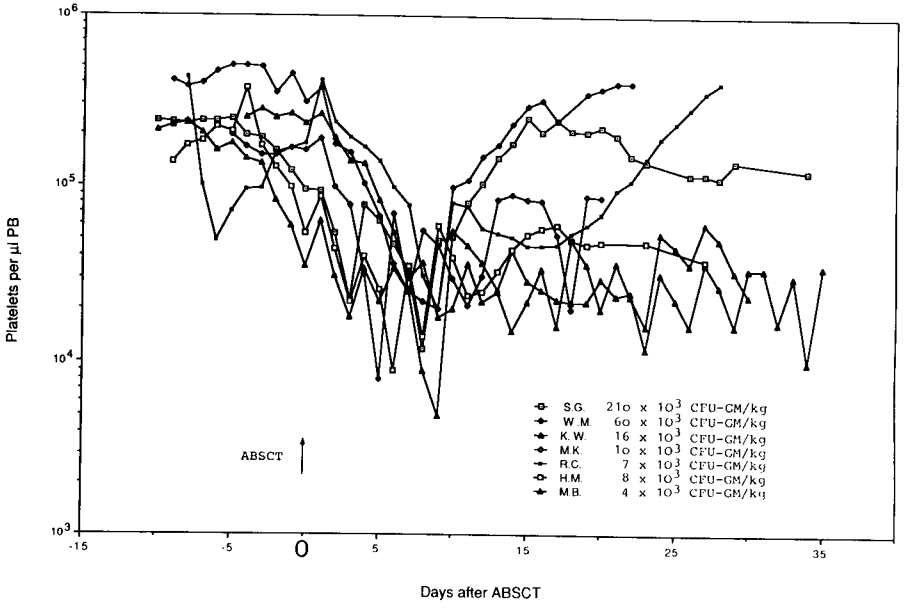

Figure 3. *Early reconstitution of platelets after autologous blood stem cell transplantation (ABSCT).*

platelets, respectively. Follow-up has been too short to assess long-term engraftment.

Patient 7 had about 600 leukocytes on day 26 and still needed regular platelet support.

DISCUSSION

To be successful, blood stem cell transplantation to restore hematopoietic function after myeloablative treatment has to fulfill two major requirements: first, hematopoietic reconstitution (including the lymphopoietic system) must be complete; and second, the reconstituted hematopoietic function must be permanent. In our two patients who received transplanted hematopoietic precursor cells in excess of 2×10^4 CFU-GM/kg b.w., hematopoietic recovery occurred very rapidly and resulted in complete and permanent trilineage reconstitution. Peripheral blood counts reached normal values within less than 2 weeks. Both patients were in a condition to be discharged from the hospital 3 weeks posttransplantation. Although the total number of CFU-GM transfused per kilogram of body weight differed by a factor of 3.5, there was no significant difference in the kinetics of cellular reconstitution (Figs 2 and 3). Follow-up at 21+ and 10+ months after ABSCT showed normal peripheral blood counts and normal bone marrow cellularity in both patients. This demonstrates that blood-derived stem cells autotransfused after myeloablative treatment are capable of sustaining long-term hematopoiesis.

In five patients transplanted with 0.4×10^4 to 1.6×10^4 CFU-GM/kg b.w., hematopoietic recovery after myeloablative therapy and ABSCT was variably delayed except in patient 6. Patients 3 and 4 stayed thrombocytopenic for 4 and 2 months after ABSCT and at last follow-up, still needed platelet support once a week. Both had about 1600-2300 leukocytes. Patients 5 and 6 who received only 0.67 and 0.75×10^4 CFU-GM/kg b.w. but high numbers of 7.5 and 5.8×10^8 mononuclear cells per kilogram of body weight had good early reconstitution, despite receiving such low numbers of CFU-GM. Long-term reconstitution remains to be evaluated.

Thus the minimal number of CFU-GM needed for safe engraftment seems to be in the range of 1×10^4/kg b.w., a stem cell dose that is confirmed by data in the canine blood stem cell transplantation model (28).

To the best of our knowledge, another 25 cases of ABSCT have been reported from seven other centers (10-19; see also the aforementioned Reiffers *et al.* chapter in this volume) (Table 2). Patients who survived ABSCT for more than 4 months showed complete hematopoietic engraftment; stable engraftment has also been noted by Reiffers and Broustet (11), Bell *et al.* (10), Juttner *et al.* (13), Castaigne *et al.* (15,16), and by Tilly *et al.* (17) with a follow-up of 4+ to 12+ months (median, 8+ months). The CFU-GM transfused per kilogram of body weight in those studies ranged from 2.3×10^4 to 23×10^4.

Table 2. Synopsis of ABSCT[a]

Reference/ Institution	Diagnosis (status prior to ABSCT)	Pretransplant Regimen	Leukocytes/kg Transfused	CFU-GM/kg Transfused	Cell Concentration After ABSCT			Follow-up
					Leukocytes 1,000/μl	PMN 500/μl	Platelets 50,000/μl	
Korbling *et al.* (9) Heidelberg, West Germany	Burkitt's lymphoma (CR1)	CY (200 mg/kg) + TBI (13.2 Gy)	7.2×10^8	21.0×10^4	d 9	d 10	d 10	Alive (21+ mo) in CR1
	Hodgkin's lymphoma (CR4)	CY (6.0 g/m²) carmustine (300 mg/m²) + etoposide (600 mg/m²)	15×10^8	6.0×10^4	d 10	d 12	d 10	Alive (10+ mo)
	AML (CR3)	CY (200 mg/kg) + TBI (13.2 Gy)	2×10^8	1.6×10^4	d 14	d 16	>d 120	Alive (4+ mo) in CR3
	Sarcoma (CR1) (Askins tumor)	TBI (12 Gy) melphalan (120 mg/m²)	6.5×10^8	1.0×10^4	d 14	d 14	>d 70	Alive (2+ mo)
	Hodgkin's lymphoma (CR3)	Mitoxantrone (24 mg/m²) carmustine (300 mg/m²) etoposide (600 mg/m²)	7.5×10^8	0.67×10^4	d 20	d 22	d 18	Alive in CR (d 37+)
	AML (CR1)	CY (200 mg/kg) TBI (13.2 Gy)	5.8×10^8	0.75×10^4	d 10	d 10	d 16	Alive in CR d 29+
	Rhabdomyo-sarcoma (CR1)	Melphalan (200 mg/m²)	5×10^8	0.4×10^4	>d 28	>d 28	>d 28	Alive (d 28+)
Bell *et al.* (10) Bournemouth, U.K.	cc-NHL (resist.)	Melphalan (140 mg/m²) + carmustine (600 mg/m²) + ara-C (400 mg/m²) etoposide (600 mg/m²)	5.1×10^8	6.0×10^4	d 10	d 10	d 14	Alive (9+ mo)

Table 2. Synopsis of ABSCT[a] (Continued)

Reference/ Institution	Diagnosis (status prior to ABSCT)	Pretransplant Regimen	Leukocytes/kg Transfused	CFU-GM/kg Transfused	Cell Concentration After ABSCT			Follow-up
					Leukocytes 1,000/μl	PMN 500/μl	Platelets 50,000/μl	
Bell *et al.* (10), Reiffers and Broustet (11); see also both chapters by Reiffers *et al.* in this volume/ Bordeaux, France	AML (Rel 1)	Etoposide (600 mg/m^2) + CY (120 mg/kg) + TBI (10 Gy)	7.8×10^8	9.5×10^4		d 16	d 35	Death in relapse (7 mo)
	AML (CR2)	Etoposide (600 mg/m^2) + CY (120 mg/kg) + TBI (10 Gy)	5.6×10^8	30×10^4	d 9	d 10	d 34	Alive in CR2 12+ mo
	AML (CR2)	Etoposide (600 mg/m^2) + CY (120 mg/kg) + TBI (10 Gy)	6.1×10^8	13.7×10^4	d 14	d 16	Immunothrombo-cytopenia (d 11)	Death due to relapse 3+ mo
	AML (CR4)	Busulphan (60 mg/kg) + melphalan (140 mg/m^2)	3×10^8	2.75×10^4	d 17	d 18	Never	Early relapse (d 45)
	AML (CR2)	Busulphan (60 mg/kg) + melphalan (140 mg/m^2)	7.6×10^8	12.4×10^4	d 12	d 14	Megakaryocyte engraftment failure	Death due to hemorrhage (d 69) without leukemia
	AML (Rel 1)	Etoposide (600 mg/m^2) + CY (120 mg/kg) + TBI (10 Gy)	10×10^8	49.7×10^4	d 8	d 9	d 11	Alive in CR 4+ mo
	AML (Rel 1)	Busulphan (60 mg/kg) + CY (200 mg/kg)	4×10^8	5.3×10^4	d 10	d 15	d 14	Alive in CR 3+ mo 55,000 platelets

Table 2. Synopsis of ABSCT[a] (Continued)

Reference/ Institution	Diagnosis (status prior to ABSCT)	Pretransplant Regimen	Leukocytes/kg Transfused	CFU-GM/kg Transfused	Cell Concentration After ABSCT			Follow-up
					Leukocytes 1,000/μl	PMN 500/μl	Platelets 50,000/μl	
Juttner *et al.* (12–14)/Adelaide, Australia	AML (Rel 1)	Melphalan (200 mg/m²)	1.3×10^8	29.0×10^4	d 14	d 14	TETE	Death in relapse (about 2+ mo)
	AML (Rel 1)	CY (120 mg/kg) + TBI (12 Gy)	3×10^8	23.0×10^4	d 16	d 16	d 16	Death in relapse (d 111)
	AML (Rel 1?)	CY (120 mg/kg) + TBI (12 Gy)	2.3×10^8	24.0×10^4	"Early trilineage engraftment"			
	AML (Rel 1)	CY (120 mg/kg) + TBI (12 Gy)	2.8×10^8	230×10^4		d 10	d 11	Alive (7+ mo)
Castaigne *et al.* (15,16)/ Paris, France	AML (CR1)	CY (100 mg/kg) + TBI (10 Gy)	1.9×10^8	2.3×10^4	d 16		d 75	Alive (8+ mo)
	AML (CR1)	CY (100 mg/kg) TBI (10 Gy)			ABSCT engraftment failure			Dead after subsequent allogeneic BMT
	ALL (Ph^1) (CR1)	CY (100 mg/kg) TBI (10 Gy)				d 21	d 19	Relapse d 60, death
	ALL (Ph^1) (CR1)	CY (100 mg/kg) TBI (10 Gy)				>d 30	>d 30	Alive d 30+
	T-ALL (CR1)	CY (100 mg/kg) TBI (10 Gy)				d 36	>d 120	Alive in CR with VOD d 120+
	ALL (CR3)	CY (100 mg/kg) TBI (10 Gy)				d 25	d 42	Relapse d 50, death

Table 2. Synopsis of ABSCT[a] (Continued)

Reference/ Institution	Diagnosis (status prior to ABSCT)	Pretransplant Regimen	Leukocytes/kg Transfused	CFU-GM/kg Transfused	Cell Concentration After ABSCT: Leukocytes 1,000/μl	PMN 500/μl	Platelets 50,000/μl	Follow-up
Tilly *et al.* (17) Rouen, France	ALL (CR1)	CY (120 mg/kg) TBI (10 Gy)		77 x 10^4		d 11	d 16	Alive in CR 6+ mo
	AML (CR2)	CY (120 mg/kg) TBI (10 Gy)		36 x 10^4		d 12	d 50	Alive in CR (4+ mo)
Kessinger *et al.* (18)/Omaha, NE, USA	Breast cancer (metastatic)	Cisplatin (125 mg/m^2) + CY (120 mg/kg) + TBI (11 Gy)	8.4 x 10^8	10.0 x 10^4	d 10	——TETE——		Death due to hepatic failure (d 12)
	Breast cancer (metastatic)	Cisplatin (125 mg/m^2) + CY (120 mg/kg) + TBI (11 Gy)	6.3 x 10^8	6.62 x 10^4		d 16	Immunothrombocytopenia 41,000 (d 43)	Death due to pneumonia (d 104)
Stiff *et al.* (19)/ Springfield, IL, USA	Small cell lung cancer ($\bar{x}$ ± SD of 3 patients)	Carmustine (900 mg/m^2) CY (4 g/m^2) etoposide (500 mg/m^2) cisplatin (100 mg/m^2)	3.8 ± 2.0 x 10^8	11.9 ± 9.9 x 10^4	d 14	d 14	>d 269 >d 129 >d 48	

[a]*This synopsis details what has been reported so far.*

Abbreviations: ABSCT, autologous blood stem cell transplantation; CR1, first complete remission; CY, cyclophosphamide; TBI, total body irradiation; CFU-GM, granulocyte-macrophage colony-forming unit; d, day; PMN, polymorphonuclear neutrophil; CR4, fourth complete remission; AML, acute myelogenous leukemia; CR3, third complete remission; cc-NHL, cytocytic non-Hodgkin's lymphoma; CR2, second complete remission; Rel 1, first relapse; TETE, too early to evaluate; ALL, acute lymphocytic leukemia; BMT, bone marrow transplantation; Ph¹, Philadelphia chromosome; T-ALL, T-cell acute lymphocytic leukemia; VOD, veno-occlusive disease.

Of particular interest is the rapid hematopoietic reconstitution after ABSCT in patients who received more than 5×10^4 CFU-GM/kg b.w.: 1000 leukocytes, including 500 granulocytes, were reached within 2 weeks after ABSCT. The rapid early rise in peripheral leukocyte counts may be explained by assuming the transfusions contained large numbers of committed progenitor cells, which, like CFU-GM in the in vitro assay, take about 10-14 days to differentiate to mature cells. Transfusing these committed progenitors, which differentiate early and subsequently appear as the first cohort of circulating neutrophils, is clinically important. It significantly reduces the times of isolation and hospitalization owing to treatment-induced neutropenia.

Long-term hematopoiesis, however, is reestablished by early pluripotent stem cells; they take longer to give rise to differentiated progeny. Hence the pattern of hematopoietic reconstitution of platelets appears to be composed of successive cohorts of cells that differentiate from various progenitor and stem cells.

Figure 4 illustrates the correlation between the numbers of mononuclear cells and CFU-GMs transfused and early hematopoietic recovery in evaluable patients from Table 2. Early hematopoietic recovery seems to correlate more with the number of mononuclear cells transfused ($r = .65$ for 1000 WBC and $r = .54$ for 50,000 platelets) than with the number of CFU-GMs transfused ($r =$

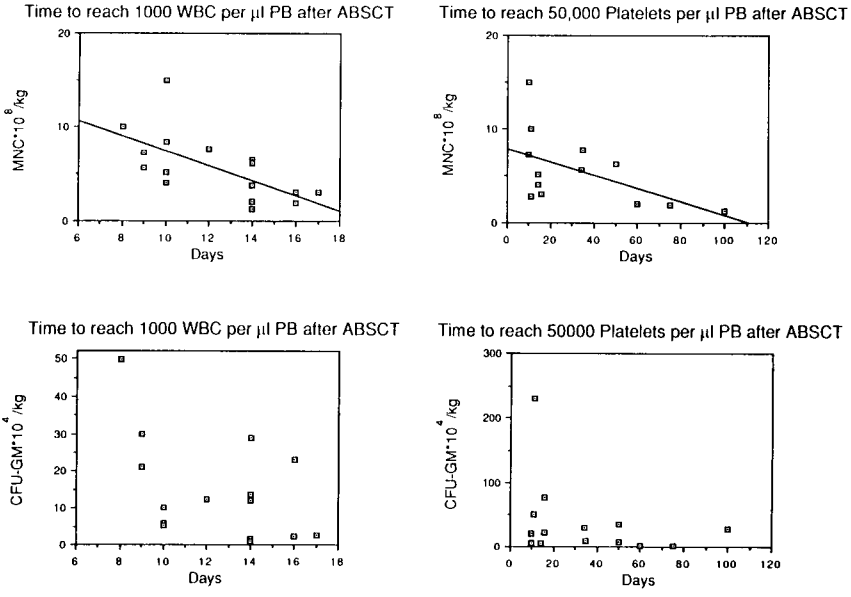

Figure 4. *Correlation between number of cells transfused and hematopoietic recovery after autologous blood stem cell transplantation (ABSCT).*

.43 for 1000 WBC and $r = .28$ for 50,000 platelets). Our experience further strongly suggests that the cytotoxic treatment prior to leukaphereses influences both the yield and the repopulating ability of progenitor and stem cells. However, although rapid early engraftment appears to be predictable by transfusing sufficient CFU-GMs, which can be assayed in vitro, the stability of long-term hematopoiesis after ABSCT cannot yet be assessed by in vitro tests but remains dependent on clinical studies.

In contrast to consistent early leukocyte reconstitution, platelet recovery after ABSCT varied (see Table 3). In our patients 1, 2, and 5, platelet recovery was very rapid. They reached 50,000 platelets within 2 weeks after ABSCT. Nine of the patients reported so far have shown this rapid platelet recovery. Delayed platelet recovery for various reasons (detailed in Table 3) occurred in our patients 3, 6, and 7 (10,13) and was reported by Reiffers *et al.* (10,11; see also the aforementioned chapter and Castaigne *et al.* [15,16], Kessinger *et al.* [18], and Stiff *et al.* [19]). Patients who received less than 2.5×10^4 CFU/GM/kg b.w. (except for our patient 6) consistently had delayed platelet recovery. Two cases of immunothrombocytopenia and one case of megakaryocytic engraftment failure were also reported.

Five of the patients with AML (10-14; see also both chapters by Reiffers *et al.* in this volume) who received ABSCT while in overt relapse eventually died in subsequent relapse. In three of their four patients, Juttner *et al.* (12-14) reported a consistent rapid early, but later incomplete, engraftment despite transfusions of $23\text{-}29 \times 10^4$ CFU-GM/kg b.w. collected in the very early first-remission phase of AML (26). The late incomplete engraftment in three of the patients may be due to underlying leukemia. Alternatively, the pattern of engraftment may be explained by the transfusion of abundant numbers of committed but insufficient numbers of pluripotent stem cells. It must be reemphasized, however, that stable long-term engraftment was achieved in all patients who survived ABSCT disease free for more than 4 months.

Table 3. Platelet Recovery After ABSCT
(Data from 32 patients shown in Table 2)

Recovery	No. of Patients
Very rapid (50,000 plts. within 16 days)	9
Slow (50,000 plts. within 34–50 days)	5
Delayed (CFU-GM transfused $< 2.5 \times 10^4$/kg)	6
Delayed without explanation (all 3 patients in one center [19])	3
Immunothrombocytopenia	2
Megakaryocytic engraftment failure	1
Early relapse	1
TETE due to early death/Data not available	3

Abbreviations: ABSCT, autologous blood stem cell transplantation; plts., platelets; CFU-GM, granulocyte-macrophage colony-forming units; TETE, too early to evaluate.

It is well known that circulating progenitor cells in humans are increased during recovery from myelosuppressive chemotherapy (25). Large numbers of CFU-GM circulate also in very early remission after induction therapy for AML (26-29). The CFU-GM compartment in these patients is preferentially expanded 10-25 fold, but CFU-GEMM are increased only about three-fold (13,28). In all centers performing ABSCT for leukemia or lymphoma (9-17; see also both chapters by Reiffers *et al.* in this volume), hematopoietic progenitor and stem cells were collected during the recovery phase after various chemotherapy regimens.

Mobilizaton of progenitor cells, however, appears to depend very much on the patients' pretreatment. In our series of heavily pretreated patients with lymphoma we could not observe an expansion of the circulating progenitor cell pool after a course of cyclophosphamide (15 mg/kg/b.w. x 4 days), a finding not previously reported in humans. Heavy and prolonged cytotoxic treatment seems to exhaust the mobilizable progenitor cell pool. This is in agreement with canine data (30-34). Consequently, circulating progenitors should be collected as early as possible after diagnosis or after achieving first complete remission.

Besides cytotoxic treatment, which causes subsequent expansion of the peripheral blood stem cell pool, a number of other approaches designed to increase the peripheral blood stem cell concentration have been reported. The administration of endotoxin (35,36), pyran copolymer (37) and related agents, or low-molecular-weight dextran sulfate has been studied in animal models, but these compounds have not yet been approved for clinical use. Other factors have not yet produced conclusive or reproducible results; they include exercise and the use of corticosteroids or activators of endogenous steroid production (ACTH, prednisone, hydrocortisone) to mobilize peripheral stem cells to overshoot after transient chemotherapy-induced myelosuppression (40-42). Recombinant human granulocyte-macrophage colony-stimulating factor may eventually prove useful.

Transplantation of circulating stem cells may have advantages over marrow-derived stem cells. For example, in patients at risk for general anesthesia, continuous-flow apheresis offers an alternative and safe way to harvest stem cells. Harvesting, processing, and freezing blood stem cells is simple and can be done in blood banks, like the handling of any other blood component. Furthermore, stem cell harvesting is feasible when the marrow collection site has been damaged by previous radiotherapy or tumor involvement.

A further factor favoring ABSCT is that hematopoietic reconstitution after myeloablative treatment and ABSCT seems to be more rapid for the WBC line, and therefore the aplasia-related risks in the early posttransplantation period are lowered, provided sufficient numbers of stem cells are transplanted. And finally, the ratio between normal hematopoietic stem cells and clonogenic tumor cells in the peripheral blood of patients with malignant

lymphohematopoietic disorders in remission may be in favor of the stem cells, although this hypothesis is yet to be proved.

ABSCT seems to be a viable treatment method in three types of settings. It can be used supportively, to augment the reconstitutive potency of quantitatively inadequate autologous marrow collection. It has demonstrated therapeutic activity against acute leukemia in CR, non-Hodgkin's lymphoma, and perhaps against resistant multiple myeloma. And it can be used prophylactically in persons who are at risk for severe radiation exposure or in those with a genetic predisposition for malignant lymphohematopoietic disorders.

This study has shown that apheresis-derived hematopoietic stem cells are able to reconstitute hematopoiesis *completely* and *permanently.* Furthermore, if sufficient numbers of stem cells are transfused (more than $1\text{-}2 \times 10^4$/kg b.w.), hematopoietic reconstitution occurs rapidly. At present, to enable hematopoietic stem cells to be collected efficiently, the patient must have been adequately pretreated cytotoxically. Other, and perhaps more effective, approaches for mobilizing hematopoietic stem cells into the peripheral blood are needed and are currently under investigation.

ABSCT after myeloablative chemoradiotherapy promises an alternative approach to autologous bone marrow transplantation that provides additional safety because of low aplasia-related risks. The possible long-term benefit to the patients who receive the transplantation must be further proven, however, in additional clinical trials.

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Toxic Deaths After Autologous Bone Marrow Transplantations: Rate and Etiology

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Toxic deaths are the major problem after bone marrow transplantation. The toxic death rate reported in the world literature after autologous bone marrow transplantation (ABMT) is in the range of 8-20%. It seems not to be related to the specific disease, i.e., it is 21% in lymphoma (1,2) and Hodgkin's disease (3), 20% in neuroblastoma (see T. Philip *et al.*, "Bone Marrow Transplantation in an Unselected Group of 65 Patients With Stage IV Neuroblastoma," in this volume), 12% in acute myeloid leukemia (4) and Burkitt's lymphoma (see T. Philip *et al.*, "Autologous Bone Marrow Transplantation in Burkitt's Lymphoma: 50 Cases in the Lyons Protocol," in this volume), 10% in Hodgkin's disease (5), and as low as 8% in breast cancer (6). However, despite controversy (1), a slightly but not significantly higher toxic death rate seems to be related to regimens incorporating total body irradiation (TBI) (Philip, unpublished data).

Our primary objectives in this retrospective study were to determine the toxic death rate in a group of 103 patients after ABMT and also to try to correlate status before ABMT with fatal complications. A secondary objective was to separate toxic deaths into early (i.e., before day 30 post-ABMT) and delayed (30-100 days post-ABMT) occurrences.

PATIENTS AND METHODS

From 1980 to 1985, 103 courses of high-dose chemotherapy followed by ABMT were administered in our unit to 100 patients with various malignancies (Table 1). Three patients received two courses each. Seventy patients were males and 30 were females; their median age was 19 ± 15 years, and 51 patients were younger than 15 years old.

The patients were categorized according to their status at grafting into one of three groups: selected poor prognosis at first complete remission (CR), 20 patients; sensitive relapse, 40 patients; and resistant relapse, 40 patients. The conditioning regimens used, which were varied, included TBI in addition to chemotherapy for 38 patients, high-dose melphalan alone or in combination, 66 patients, and high-dose cyclophosphamide (with TBI or in combination), 29 patients. For each patient, a vascular access was fitted with two silicone rubber catheters, the first one for parenteral nutrition, the second one for transfusions, antibiotics, and other possible supportive therapies.

Fifty-eight courses were administered in a sterile-care unit with laminar air flow and 45 in conventional rooms, but all the patients were treated by the same team and received identical supportive care. When a patient developed a temperature of 38° C or greater, he was examined for signs of infection; blood and urine cultures were taken whenever antibiotic therapy was instituted. The initial antibiotic regimen was based on the previous bacterial results (oral, gut, skin) obtained routinely twice a week. The usual patient surveillance also includes daily ionograms and blood counts and, twice weekly, liver function tests, a urine chemical study, and chest x rays. Critically ill patients were transferred to the intensive care unit.

To optimize the study of adverse reactions, postmortem examination was performed, if possible, for every patient who died, early or later, after ABMT.

Table 1. Types of Malignancies

Tumor Type	Number
Lymphomas	41
Neuroblastomas	15
Germinal tumors	11
Ewing's tumors	10
Soft tissue sarcomas	7
Small cell lung cancers	5
Other tumors	11

RESULTS

Fifty-nine patients went into CR after ABMT, 23 achieved partial remission (PR), and 17 did not respond to chemotherapy; four courses were unevaluable. Overall, the long-term persisting CR rate was 23% (23 patients), with a median continuous complete remission follow-up of 34± months (range, 12-67 months).

Death was considered to be toxic when not a result of the tumor. Toxic deaths were separated into early toxic deaths (before the recovery of a neutrophil count of 500/ml, i.e., 0-30 days in general) and delayed toxic deaths (after recovery from granulocytopenia).

Despite the difficulty of establishing the accurate cause of every death, we chose from among the several potentially lethal events for each patient a primary cause of death. The fatal complications in the early and late periods are presented in Tables 2 and 3. Other events are also specified.

The fatal complications were: aspergillosis (eight patients), veno-occlusive disease (VOD) (five patients), viral interstitial pneumonia (three patients), central nervous system hemorrhage (three patients), cyclophosphamide-related cardiac failure (two patients), systemic candidiasis (two patients), and toxic hepatitis (one patient). The cause of death could not be determined for two patients.

The status of patients at the time of toxic death differs between the early and late periods. From 0 to 30 days after ABMT, toxic death occurred in 19 patients. Their pre-ABMT statuses were resistant relapse (RR) in 11 patients, sensitive relapse (SR) in 6, and CR in 2. Among these patients, seven were in CR at the time of death. From days 30 to 100 after ABMT, toxic death occurred in nine patients. Their pre-ABMT status was RR in two patients, SR in five, and CR in two. Six patients were in CR at the time of death.

DISCUSSION

Our toxic death rate is within the range of other studies or a little higher, perhaps because of the high proportion of patients in RR who had a low Karnofsky scale.

The principal cause of death appears to be infectious in origin. Aspergillosis occurred in nine patients, eight of whom died despite specific early therapy with amphotericin B. The cause of failures may be explained by the frequency of disseminated forms of aspergillosis in six of the nine patients and multiresistant *Aspergillus* in the other three. To minimize the incidence of this often fatal disease, we have treated all patients in a laminar air flow room since 1985, and no case of aspergillosis has developed since then. Candidiasis appears to be difficult to diagnose, and our two cases of fatal candidiasis were only suspected during the patients' lifetime. Only one patient received a short course of amphotericin B, without evident result. Now that candidal antigens

Table 2. Deaths During Granulocytopenia

Patient/ Age/Sex	Diagnosis	TBI	Status Before ABMT	Status at Death	Principal Cause of Death	Other Events
1/33/M	Germinal tumor		RR	CR	Septicemia	Pneumonia
2/47/M	Lung cancer		RR	CR	Aspergillosis	
3/9/F	Ewing's tumor	+	RR	PR	Septicemia	Pneumonia
4/7/M	Lymphoma		SR	NE	Viral pneumonia	
5/5/M	Lymphoma	+	SR	CR	Toxic hepatitis	
6/52/M	Lung cancer	+	SR	PR	Cardiac failure	
7/33/M	Lymphoma	+	CR	CR	Aspergillosis	CNS bleeding
8/4/M	Ewing's tumor		RR	PR	Viral pneumonia	
9/34/F	Breast cancer	+	RR	PR	CNS bleeding	Pneumonia
10/6/F	Neuroblastoma		SR	CR	Aspergillosis	
11/19/M	Lymphoma		RR	NE	Aspergillosis	
12/15/M	Lymphoma		RR	PR	VOD	Enteritis
13/8/M	Neuroblastoma	+	CR	CR	VOD	ARF
14/57/M	Lymphoma		SR	NE	Aspergillosis	
15/12/F	Soft tissue sarcoma		RR	PR	Candidiasis	
16/22/M	Germinal tumor		RR	PR	Aspergillosis	
17/20/F	Germinal tumor		SR	NE	CNS bleeding	
18/57/M	Lymphoma		RR	PR	Unknown	ARF, GI hemorrhage
19/20/M	Hodgkin's disease		RR	NE	Cardiac failure	

Abbreviations: TBI, total body irradiation; ABMT, autologous bone marrow transplantation; CR, complete remission; PR, partial response; SR, sensitive relapse; RR, resistant relapse; NE, not evaluable; VOD, veno-occlusive disease; CNS, central nervous system; ARF, acute renal failure; GI, gastrointestinal.

can be detected, immunohistochemically, we hope for earlier diagnoses. Viral diseases seem to be less important, and we have observed no fatal interstitial pneumonia despite our using nonselected transfusion products. Similarly, even without any prophylaxis against *Pneumocystis carinii,* we observed only two cases of pneumonia after ABMT.

Death by septicemia is rare (in 2 of 58 patients). The first was caused by fulminant alpha *Streptococcus* infection in a young patient, and the second by a multiresistant *Pseudomonas* sepsis in another patient.

Table 3. Deaths After Recovery From Granulocytopenia

Patient/ Age/Sex	Diagnosis	Status Before ABMT	Status at Death	Delay of Death/ABMT (days)	Principal Cause of Death	Other Events
1/28/M	Lymphoma	SR	CR	32	Aspergillosis	CNS bleeding
2/16/F	Lymphoma	SR	CR	44	VOD	CNS bleeding
3/1/M	Medullo-blastoma	CR	CR	32	CNS bleeding	Pneumonia
4/26/M	Ewing's tumor	RR	PR	44	Candidiasis	
5/3/M	Neuroblastoma	CR	CR	36	VOD	CNS bleeding
6/39/M	Lung cancer	SR	PR	31	VOD	CNS bleeding
7/19/F	Soft tissue sarcoma	SR	CR	36	Aspergillosis	CNS bleeding
8/30/M	Germinal tumor	RR	PR	37	Viral pneumonia	
9/1/M	Medullo-blastoma	SR	CR	62	Sudden death unexplained	

Abbreviations: ABMT, autologous bone marrow transplantation; CR, complete remission; PR, partial response; SR, sensitive relapse; RR, resistant relapse; VOD, veno-occlusive disease; CNS, central nervous system.

We now consider deaths of noninfectious origin to be the major problem. Since 1985 we have observed only eight toxic deaths after 77 courses of ABMT, and six of the eight were of noninfectious origin. Cyclophosphamide cardiomyopathy occurred in two high-risk patients. One had received previous thoracic irradiation and a high cumulative dose of Adriamycin (doxorubicin); the other developed atheromatous coronaropathy. The complication was always fatal. VOD was responsible for the deaths of three patients in CR. No treatment was efficient in the acute phase of the VOD (five patients). Portocaval shunt or hepatic transplantation, which has been proposed for subacute VOD (7), was not considered for the acute phase. Preventive therapies have never been assessed, except in animals, but heparin seems to be a possible preventive agent against the damage of hepatic irradiation (8). We plan to evaluate in a randomized study the possible advantage of heparin administered during conditioning regimens as a preventive agent of VOD.

We can conclude that 1) the risk of toxic death is higher for patients in RR, but it is present for patients in SR or CR or responsive to the conditioning regimen; 13 of 28 toxic deaths occurred during CR in our study; 2) a well-

defined antibiotic strategy and the systematic use of laminar air flow procure a good control of infectious complications; and 3) the incidence of noninfectious complications, like VOD, appears now to be our principal problem.

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Infectious Complications of Autologous Bone Marrow Transplantation

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Infectious complications are a major concern following autologous bone marrow transplantation (ABMT). Numerous intrinsic and extrinsic factors combine to place patients at risk for a variety of infectious complications. Despite the increased use of ABMT in the treatment of refractory malignancies, the scope of infectious complications remains poorly characterized. As a consequence, patients receiving ABMT have been treated in accordance with principles derived from the care of patients undergoing allogeneic bone marrow transplantation, in whom infectious complications have been more carefully studied (1). Such an analogy may not be warranted. Patients receiving ABMT tend to have shorter periods of neutropenia, do not require prophylactic posttransplantation immunosuppressive therapy, and do not develop graft-versus-host disease. These differences may result in a different spectrum of infectious complications.

Our study reviews the courses of 35 patients with a variety of underlying malignancies who underwent ABMT. We reviewed the methods of infection prophylaxis employed and recorded the nature of infectious complications encountered. We then compared our findings to the established patterns of infection seen in patients who receive allogeneic bone marrow transplanta-

tion. In this chapter we discuss the implications of our findings for the development of prophylaxis and treatment principles for patients undergoing ABMT.

METHODS

We retrospectively reviewed the charts of all patients who received ABMT at the University of Oklahoma Health Sciences Center between January 1983 and July 1986. All patients were evaluated clinically prior to transplantation. Fever was not an absolute contraindication to transplantation. Hickman central venous catheters were placed in all patients prior to transplantation. Patients were treated in ordinary private hospital rooms; handwashing and masks were the only isolation precautions employed. Antibacterial, antifungal, or antiviral chemoprophylaxis was employed at the discretion of the attending physician.

At the onset of fever, blood and urine cultures were routinely obtained. Other cultures were obtained as clinically indicated. All patients then received broad-spectrum antibacterial therapy.

The patients' courses from the time of admission for ABMT to the time of recovery of marrow function and discharge from the hospital or until death were reviewed. Complications possibly or definitely related to infection, as defined below, were tabulated. Oral mucositis or diarrhea was recorded if the presence of either was noted in the physicians' progress notes on 1 or more days. Fever was defined as an oral temperature recorded at greater than or equal to 38.5°C. The identification of any bacterial growth from a blood culture in a febrile patient was considered significant bacteremia. Localized bacterial infections were determined by clinical definition; 50% were confirmed microbiologically. Invasive fungal infection was defined as isolation of a fungus from blood or a normally sterile body fluid or histopathologic evidence of invasive fungal infection. The diagnosis of mucocutaneous fungal or viral infection required the presence of lesions with a characteristic clinical appearance, with or without microbiologic confirmation (obtained for 40%). A course was categorized as unexplained febrile if no definite infections were clinically or microbiologically detected; patients with these courses may have had mucositis, diarrhea, or both.

RESULTS

The patients' age, sex, underlying diseases, and pretransplantation laboratory data are shown in Table 1. All patients received high-dose chemotherapy; eight also received total body irradiation. Antimicrobial prophylaxis was administered at the discretion of the attending physician; the regimens employed are shown in Table 2. Patients were transplanted with $2.27 \pm 0.14 \times 10^8$ autologous, cryopreserved nucleated marrow cells per kilogram. The

Table 1. Patient Characteristics and Pretransplantation Laboratory Data

Mean age (range), yrs	34.2 (2–55)
Men/women	16/19
Underlying disease	
Leukemia/lymphoma	12
Acute myelogenous leukemia	4
Acute lymphoblastic leukemia	2
Lymphoma	6
Other malignancy	23
Breast carcinoma	9
Melanoma	6
Testicular carcinoma	2
Malignant fibrous histiocytoma	2
Neuroblastoma	1
Ewing's sarcoma	1
Adenocarcinoma of colon	1
Undifferentiated sarcoma	1
Mean (± SEM) hemoglobin (g/dl)	10.88 ± 0.31
Mean WBC count ($\times 10^3/mm^3$)	8.85 ± 1.43
Mean platelet count ($\times 10^3/mm^3$)	294 ± 23
Mean albumin (g/dl)	3.58 ± 0.09
Mean creatinine (mg/dl)	0.88 ± 0.04

Table 2. Antimicrobial Prophylaxis Employed

		Day[a] Begun		Days Duration	
	n	Mean	Range	Mean	Range
Antibacterial					
Trimethoprim/sulfamethoxazole	18	−6.9	−17 – 0	5.4	3–10
Other	2	−8	−10 – −6	24	10–38
Antifungal					
Oral nonabsorbable	25	−2.4	−13 – +6	26.6	3–80
Ketoconazole	1	−4	—	13	—
Antiviral					
Acyclovir i.v.	10	−3	−14 – +9	21.8	9–39

[a]*From day 0, the day of bone marrow transplantation.*

patients were granulocytopenic (WBC < 200/mm^3) from 1 to 39 days (mean, 13.6 ± 1.36 days).

The infectious complications encountered are shown in Table 3. Four patients were febrile at the time of transplantation, two of whom had infections at the Hickman catheter exit site. All of the remaining patients became febrile during their posttransplantation course. Fever occurred within 7 days of transplantation in 30 of the 35 patients (86%). A microbiologically confirmed or clinically apparent infection, other than mucositis or diarrhea, was found in 23 of the 35 patients (66%).

All of the patients received broad-spectrum antibacterial therapy (mean duration, 17 days). This usually consisted of the combination of an extended-spectrum beta-lactam antibiotic and an aminoglycoside, most commonly ticarcillin and tobramycin. Eleven patients received systemic antifungal therapy: four ketoconazole, six amphotericin B, and one ketoconazole followed by amphotericin B. Thirteen patients received systemic antiviral therapy with acyclovir.

Bacterial infection occurred in 21 patients (60%). Sixteen patients had bacteremia and nine had localized bacterial infections. Thirteen gram-positive bacteria were isolated from the blood of 10 patients: streptococci 4, *Staphylococcus aureus* 2, *S. epidermidis* 2, *Corynebacterium* JK 2, and peptostreptococcus and diphtheroid 1 each. Eight gram-negative bacteria

Table 3. Infectious Complications Encountered[a]

	n	%
Mucositis	20	57.1
Diarrhea	25	71.4
Fever ≥ 38.5° C	35	100
Any bacterial infection	21	60.0
Gram-positive bacteremia	10	28.6
Gram-negative bacteremia	7	20.0
Localized bacterial infection	6	17.1
Mucocutaneous fungal infection	12	34.3
Invasive fungal infection	2	5.7
Mucocutaneous viral infection	13	37.1
Systemic viral infection	1	2.8
New pulmonary infiltrate	7	20.0
Unexplained febrile course	12	34.3

[a]*Because some patients had more than one complication, the total is > n = 35 and 100%.*

were isolated from blood: *Pseudomonas aeruginosa* 4, *Klebsiella pneumoniae* 3, and *Escherichia coli* 1. The local sites of infection were the Hickman catheter exit site in six patients and a paranasal sinus, a pilonidal cyst, and a perirectal abscess in one each. Three of the Hickman catheter exit site infections were associated with bacteremia.

Eighteen patients received trimethoprim-sulfamethoxazole (160/800 mg orally twice daily) as prophylaxis for bacterial infections (Table 2). Seventy-five percent of the group who received this prophylactic regimen developed bacterial infection compared to 40% of the group not receiving any antibacterial prophylaxis (chi-square test, $P = .036$). There was no significant difference in the incidence of fungal or viral infections between those who did or did not receive trimethoprim-sulfamethoxazole.

Mucocutaneous fungal infections were common, occurring in 12 patients (34.3%). Nine patients developed oropharyngeal candidiasis, two developed *Candida* vaginitis, and one had perineal candidiasis. However, there were only two cases of invasive fungal disease. *Candida albicans* was isolated from the blood of one patient on the 31st posttransplant day and *C. parapsilosis* was isolated from the blood of a second patient on the third posttransplant day. Additionally, seven patients with persistent unexplained fever were given an empiric course of amphotericin B therapy, but none had defervescence clearly attributable to systemic antifungal therapy.

A majority of patients (26/35) received oral nonabsorbable antifungal agents as attempted prophylaxis for fungal infection. There was no significant association between the use of this therapy and the development of mucocutaneous fungal infection. The two episodes of fungemia developed in patients who were receiving antifungal prophylaxis, but the incidence of this complication is too low to allow meaningful analysis of the efficacy of prophylaxis.

There were 13 cases of mucocutaneous viral infection. Six patients had culture-confirmed oropharyngeal herpes simplex virus infection. Six others had clinically characteristic oropharyngeal herpes simplex virus infection and one had localized herpes zoster. One initially seronegative patient developed a high positive titer of antibody to cytomegalovirus and was therefore considered to have had systemic cytomegalovirus infection.

Ten patients received antiviral prophylaxis with intravenous acyclovir (15 mg/kg/d). None of these patients developed evidence of mucocutaneous or systemic herpetic infection. Twenty-five patients did not receive such prophylaxis, and 13 of this group developed mucocutaneous herpes virus infection. Thus acyclovir prophylaxis was shown to significantly reduce the incidence of mucocutaneous herpes virus infection (chi-square test, $P = .004$).

Seven patients developed new pulmonary infiltrates in the posttransplantation period. The infiltrate was not clearly attributable to an infection in any of these cases. Two of the patients underwent bronchoscopy, but this did not lead to definitive diagnosis in either. The new pulmonary infiltrate resolved in four patients (all received broad-spectrum antibacterial therapy). The

infiltrate in one patient was attributed to hemorrhage and in another, to pleural malignancy. One patient was found to have fibrosing alveolitis at postmortem examination.

There were six deaths in the posttransplantation period monitored in this study. Only one of these was attributable to infection: a patient who died of septic shock syndrome with blood cultures positive for a multidrug-resistant *K. pneumoniae*. Two of the deaths were related to recurrent malignancy. One death resulted from hemorrhage, for one sudden death no apparent cause was detected at postmortem examination, and one death was associated with a progressive interstitial pneumonitis found to be fibrosing alveolitis at postmortem examination (as described above).

DISCUSSION

The patterns of infections that emerged in the patients undergoing ABMT in our study share similarities but also manifest important differences from those previously reported in patients undergoing allogeneic bone marrow transplantation. Bacterial infections were common (60%) in our patients. This is similar to the rate of bacterial infection reported in the first 30 days after allogeneic bone marrow transplantation (1). The distribution of gram-positive and gram-negative bacterial isolates is similar to that reported in other series of patients with profound neutropenia (2).

Mucocutaneous fungal infections were common (34.3%) in our patients, about as common as reported in allogeneic transplantation patients (1). However, invasive fungal disease was rare (5.7%). This contrasts sharply with a reported 33% incidence of invasive fungal infection in patients undergoing allogeneic bone marrow transplantation. This may have important implications for the employment of empiric systemic antifungal therapy, which has become standard management with allogeneic bone marrow transplantation (1), but may not be beneficial with ABMT.

Mucocutaneous herpes simplex virus infections were common (approximately 50%) in patients undergoing ABMT and not receiving antiviral prophylaxis, an incidence similar to that reported in allogeneic bone marrow transplantation (1). We are unable to retrospectively determine the historical or serologic status of these patients with respect to herpes simplex virus. Our study confirms the efficacy of acyclovir as prophylaxis for mucocutaneous herpes simplex virus infections in immunocompromised hosts (3,4) and extends its usefulness to the ABMT patient population.

The increase in bacterial infections in patients given trimethoprim-sulfamethoxazole prophylaxis found in this study must be interpreted with caution because of the retrospective nature of our study. Additionally, the timing and duration of administration of this prophylactic regimen varied greatly over the period of our study. Numerous prospective studies of the efficacy of prophylactic trimethoprim-sulfamethoxazole have been recently

reported (5-8). Young's analysis of the conflicting results of these studies is a useful review (9). Our findings, in addition to the recently reported experiences of the EORTC International Antimicrobial Therapy Project Group (10) and Henry *et al.* (11), make the usefulness of this prophylactic regimen appear quite uncertain.

We conclude that infectious complications in patients undergoing ABMT are substantially different from those reported in other immunocompromised patient populations. We are performing multivariate analysis of our data to further elucidate factors associated with the development of infectious complications in conjunction with ABMT. Further prospective study of the issues raised by this type of retrospective analysis are required to develop specific guidelines for infection prevention and treatment in this unique patient population.

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Infectious Complications of Autologous Bone Marrow Transplantation in Children With Neoplastic Diseases

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Modern regimens of massive antitumor therapy followed by autologous bone marrow transplantation (ABMT), causing a prolonged period of granulocytopenia, render patients highly susceptible to bacterial and fungal infections. Moreover, as occurs with patients who undergo allogeneic bone marrow transplantation, these procedures may also be responsible for late episodes of viral and protozoal infections (1). However, although the overall mortality from infection varies from 6% to 33% in comparable series (2-5), very few comprehensive studies have been conducted so far that deal specifically with infection.

In order specifically to study infections associated with ABMT and to prepare a protocol for their management, we have prospectively evaluated all febrile episodes that developed in 42 patients, 25 boys and 17 girls aged 7 months to 17 years (median, 5 years) who, from October 1984 to September 1986, underwent ABMT for the treatment of various types of neoplastic diseases.

MATERIAL AND METHODS

Some characteristics of ablative treatment as well as details of our patient care are published elsewhere in this book; therefore in this chapter we only summarize data concerning infection control.

Before administering ablative therapy, we evaluated pharyngeal, intestinal, and urinary flora for the presence of "difficult" bacterial and fungal agents; these included *Pseudomonas* spp., *Salmonella* spp., and enteropathogenic *Escherichia coli* in stool and urine samples and *Staphylococcus*, streptococci (groups A, B, and D), *Haemophilus influenzae*, enterobacteria, and fungi on throat swabs. If any one of these pathogens was found, antibiotic prophylaxis was started in an attempt to eliminate it. In addition, before and after ABMT, we evaluated sera for herpes simplex virus, cytomegalovirus (CMV), *Toxoplasma gondii*, and *Legionella* spp.

Every febrile episode was defined according to the criteria of the International Antimicrobial Therapy Cooperative Group of the European Organization for Research on Treatment of Cancer (6) as a microbiologically documented infection (MDI) with or without septicemia or as a clinically documented infection (CDI). CDI included pneumonias for which no culture specimen was available and severe necrotizing mucositis from which only a mixed flora was isolated. Episodes lacking in clinical or microbiologic documentation were defined as possible infections (PI) or doubtful infections (DI). As a superinfection we defined all MDI and CDI that developed after at least 24 hours of recovery (defervescence) from the previous episode.

At the development of fever (at least three subsequent measurements >38°C or a single measurement >39°C), culture specimens were taken and an empirical antibiotic treatment was initiated combining amikacin and ceftazidime with or without vancomycin. With the aim of detecting bacterial, viral, and fungal superinfections, the microbiologic surveillance was continued during the episodes by both direct (culture) and indirect (counterimmunoelectrophoresis) methods. The microbiologic procedures were the classic ones (7), and blood cultures were incubated under both aerobic and anaerobic conditions.

As far as predisposing factors to infection are concerned, we assumed that the different ablative treatments used in treating various underlying diseases (Table 1) caused a similar toxicity. In evaluating the duration of fever and granulocytopenia, however, patients who received total body irradiation (TBI) were considered separately from those who did not receive it (non-TBI).

RESULTS

Consequent to the ablative treatment, all patients experienced a prolonged period of granulocytopenia (< 1000 polymorphonuclear neutrophils [PMN]/μl) that lasted a mean of 21 days (range, 5-55 days) in patients after

Table 1. ABMT in Children With Neoplastic Diseases

Ablative Therapy	Doses	Underlying Disease	No. of Patients	
Vincristine Melphalan TBI	4 mg/m² 140 mg/m² 3.33 Gy × 3 days	Neuroblastoma Ewing's sarcoma	23 2	25
Vincristine Melphalan Lung irradiation	4 mg/m² 180 mg/m² 14 Gy	Nephroblastoma		5
Peptichemio	900 mg/m²	Neuroblastoma Rhabdomyosarcoma	3 1	4
Vepeside (etoposide) Melphalan	1 g/m² 180 mg/m²	Acute non lympho-blastic leukemia		3
Teniposide (VM-26) Adriamycin Belustine (lomustine)	1 g/m² 45 mg/m² 300 mg/m²	Neuroblastoma		2
Cyclophosphamide TBI	1800 mg/m² 3.33 Gy × 3 days	Acute lymphoblastic leukemia		1
Melphalan Vincristine Melphalan	180 mg/m² 4 mg/m² 180 mg/m²	Rhabdomyosarcoma Nephroblastoma		1 1
		Total patients		42

Abbreviations: ABMT, autologous bone marrow transplantation; TBI, total body irradiation.

TBI and of 19.5 days (range, 16-25 days) in patients without TBI. In all patients but one, such a period was complicated by a febrile episode (95% morbidity). We observed an interesting correspondence between the median duration of both fever (>38°C) and neutropenia. In fact, considering as "day 0" the day in which bone marrow was reinfused, the PMN count dropped below 100/μl on day 3 (range, 1-5 days) in patients who received TBI and on day 5.5 (range, 2-7 days) in those who did not, while patients in both groups became febrile on day 3.5 (range, 1-9 days). The PMN count increased above 100/μl on day 11 (range, 4-22 days) in both groups, and fever disappeared on day 13.5 (range, 9-31 days) in those with TBI and on day 12 (range, 10-20 days) in those without TBI. Finally, the median duration of fever was 10.5 days (range, 5-23 days) in patients who had TBI and 8.5 days (range, 3-18 days) in those who did not, while neutropenia between 100 and 500 PMN/μl lasted 9 days (range, 2-29 days) with TBI and 7 days (range, 3-14 days) without TBI.

Table 2 shows the characteristics of the febrile episodes. MDI (48%) and CDI (19%) were documented in 67% of all febrile episodes, while 33% of them

Table 2. Fever in Autologous Bone Marrow Transplantation

Category	No. of Patients	Infection	
		Type	No.
MDI	20	Septicemias	11
		Localized	9
CDI	8	Pneumonias	2
		Necrotizing stomatitis	5
		Necrotizing stomatitis and vulvitis	1
PI	14		
Total	42[a]		

[a]*One patient was affected simultaneously by septicemia and urinary tract infection.*
Abbreviations: MDI, microbiologically documented infection; CDI, clinically documented infection; PI, possible infection.

remained unexplained (PI). No doubtful infections were observed. Of MDI, 55% were septicemias and 45% localized infections (4 of 9 urinary tract infections and 5 of 9 necrotizing stomatitis).

Table 3 shows the MDI-causing pathogens. Gram-positive bacteria represented 60% of all isolates (14 of 23) and gram-negative, 35% (8 of 23). In one patient the infectious episode was primarily sustained by a reactivated

Table 3. Microbiologically Documented Infections in Children Undergoing Autologous Bone Marrow Transplantation

Infection	No. of Cases	Organism
Septicemias	11	2 *S. aureus*
		1 *S. epidermidis*
		4 *Strept. sanguis*
		1 *Strept. mitis*
		1 *E. coli*
		1 *E. coli* + *Corynebact.* spp.
		1 *E. coli* + *Haemophilus influenzae*
Urinary tract	4	*Strept. faecalis*
		E. coli
		E. coli + *Strept. faecalis*
		Proteus spp.
Necrotizing stomatitis	5	*S. aureus*
		S. aureus
		Anaerobic streptococci
		Enterobacter cloacae
		Herpes simplex virus (reactivation)
Localized	9	

herpes simplex virus. While they were persistently neutropenic, 9 of 41 patients (22%) developed a further febrile episode, which was due to a bacterial infection in three instances (one pneumonia, one urinary tract infection, and one septicemia), to a viral infection in two instances (herpes simplex virus stomatitis), and to a fungal infection in three instances (one *Trichosporon beigelii* and two *Candida albicans* septicemias). In one patient the superinfection was mixed in both etiology and localization (herpes simples virus stomatitis and *E. coli* urinary tract infection).

The outcome was successful in all patients but two; one died of a bacterial pneumonia (*Enterobacter cloacae, Streptococcus faecalis,* and group G *Salmonella*) that complicated a previous pneumonia of unknown origin. The second patient died of noninfectious causes. Therefore the overall mortality was 4.7%, but the mortality from infection was 2.3%. Late infection was rare. Only 1 of the 31 patients who had reached a reasonable length of follow-up (100 days) experienced infectious problems (herpes zoster successfully treated with acyclovir). No patient developed interstitial pneumonitis.

The serologic surveillance for CMV indicated that 20 of 31 patients (65%) were seropositive before ABMT. None had clinical evidence of CMV reactivation. Out of 11 patients who were CMV-negative, 10 were still negative after ABMT, while the serum of 1, the only 1 who received granulocyte transfusions, became positive for CMV. However, even this patient did not show any clinical evidence of CMV infection, and urine cultures remained persistently negative.

DISCUSSION

Infection remains a major problem in patients undergoing ABMT. However, mortality owed to infection (at least in our series) is, so far, acceptable.

In this series, nearly 60% of febrile episodes resulted from a clinically or microbiologically documented infection, while 40% of them, even if strongly compatible with an infectious etiology, remained substantially unexplained. This is in accordance with the experience of the authors who have dealt with the problem of infections in neutropenia and with our previous works (8-11). Gram-positive microorganisms predominated, especially among septicemia-causing pathogens, and we believe that some of these, especially streptococci, derive from the oral flora and probably penetrate the bloodstream through oral mucosal lesions.

As shown by the correspondence of the febrile curve with the evolution of the PMN count, bone marrow recovery is crucial for a satisfactory outcome from the infectious episode. Antibiotics may be only the bridge upon which the patient can cross the period of severe immunosuppression (12).

As already documented by others (13), herpes simplex virus has often

been found, especially as a superinfecting pathogen; it is quite possible, therefore, that an acyclovir prophylaxis is a useful consideration. Respiratory infections occurred in two of our patients. Both developed pneumonia during the first month after ABMT. One died as a direct consequence of the pneumonia (bacterial pulmonary superinfection on a previous pneumonia of unknown origin), and the second died as a result of multiple organ failure while his pneumonia was improving. In our experience, lat interstitial pneumonia and CMV infection or reinfection has not occurred as a complication of ABMT.

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Newer Antibiotic Regimens in Cancer Patients

Kenneth V. I. Rolston

The use of empiric antimicrobial therapy for the management of febrile episodes in neutropenic cancer patients is now accepted as standard practice. Bacterial infections in this group of patients are caused by a variety of gram-positive and gram-negative organisms that form the endogenous flora of the skin, gastrointestinal tract, and oropharynx (1). In order to provide broad-spectrum coverage before the identification of the causative pathogen(s), empiric regimens have traditionally consisted of combinations of two (or more) antimicrobial agents (2). The agents commonly used are the aminoglycosides (gentamicin, tobramycin, amikacin), the antipseudomonal penicillins (ticarcillin, mezlocillin, piperacillin), and the extended-spectrum or third-generation cephalosporins (moxalactam, cefoperazone, ceftazidime). Several prospective trials using a variety of such combinations have demonstrated overall clinical responses in 70-80% of patients (3-6).

A number of recent developments have prompted clinical investigators to reevaluate the empiric regimens frequently employed in neutropenic patients. These developments include: a) the availability of several newer agents such as the monobactams, the carbapenems, and the quinolones, which might be equivalent or superior to older agents or combinations; b) increasing resistance of common gram-negative and gram-positive pathogens to older agents; c) a change in the pattern of organisms causing infections in cancer patients; and d) the escalating cost of medical care and

the need to reduce these costs. Thus, it is important to continue to evaluate newer agents and regimens and to compare them to standard ones, in an effort to provide optimum therapy under current conditions.

COMBINATION THERAPY

Antimicrobial combinations are used for the following reasons: a) to provide a broad antimicrobial spectrum; b) to reduce the incidence of the emergence of resistant organisms; c) to reduce toxicity (by giving reduced doses of toxic drugs); and d) for potential synergistic interaction.

Various antibiotic combinations have been evaluated and are listed in Table 1. Many investigators prefer the use of aminoglycoside-containing combinations because of the greater chances of achieving synergy (7). However, aminoglycosides are not very effective agents in neutropenic patients, and some investigators believe that β-lactam is the effective agent in such combinations, whether or not synergy is present (8). Most aminoglycoside-containing combinations have been associated with an overall response rate of between 70% and 80% (9).

Other problems associated with aminoglycoside therapy include the high incidence of adverse reactions (oto- and nephrotoxicity), the need to monitor peak and trough levels, the need to monitor renal function, and the frequent use of other nephrotoxic agents (amphotericin B, cisplatin, cyclosporin, vancomycin), which may preclude the use of aminoglycosides. Combinations that do not include an aminoglycoside are also listed in Table 1.

Double β-Lactam Combinations

Combinations of antipseudomonal penicillins with extended-spectrum cephalosporins have also been extensively evaluated in neutropenic cancer patients. The carboxypenicillins, carbenicillin and ticarcillin, and the ureidopenicillins, mezlocillin, azlocillin, and piperacillin, are the agents used most often. Timentin (ticarcillin + clavulanic acid [a β-lactamase inhibitor]) is also a potentially useful compound (10). The cephalosporins used most often have been moxalactam, cefoperazone, and ceftazidime (11-13). Double β-lactam combinations, like aminoglycoside-containing combinations, have also been associated with 70-80% response rates. However, the incidence of oto- and nephrotoxicity has been much lower.

Miscellaneous Combinations

In recent years, gram-positive organisms such as coagulase-negative staphylococci, CDC-JK diphtheroids, and *Bacillus* spp. have emerged as significant pathogens in some institutions. This has made it necessary to provide initial empiric coverage with agents such as vancomycin which is active against a majority of gram-positive organisms. This approach has been shown to decrease the morbidity associated with gram-positive infec-

Table 1. Therapeutic Options in Neutropenic Patients

Aminoglycoside-containing Combinations
Aminoglycoside + carboxypenicillin or ureidopenicillin
Aminoglycoside + extended-spectrum cephalosporin
Aminoglycoside + penicillin + cephalosporin
Aminoglycoside + monobactam (aztreonam)[a]
Aminoglycoside + carbapenem (imipenem)[a]
Aminoglycoside + TMP/SMX
Aminoglycoside + timentin (ticarcillin = clavulanic acid)
Other Regimens
Double β-lactam combinations
Vancomycin + penicillin or cephalosporin or monobactam[a]
TMP/SMX + penicillin (or cephalosporin)
Monotherapy (single-agent therapy)
Extended-spectrum cephalosporin[a]
Carbapenems[a]
Quinolones (ciprofloxacin)[a]
Combinations with β-lactams/aminoglycosides
Single agents

[a]*Need further clinical investigation.*
Abbreviation: TMP/SMX, trimethoprim/sulfamethoxazole.

tions; however, whether there is a reduction in the overall mortality is not yet clear (14). Vancomycin can either be added to standard aminoglycoside-containing or double β-lactam combinations, or can be used in combination with penicillins, cephalosporins, or newer agents such as the monobactams (aztreonam) (15).

Agents such as trimethoprim/sulfamethoxazole (TMP/SMX) can be used in combination with β-lactams or aminoglycosides (16). However, the widespread use of TMP/SMX for prophylaxis has limited its use as an effective therapeutic agent.

MONOTHERAPY

With the availability of broad-spectrum agents such as cefoperazone, ceftazidime, and the penem primaxin, the question of monotherapy (or single-agent therapy) needs to be reexamined. Studies done to date indicate that these agents could be used alone for the initial empiric therapy of febrile episodes in neutropenic patients (17-20). Further studies, however, are being conducted to determine their role in comparison to standard regimens, particularly in the treatment of infections caused by *Pseudomonas aeruginosa* and in polymicrobial infections. The emergence of resistant organisms might also limit this approach. These questions should be answered in the forthcoming years.

The 4-quinolone derivatives are newly developed antimicrobial agents

with a broad antimicrobial spectrum including most gram-positive and gram-negative organisms (21). They have the added advantage of being available for oral administration since they are well absorbed from the gastrointestinal tract. They have not yet been fully evaluated in neutropenic patients, but if found to be effective, could result in considerable reduction in the costs associated with antibiotic therapy.

SUMMARY

Many therapeutic options are available for the management of infections in neutropenic patients. No single approach can be considered universally applicable since, very often, local factors (different microflora and resistance patterns) are quite variable in different institutions. Newer agents, which are potentially more potent and less toxic, might improve the overall clinical outcome of neutropenic patients in the near future. The emergence of resistant organisms may limit the usefulness of some of these newer agents. Therefore, greater efforts need to be directed toward the prevention of infectious complications in neutropenic patients.

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The Empiric Use of Amphotericin B for Unexplained Neutropenic Fever: Toxicity and Efficacy

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Fevers developing in neutropenic patients with acute leukemia who are undergoing intensive chemoradiotherapy generally are treated with broad-spectrum antibacterial agents. Because patients with prolonged neutropenia have a significant risk of invasive fungal infections, and because fungal infections are difficult to diagnose antemortem, empiric therapy with amphotericin B has been widely used (1-3). Several studies have shown a reduction in clinically or autopsy-documented fungal infections when amphotericin B was used to treat unexplained neutropenic fevers (4-7). In a previous study from our institution (8), prompt defervescence after empiric amphotericin B administration has been documented also in most patients randomized to receive amphotericin B therapy after 5 days of unexplained neutropenic fever. Here we report our experience with amphotericin B empirically given in intensively treated cancer patients over a 3-year period on one inpatient unit. Amphotericin B therapy for patients with unexplained neutropenic fevers, administered at relatively rapid infusion rates, resulted in resolution of fever in the majority of patients and was well tolerated.

MATERIALS AND METHODS

Patients

The hospital records of 107 patients representing 138 consecutive admissions to the Ireland Cancer Center inpatient unit were reviewed. Median patient age was 42 years, with a range of 17-68 years. Disease categories are listed in Table 1. Sixty-eight treatment courses involved intensive chemotherapy without bone marrow support, and 70 treatment courses were associated with intensive chemoradiotherapy and bone marrow transplantation.

Supportive Care

Supportive care measures included oral therapy with trimethoprim-sulfamethoxazole, one double-strength tablet twice daily at the start of antineoplastic therapy, and either oral Mycostatin suspension or clotrimazole troche therapy at the development of neutropenia (polymorphonuclear leukocytes < 500/μl). Neutropenic fevers, defined as single oral temperature elevations of greater than or equal to 38.2° C in patients with neutrophil counts of less than or equal to 500/μl, were treated with broad-spectrum antibacterial agents after blood, urine, sputum, stool, and other appropriate cultures were obtained. The most commonly employed antibacterial agent regimen included intravenous nafcillin (2 g every 4 hours), tobramycin (5 mg/kg/day), and ticarcillin (300 mg/kg every 4 hours). Vancomycin (500 mg intravenously every 6 hours) was substituted for nafcillin when central venous catheter-related infections occurred, or when semisynthetic penicillin-resistant staphylococci were identified on culture. Tobramycin dosages were measured and adjusted routinely to keep serum concentrations in therapeutic

Table 1. Disease Status

Disease	No. of Patients (n = 107)
Acute nonlymphocytic leukemia	24
Acute lymphoblastic leukemia	11
Hodgkin's disease	8
Non-Hodgkin's lymphoma	8
Malignant melanoma	22
Small cell carcinoma—lung	6
Non-small cell carcinoma—lung	6
Colorectal carcinoma	6
Carcinoma—breast	2
Other	14

and safe ranges. Penicillin-allergic patients usually were treated with vancomycin, tobramycin, and a third-generation cephalosporin such as ceftazidime.

Amphotericin B Therapy

Based on data that demonstrated prompt defervescence after empiric administration of amphotericin B in persistently febrile neutropenic patients (8), amphotericin B was added routinely after a patient had experienced 4-5 days of unexplained neutropenic fever. Amphotericin B was used also when unexplained fever recurred in neutropenic patients who had previously become afebrile after broad-spectrum antibacterial agents had been given, and whenever a fungal infection was documented or suspected clinically.

Amphotericin B was administered as a slow infusion of a 1-mg or 10-mg test dose, followed on most occasions by the infusion of an additional dose of amphotericin B to constitute the entire maintenance dose on the same day. Daily maintenance doses consisted of 0.6 or 0.7 mg/kg of actual body weight. Amphotericin B was mixed in 150-250 ml of 5% dextrose in 0.9% saline, 500-1000 U of heparin, and 100 mg of hydrocortisone hemisuccinate, and was infused through a central venous catheter over 1½-2 hours. Patients received premedication consisting of 650 mg of acetaminophen by mouth and 50 mg of diphenhydramine hydrochloride intravenously. Meperidine, 25 to 50 mg intravenously, was added as needed to treat rigors (9). In some patients, amphotericin B therapy was modified during the treatment course so that it was administered every other day. Amphotericin B doses were not modified in cases of renal insufficiency because of the drug's lack of significant renal excretion. Total amphotericin B doses were determined according to indication and the patient's clinical response. Patients with documented fungal infections received a total dose of 1.0-1.5 g. In patients in whom fungal infections were not documented but fever resolved after amphotericin B administration, at least a 300-mg total dose was given. For patients who did not have documented fungal infections or who did not respond clinically, amphotericin B therapy was discontinued when neutropenia resolved.

Clinical response to amphotericin B antifungal drug treatment was defined as resolution of unexplained fever (for at least 72 consecutive hours) during amphotericin B therapy. Renal toxicity was defined as a rise in serum creatinine of greater than or equal to 0.5 mg/dl if the baseline creatinine (immediately before amphotericin B or antibacterial agents) was less than or equal to 3.0 mg/dl, or a rise of greater than or equal to 1.0 mg/dl if the baseline creatinine was more than 3.0 mg/dl (10).

RESULTS

Seventy-five patients receiving 87 separate courses of amphotericin B were evaluated for toxic reactions and treatment efficacy. Thirty-two patients who received 51 comparable intensive chemoradiotherapy courses did not

receive amphotericin B, and 35 of these treatment courses were complicated by neutropenic fever that required broad-spectrum antibacterial agents. Median duration of neutropenia for all patients was 18 days (range, 9-80 days). Patients who received amphotericin B had a median of 18 days (range, 10-80 days) of neutropenia compared to a median of 17 days (range, 9-32 days) in those who did not receive this drug ($P > .1$, NS; Student's t test). In eight patients, fungal blood cultures were positive. Three patients had fungemia with *Candida albicans*, two had *C. tropicalis*, one had *Torulopsis glabrata*, one had noncandidal yeast not further identified, and one had *C. krusei* and *Saccharomyces cerevisiae*. Positive throat, stool, and urine cultures were seen in 28, 32, and 9 patients, respectively.

Median duration of unexplained neutropenic fever before amphotericin B administration was 5 days and ranged from 0 to 32 days. Median total dose of amphotericin B was 460 mg (range, 10-2450 mg). Fifty-eight of 87 treatment courses (66.7%) were associated with fever resolution in a median of 3 days (range, 1-18 days); this defervescence corresponded to an amphotericin B dose of 135 mg (range, 10-900 mg). Of 15 patients with positive fungal blood cultures, or the same fungus culture from at least two other unrelated sites, 12 (80%) had resolution of their fever during amphotericin B administration.

Adverse reactions to amphotericin B are listed in Table 2. Most patients experienced rigors, which usually responded to meperidine. Only 24% of patients developed fever that could be attributed to the amphotericin B. Hypotension (systolic blood pressure < 90 mm Hg) was uncommon and resolved in all cases by slowing the infusion rate and administering crystalloid fluids. Bronchospasm was observed infrequently but was severe enough in two patients to necessitate permanent discontinuation of the amphotericin B. Forty-five of 87 treatment courses were associated with a significant rise in creatinine, the median rise being 0.5 mg/dl (range, 0.0-2.6 mg/dl). By comparison, similarly treated patients who received broad-spectrum antibacterial drugs for neutropenic fever, but not amphotericin B, had a median creatinine rise of 0.1 mg/dl, with a maximum value of 2.9 mg/dl. No patient underwent hemodialysis for renal failure. Marked potassium and magnesium

Table 2. Amphotericin B Adverse Reactions

Reaction	No. of Treatment Courses (n = 87)	% of Treatment Courses
Rigors	78	90
Fever	21	24
Bronchospasm	8	9
Hypotension	8	9
Increased creatinine	45	52

renal loss was observed in most patients, but complicating factors such as use of cisplatin or semisynthetic penicillin (ticarcillin, carbenicillin, or mezlocillin), or lysozymuria associated with acute nonlymphocytic leukemia, made assessment of amphotericin B-induced renal tubular damage difficult (11-13). Pulmonary infiltrates occurred in 23 patients for a variety of reasons. None of the patients had, however, received WBC transfusions. In three of those patients, diagnostic open-lung biopsy was performed to aid in management. One patient was found to have *C. tropicalis* as the primary pathogen; the other two lung biopsy specimens were negative for fungus. Seventeen patients, nine of whom had received amphotericin B, died during their hospitalization. At postmortem exam, four patients had documented fungal infections, including one each of *Aspergillus* sp., *C. albicans, C. tropicalis,* and *C. krusei.* Three of the four patients had responded previously to amphotericin B, as evidenced by prompt defervescence, and they were still receiving amphotericin B at the time of their deaths.

DISCUSSION

Amphotericin B is widely used empirically to treat unexplained neutropenic fevers in acute leukemia patients undergoing intensive chemoradiotherapy regimens. Although improved survival has not been documented, amphotericin B therapy has resulted in clinical improvement and is associated with a reduced risk of fungus-related deaths (4-8). Concerns regarding indications for use, undetermined end point for total doses, and toxicity continue to limit its application.

We report our experience with 107 patients undergoing 138 treatment courses; amphotericin B was administered in 87 of the treatment courses. Although rigors occurred in almost all patients, meperidine therapy usually was adequate to control symptoms. Bronchospasm, hypotension, and fever were uncommon and rarely required discontinuation or dosage adjustment. Bronchospasm generally responded to prophylactic inhaled sympathomimetic agents, and in only two patients was it severe enough to lead to permanent discontinuation. All patients with hypotension responded to fluid administration and slowing of the infusion rate. Renal toxicity was frequent but mild, reversible, and no patient required peritoneal or hemodialysis. Toxic effects were acceptable, moreover, despite infusion rates that were considerably more rapid than are usually described.

Resolution of unexplained neutropenic fever occurred in 66.7% of 87 amphotericin B courses; these responses usually were observed during the first 3 to 4 days of therapy. Although autopsy data are limited, invasive fungal infections were documented in only four patients at postmortem exam. Furthermore, of three open-lung biopsies performed, only one showed evidence of invasive fungal infection. All other pulmonary infiltrates resolved with appropriate empiric antifungal therapy. None of the amphotericin B-

treated patients received WBC transfusions, which have been associated in one series with an increased risk of pulmonary infiltrates (14). Pulmonary toxicity, however, has been attributed to amphotericin B even in the absence of WBC transfusion and may have contributed to the abnormal x-ray findings in some of our patients (15).

Although the efficacy of empiric amphotericin B therapy in reducing the risk of invasive fungal infections cannot be assessed directly in this study, our experience is consistent with other published data that demonstrate frequent clinical improvement after amphotericin B administration. Many of our patients had intensive treatment for solid tumors, and thus belonged to a group not usually at risk for invasive fungal infection during therapy. Toxicity, moreover, was acceptable in our patients despite the use of rapid infusion rates since only two patients required discontinuation of antifungal drug owed to adverse reactions. Empiric amphotericin B therapy for unexplained neutropenic fever is a safe and effective treatment for patients undergoing intensive antitumor chemoradiotherapeutic regimens. We are conducting additional studies of the role of surveillance fungal cultures or fungal serologic studies to define more specific indications for amphotericin B therapy in neutropenic patients.

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The Role of Intravenous Immune Globulin in Autologous Bone Marrow Transplantation

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Immune globulin has been used primarily as replacement therapy for congenital and acquired immunodeficiencies (1). In addition, using selected donors, it can render passive immunity to a variety of viral diseases, including hepatitis B (2,3), herpes zoster, variola, and rabies (4,5). Since the introduction of serum immune globulin for intravenous use (IVIg) (6), protection against viral infection in conjunction with renal transplantations has been possible (7). Other therapeutic considerations have included suppression of alloimmune platelet destruction (8,9) and suppression or prevention of life-threatening bacterial infections (10-12). Of most interest is the reduced incidence of cytomegalovirus (CMV) pneumonitis in patients who have received allogeneic bone marrow transplants (13-18). However, IVIg did not as consistently reduce the incidence of other interstitial pneumonitides (IP), and CMV infections, nor was longevity increased markedly. Because of its overall safety (19,20) and the rarity of anaphylactic reactions (21), the use of IVIg in allogeneic transplantation has been generally accepted.

Its role in autologous bone marrow transplantation (ABMT), however, has not been explored as thoroughly. The immunologic deficiencies (22-26) and infectious complications (27) of patients following allogeneic bone marrow transplantations are well known, but they are not as well documented

in autologous transplant recipients. One recent study of 70 patients with leukemia or lymphoma who received ABMT showed a 10% incidence of IP; three were due to CMV, one to *Pneumocystis carinii*, and three were idiopathic. The incidence seemed to be correlated with prior radiation (28). Another review identified CMV pneumonitis in 1 of 14 patients after ABMT, a patient who received granulocyte transfusions (29). We have recently compiled data on 34 patients who received ABMT as treatment for a variety of malignancies; half of them received prophylactic IVIg (manuscript in preparation). One patient who did not receive IVIg died of transfusion-related CMV pneumonitis, and three had self-limited nonpneumonitic infections; only one in the latter group had received IVIg. The infection rate is obviously too low to detect a role for IVIg in CMV prophylaxis.

Nevertheless, immunologic problems still exist in conjunction with ABMT, including other infections, fevers, and gastrointestinal difficulties. In our patient population, over half of the ABMT recipients have an episode of fever with bacteremia some time during treatment (unpublished data). Gastrointestinal problems, including debilitating mucositis and diarrhea, occur in up to 45%. Especially when radiotherapy is combined with intensive chemotherapy, diffuse mucosal changes have occurred within 10 days of initiation of therapy. Changes include severe cellular atypia in villous and crypt epithelium and eventually necrosis of damaged tissue; repair begins between days 16 and 20. Damage to the mucosal barrier and decreased intestinal motility, mucus production, and acid production (30,31) increase the potential for bacterial attachment to the mucosa (32). Furthermore, this damage to the mucosal barrier, immunodeficiency and neutropenia resulting from the cytoreductive therapy, and changes in flora due to antibiotic therapy may place the marrow recipients at high risk of developing potentially severe gastrointestinal and systemic infections, a large proportion of which arise from abdominal sources (33). Enteric viruses, including rotaviruses (34), adenoviruses (34,35), CMV (34-37), Coxsackie A1, and herpes simplex (38,39), have been problematic in causing enteritis among patients who receive transplants; it is unclear how many of these viruses are acquired (35) or reactivated (34) in the hospital.

Theoretically, therefore, orally administered immune globulin could serve a prophylactic function. Locally produced antiviral IgA appears to be critical protection from bowel and systemic infections (40), and it, like all other antibodies, is deficient in the posttransplant period. Research has shown that intestinal antibody helps to inhibit the adherence of *Vibrio* bacteria to rabbit intestinal mucosa (41). In another study, a systemic antibody response alone to polio vaccine, without a concomitant response in the intestinal tract, did not necessarily inhibit continued intestinal carriage of the organism (40). These two studies point to a role for exogenous oral immune globulin in prophylaxis against enteric infections. Since patients' acid production is reduced when the immune globulin is given orally, it is not

broken down; antigenically intact Ig appears in the stool (42). In one pilot study, six patients given oral globulin following ABMT suffered from no bacteremia, diarrhea, or enteritis (43).

Today, more cytotoxic regimens are being used with ABMT, and more ex vivo marrow manipulation is being done prior to transplant. Therefore, immunologic and physical barriers to infections may become severely compromised. IVIg can play a role in suppressing bacterial infections, whether systemic or in the intestinal tract. Therefore studies with large numbers of patients are still needed to define whether IVIg, administered intravenously, orally, or by both routes, may indeed have a role in preventing the complications of ABMT.

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The Possible Therapeutic Value of Natural Killer Cells in Suppressing Residual Leukemic Cells After Autologous Bone Marrow Transplantation

Eva Lotzova and Karel A. Dicke

Natural immunity, as represented by natural killer (NK) cells, appears to be the first line of defense against cancer (1,2). Leukemia and lymphoma targets emerge as the most sensitive neoplasias to NK cell attack (3-5), suggesting the primary role of NK cells in protection against malignancies of hematopoietic and lymphoid origins. This hypothesis is in accordance with deficient NK cell function in patients with leukemia and preleukemic disorders, high occurrence of lymphohematopoietic malignancies in individuals with low NK cell activity (4-6), and reinstallation of antileukemic activity in NK cell-deficient individuals by transfer of NK cells in vivo or their stimulation with various biological response modifying agents (5-7).

If NK cells are involved in immunosurveillance against leukemia, why, then, do leukemic cells escape the NK cell scrutiny? One possible explanation for this phenomenon might be the low NK cell cytotoxic function in bone marrow (8-10), the tissue in which most of the leukemias originate. Consequently, the absence of an appropriate NK cell surveillance mechanism in this tissue may allow uncontrolled proliferation of leukemic cells and their spread to the periphery. A similar mechanism may be responsible for recurrence of

leukemia after remission has been induced by chemotherapy. Specifically, low bone-marrow NK cell activity may not be efficient in controlling the growth and expansion of residual leukemic cells that survive the chemotherapy.

Since bone marrow compartment may be a critical environment for the origin and expansion of various types of human leukemia, generation of NK cell activity in this tissue may have a great therapeutic value. This possibility encouraged us to study the role of interleukin 2 (IL2) in activating NK cell cytotoxic function in human bone marrow. As we demonstrated previously, the IL2 generates significant levels of antileukemic activity in human peripheral blood (5,7).

NK CELL CYTOTOXICITY IN HUMAN BONE MARROW AFTER CULTURE WITH IL2

To determine the effectiveness of IL2 in generating NK cell cytotoxic activity in bone marrow, we cultured the bone-marrow mononuclear cells of normal donors and leukemic patients with recombinant IL2 for various time intervals and then tested such cultured cells for cytotoxicity against leukemic cell line K-562 (established from a patient with chronic myelogenous leukemia [CML] in blast crisis), which is highly sensitive to NK cell lysis, and against solid tumor cell line OV-2774 (established from a patient with ovarian cancer), which is relatively resistant to NK cell attack (11). Results of these investigations demonstrated that the culturing of bone marrow cells with IL2 generated significant levels of NK cell cytotoxic activity in cells from normal donors as well as from patients with various types of leukemia, including those with acute myelogenous leukemia (AML) and CML, respectively, acute and chronic lymphoid leukemias (ALL and CLL, respectively), and preleukemia (PRL). Representative experiments of these studies appear in Tables 1 and 2. Even though NK-cell cytotoxic potential was induced in bone marrow of both groups of individuals, bone marrow cells of leukemic patients required a longer time in culture with IL2 to generate substantial levels of cytotoxic activity. A similar phenomenon had been noted earlier in peripheral blood of leukemic patients (8). The observation may be important when adoptive therapy with IL2-activated killer cells of leukemic patients is considered.

To achieve the optimal antileukemic effect, 3-4 days of stimulation with IL2 in vitro (as is done in current protocols) may not be sufficient to activate killer cells in peripheral blood and bone marrow of leukemic patients.

Characterization of IL2-Activated Bone Marrow-Derived Cytotoxic Cells

After the encouraging observation that substantial levels of tumor-directed cytotoxicity could be generated from functionally inert bone marrow after activation with IL2, we analyzed the type of effector cells involved in

Table 1. Induction of NK Cell Cytotoxic Activity in Bone Marrow of Normal Donors by Interleukin 2

Donor Number	Time in Culture[a] (days)	Percentage of Cytotoxicity[b]	
		K-562	OV-2774
1	0	4.9	0.2
	6	61.9	40.3
2	0	2.4	0.4
	6	26.5	8.2
	11	33.8	27.9
3	0	-0.7	-1.7
	7	19.3	22.5
	14	56.6	47.4
4	0	1.6	0.6
	7	59.6	57.3
	14	79.3	61.6

[a]*Bone marrow mononuclear cells were cultured with 10^3 units of recombinant interleukin 2.*

[b]*Cytotoxicity was tested at 1:12 target-to-effector cell ratio in a 3-hour ^{51}Cr-release assay as described previously (4).*

Table 2. Induction of NK Cell Cytotoxic Activity Against K-562 in Bone Marrow of Leukemic Patients

Type of Leukemia	Time in Culture[a] (wks)	Cytotoxicity[b] (%)
AML	0	1.7
	1	4.1
	2	18.9
CML	0	9.7
	1	22.5
	2	28.5
ALL	0	0.1
	1	7.3
	5	69.7
CLL	0	1.2
	2	6.0
	4	40.5
PRL	0	-0.4
	1	48.9
	4	71.0

[a]*Bone marrow mononuclear cells were cultured with 10^3 units of recombinant interleukin 2.*

[b]*Cytotoxicity was tested at target-to-effector cell ratios of 1:50 (AML), 1:25 (CML and CLL), and 1:12 (ALL and PRL).*

Abbreviations: AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphoid leukemia; CLL, chronic lymphoid leukemia; PRL, preleukemia.

cytotoxic function. We used a two-step complement-dependent cytotoxicity test and monoclonal antibodies directed against NK cell- and T cell-associated structures. These studies demonstrated that bone-marrow cytotoxic activity was mediated by NK cells of the CD16 and NKH1 phenotypes, as demonstrated by abrogation of cytotoxic activity when IL2-activated killer cells were treated with Leu-11 and NKH1 antibodies (Table 3). Removal of T cells did not affect cytotoxic function of bone-marrow killer cells.

CONCLUSIONS AND POSSIBLE THERAPEUTIC APPLICATION

Our investigations indicated that IL2 is effective in inducing cytotoxic function in human bone marrow and that the IL2-dependent killer cells were NK cells and not T cells. These observations are in accordance with our and other investigators' studies implicating NK cells (and not T cells) in the major antitumor effect in peripheral blood after stimulation with IL2 (5,12).

Generation of NK cell activity in human bone marrow after culture with IL2, together with our previous demonstration of the ability of IL2-activated NK cells to kill fresh leukemic cells (5,7), naturally triggered our interest in the therapeutic application of IL2 and IL2-activated NK cells in therapy for leukemia. In a realistic consideration of the therapeutic value of both modalities for leukemia, one must emphasize, however, that these approaches (as all other immunotherapeutic approaches) are most likely to be effective for

Table 3. Characterization of Interleukin 2-Induced Cytotoxic Cells in Bone Marrow of Normal Donors and Leukemic Patients

Type of Donor	Treatment of Effector Cells[a]	Cytotoxicity[b] (%)	Cytotoxicity Inhibition (%)
Normal donor	None	55.0	
	RC	48.1	
	Leu-1	51.5	
	OKT3	59.2	
	OKT4	55.4	
	OKT8	36.6	23.9
	Leu-11	18.2	62.0
	NKH1	5.3	89.0
Leukemic patient	None	50.4	
	RC	45.9	
	Leu-1	54.3	
	Leu-11	0.1	99.8

[a] *Monoclonal antibodies were treated using a two-step complement-dependent assay, as described previously (5,7). RC=rabbit complement.*

[b] *Cytotoxicity of cultured cells of normal donor (11 days) or leukemic patient (19 days) was tested against K-562 at target-to-effector cell ratios of 1:25 and 1:12 (for cells from leukemic and normal donors, respectively) in a 3-hour ^{51}Cr-release assay.*

patients with minimal residual disease. Two groups of leukemic patients may be eligible for such therapy—those in remission and those undergoing autologous bone marrow transplantation. In both conditions, NK cells may restrict eventual proliferation of residual leukemic cells that survived chemotherapy or radiotherapy.

The therapeutic approach using NK cells may be implemented in two ways. In patients who have regained some immunocompetence (patients in remission), IL2 administration in vivo may represent the effective therapeutic approach. Alternatively, in patients whose immune system has been severely depleted or in whom the presence of suppressor cells or their factors may interfere with NK cell activation by IL2 in vivo, adoptive transfer of in vitro IL2-activated and propagated effector cells along with IL2 may be preferred.

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Autologous Transplantation of Circulating Stem Cells in Acute Nonlymphocytic Leukemia

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Autologous cryopreserved marrow cells have been used for several years to restore hematopoiesis after myeloablative chemoradiotherapy. More recently, the possibility of hematopoietic engraftment of peripheral blood stem cells (PBSC) has been considered in patients with acute leukemias (1-4), non-Hodgkin's lymphomas (5,6), or solid tumors (7). Since the number of PBSC is often very low, however, the collection of sufficient circulating progenitors may be difficult. In patients with acute nonlymphocytic leukemia (ANLL), we previously reported that an overshoot in colony-forming units of granulocyte-macrophage stem cells (CFU-GM) occurs during recovery from myelosuppressive chemotherapy. Performed during this period, leukaphereses enabled us to collect a mean of 15.2×10^4 CFU-GM cells/kg per leukapheresis from 11 patients in first complete remission (CR) and 4.4×10^4 CFU-GM cells/kg from 6 patients in second or subsequent CR (8). Thus, the number of CFU-GM cells collected by means of four to six leukaphereses was equivalent or greater than that usually obtained from a marrow harvest. To evaluate the hematopoietic capacity of PBSC, we performed autologous PBSC trans-

plantations in seven adult patients with resistant ANLL. A rapid and sustained erythroid and granulomonocytic engraftment was obtained in every case, but megakaryocytic engraftment was observed in only five of the seven patients.

PATIENTS AND METHODS

PBSC Collection

Blood-derived stem cells were collected in seven adult ANLL patients (mean age, 26 years [range, 15-48 years]; male:female ratio, 4:3). Leukaphereses were performed during first CR (three patients), second CR (three patients), or fourth CR (one patient) during marrow recovery following chemotherapy given as induction treatment to five patients, and as consolidation or intensification treatment of two patients. Five to seven leukaphereses (median, 5.9 leukaphereses) per patient were done during a 12.7-day period (range, 10-18 days); in six cases, a continuous flow cell separator (Cs 3000) was used. A mean of 28.7×10^4 CFU-GM cells/kg per patient was collected. Harvested cells were cryopreserved with dimethyl sulfoxide and stored in liquid nitrogen according to previously described methods (9).

Autologous Transplantation

Patients underwent autologous PBSC transplantation during first relapse (two patients), second CR (four patients), or fourth CR (one patient). The median duration of CR before transplantation was 9 months (range, 2-40 months). Five patients were prepared with etoposide (VP-16-213, 300 mg/m^2/day i.v. for 2 days), cyclophosphamide (60 mg/kg/day i.v. for 2 days), and total body irradiation (TBI, 5 Gy × 2 or 4 Gy × 3). Two patients received busulfan (4 mg/kg/day per os for 4 days); in one of these patients this was followed by cyclophosphamide (50 mg/kg/day i.v. for 4 days) and in the other by melphalan (140 mg/m^2 i.v.). After this conditioning regimen, patients received transfusions of all previously collected buffy coat cells—a median of 6.3×10^8 (range, 2.95-10 cells) thawed nucleated cells/kg containing a median of 17.6×10^4 (range, 2.75-49.7 cells) CFU-GM cells/kg. These numbers correspond to 85% and 66%, respectively, of nucleated and CFU-GM cells collected.

RESULTS

Hematopoietic Recovery (Table 1)

In every patient, reticulocytes were present during the 3 weeks following transplantation. Median times required to recover to 0.5 polymorphonuclear cells (PMN) × 10^9/l and to 10^9 leukocytes/l were, respectively, 14.5 and 12.4 days. Patients who received autografts of more than 10×10^4 CFU-GM cells/kg recovered more rapidly than other patients, median time to reach 0.5×10^9 PMN/l being, respectively, 12.2 and 17.7 days (*P*, NS).

Table 1. Hematopoietic Recovery After Autologous Transplantation of Circulating Stem Cells in ANLL Patients

Sex/Age	No. of CFU-GM Infused/ No. of CFU-GM Collected × 10^4/kg (% of Recovery)	Number of Days to Recover to		
		0.5 × 10^9 PMN/l	1 × 10^9 WBC/l	50 × 10^9 Plt/l
F/17	49.7/53 (94%)	9	8	11
M/36	30/43.8 (68%)	10	9	34
F/15	13.7/10.1 (135%)	16	14	10+
M/48	12.4/30 (41%)	14	12	—
M/19	9.5	20	17	35
M/25	5.3/16.3 (33%)	15	10	14
F/21	2.75/18.7 (15%)	18	17	—
Mean	17.6/28.7	14.5	12.4	—

Abbreviations: ANLL, acute nonlymphocytic leukemia; CFU-GM, colony-forming units of granulocyte-macrophage stem cells; PMN, polymorphonuclear cells; Plt, platelets.

Megakaryocyte engraftment was observed in five of seven patients a median of 20.8 days after transplantation. Severe thrombocytopenia as a result of megakaryocytopenia recurred in three of these patients. In one patient, thrombocytopenia was followed by the onset of leukemic relapse; in the other two patients, thrombocytopenia was transient, lasting only 5 and 8 weeks, respectively. These patients showed no evidence of cytomegalovirus or other viral infections. Two patients failed to develop more than 50×10^9 platelets/l; one experienced a leukemic relapse 45 days after transplantation, and the other died of gastrointestinal hemorrhage 69 days after transplantation without marrow megakaryocytes at the time of death. These two patients had received transplants of fewer than 10×10^4 CFU-GM cells/kg, which represents less than 50% of the CFU-GM cells collected.

Median time to recover to 10^9 lymphocytes/l was 14.9 (range, 9-20) days. There was an inversion in the T4:T8 ratio in 17 of the 19 tests performed during the first 3 months following transplantation. Afterward the T4:T8 ratio was more than 1 in every test.

Survival

In the patient who underwent transplantation in second CR and died of hemorrhage, no leukemic cells were found at postmortem examination. Three patients who received transplants during first relapse, and during second or fourth CR had leukemic relapses 45, 105, and 180 days, respectively, after autologous transplantation and died a few weeks later. Three other patients who had received transplants during first relapse (one) or second CR (two) are alive in continuous complete remission 2.5, 3.5, and 12 months, respectively, after transplantation.

DISCUSSION

The hematopoietic capacity of PBSC has been demonstrated in several animal models. In patients with hematologic malignancies or solid tumors, PBSC have been shown capable of restoring hematopoiesis after supralethal therapy. We probably performed the first successful transplantation of PBSC in a 19-year-old patient with ANLL in first relapse in May 1984 (10). Since then, more than 20 patients have received such transplants, and no doubt remains that hematopoietic reconstitution can be achieved by PBSC transplantation.

In this study, seven ANLL patients received autologous transplants of circulating stem cells. Five experienced prompt and sustained engraftment of erythrocytic and granulocytic precursors. Engraftment was faster in patients transfused with larger numbers of CFU-GM cells, a "cell-dose" effect recently reported by To *et al.* (11) in a review of data concerning 14 patients from different centers who received transplants for various types of hematologic malignancies or solid tumors. This effect was also reported in patients

who underwent transplantation of Philadelphia chromosome-positive PBSC for chronic granulocytic leukemia in transformation (12).

Delayed platelet recovery after PBSC transplantation was reported both in dogs (11) and in humans (1-5). Two of our patients failed to achieve megakaryocyte engraftment. In one of these, as described in other reports (13), the follow-up after transplantation was very short, and it is difficult to conclude that it was a true graft failure. In the other patient, no megakaryocytes were seen on marrow smears until the patient's death 2 months after transplantation.

The absence of platelet engraftment or the delayed platelet recovery is probably explained by insufficient numbers of megakaryocyte precursors and totipotent stem cells in transfused buffy coat cells. That could be caused either by less expansion of the latter progenitors than that of CFU-GM cells during the period of leukapheresis (14) or by a selective cryoinjury of the megakaryocyte precursors, which could be more sensitive to this injury than CFU-GM cells. CFU-GM recovery was only 15% and 41% in the two patients who had no megakaryocyte engraftment, which suggests that cryopreservation was in some way toxic in these patients and resulted in destruction of megakaryocyte precursors.

Because of the delayed platelet recovery observed in some patients, the theory that PBSC may reconstitute hematopoiesis as well as marrow cells has not yet been proved. The use of PBSC rather than marrow cells would be preferable, however, if it were confirmed that PBSC are less contaminated by residual leukemic cells (15). Further studies are needed to test this hypothesis.

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Restoration of Hematopoietic Function With Autologous Cryopreserved Peripheral Stem Cells

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Identification of circulating hematopoietic stem cells in the peripheral blood of laboratory animals (1) and man (2) generated interest in the possibility of using these cells to restore hematopoiesis following marrow aplasia. Advantages of using peripheral stem cells rather than marrow stem cells for autologous transplantation include eliminating the need for anesthesia during marrow collection and avoiding the discomfort that follows multiple needle insertions into bone to collect marrow. Patients with unacceptable anesthetic risks and those with prior pelvic radiation or metastatic disease in their bone marrow would not be disqualified from receiving marrow-ablative therapy for their malignancy.

Since circulating stem cells are much less concentrated than marrow stem cells (2), necessary methods using cytophoresis and cryopreservation techniques were devised to collect and preserve circulating stem cells in numbers adequate to ensure engraftment (3,4).

In 1981, Goldman *et al.* reported performing 20 autologous peripheral stem cell transplantations for patients with transformed chronic myelocytic leukemia (CML) (5). Autologous peripheral blood stem cells were collected

and cryopreserved early in the disease course. When the leukemias transformed into an acute phase, all patients were treated with cytotoxic drugs and some received whole body irradiation; this therapy was followed by infusion of the previously collected stem cells. Engraftment was demonstrated in 19 of these patients by a return of the disease to the chronic phase. Later this series was expanded to 50 patients; 94% demonstrated engraftment (6).

Although autologous peripheral stem cell transplantation resulted in engraftment in patients with transformed CML, two reports of unsuccessful syngeneic peripheral stem cell transplantations appeared in 1979 and 1980 (7,8). The number of circulating stem cells in patients with CML was known to be greatly increased over the number in a normal population (6). Therefore concern continued about the ability of peripheral hematopoietic stem cells from nonleukemic patients to restore hematopoietic function until successful autologous peripheral stem cell transplantations were reported some years later for patients without CML (9-15).

Autologous peripheral stem cell collections have been described in two distinctly different settings. In the first, peripheral stem cells are collected at a time when their numbers are augmented. The number of granulocyte-macrophage colony-forming units is known to be 25-fold higher than in normal subjects in patients with acute myelogenous leukemia just as marrow recovery begins following induction therapy (16). Peripheral stem cells collected at that time have restored hematopoietic function following high-dose therapy for patients with acute leukemia and lymphoma (9,12-15).

In the second setting, peripheral stem cells have been collected when marrow function was in a relatively steady state and no augmentation of numbers was anticipated; these cells have restored hematopoietic function following high-dose therapy for patients with lymphoma and breast cancer (10,11). This report updates our experience with transplantation of autologous peripheral stem cells collected at a time when augmentation of their numbers was not anticipated.

PATIENTS AND METHODS

Between June 8, 1984, and October 31, 1986, 10 patients received autologous peripheral stem cell transplants. Six patients had advanced breast cancer, three had refractory Hodgkin's disease, and one had refractory large cell lymphoma. Characteristics of the transplanted cells are listed in Table 1.

Patients were eligible for this study if the only feature disqualifying them for high-dose cancer therapy and autologous marrow transplantation was metastatic malignancy in bone marrow. Methods used for peripheral stem cell collection and cryopreservation have been published (11). Each patient underwent eight 4-hour leukapheresis procedures no more than three times weekly. Autologous marrow was harvested and cryopreserved to serve as a backup in the event engraftment with peripheral stem cells did not occur or was not durable.

Table 1. Cell Characteristics

Patient	No. Collected Mononuclear Cells $\times 10^8$/kg	No. Thawed CFU-GM $\times 10^4$/kg	No. Thawed CFU-GEMM $\times 10^4$/kg
1	8.4	89.0	12.6
2	6.3	6.6	1.4
3	6.6	2.4	0.4
4	11.3	5.3	0.3
5	9.5	2.3	1.6
6	10.9	98.6	4.0
7	4.9	13.2	20.3
8	10.3	6.3	10.5
9	8.0	NA	NA
10	15.8	10.4	5.64

Abbreviations: CFU-GM, granulocyte-macrophage colony-forming units; CFU-GEMM, human pluripotent stem cell colony-forming units.

The high-dose therapy these patients received varied according to the underlying disease and prior treatment. Four patients with breast cancer received cisplatin (125 mg/m^2) in a 24-hour intravenous infusion followed by two consecutive daily intravenous doses of cyclophosphamide (60 mg/kg). One day after the last dose of cyclophosphamide, total body irradiation (TBI) was administered in five daily fractions of 2.2-2.5 Gy. Two patients with breast cancer received etoposide (150 mg/m^2) intravenously every 12 hours for six doses, rather than TBI, followed by cisplatin and cyclophosphamide. Three patients with Hodgkin's disease received cyclophosphamide (1.5 g/m^2), etoposide (150 mg/m^2) intravenously, and carmustine (300 mg/m^2) intravenously. The cyclophosphamide dose was repeated daily for a total of four doses and the etoposide dose was repeated every 12 hours for a total of six doses. The patient with large cell lymphoma received cytarabine (3 g/m^2) intravenously every 12 hours for six doses. Two days following the last dose of cytarabine, cyclophosphamide (90 mg/kg) was infused intravenously. Two days after the cyclophosphamide, the patient received TBI (9 Gy fractionated) for five doses every 12 hours.

Following therapy, autologous peripheral stem cells were thawed and immediately infused intravenously over a 1-4 hour period.

RESULTS

All patients demonstrated engraftment, and all grafts were durable. Two patients died 12 days following transplantation and could not be evaluated for

Table 2. Hematopoietic Recovery Level, Day Following Autologous Peripheral Stem Cell Transplantation

Patient	1st WBC	Granulocytes >500 mm^3	10 g/dl Hg	Platelets >20,000 mm^3	Platelets >50,000 mm^3
1	8	—	—	—	—
2	8	17	34	—	70
3	11	37	47	25	59
4	7	16	23	15	32
5	9	58	58	36	48
6	8	26	26	25	45
7	9	27	21	21	49
8	9	—	—	—	—
9	7	19	14	14	21
10	8	11	11	—	25

effective trilineage engraftment; bone marrow examined at autopsy showed all cell lines were present. Characteristics of hematopoietic recovery are listed in Table 2. Patient deaths were recorded on days 12, 12, 104, 156, and 425 following transplantation. Surviving patients are alive 25, 36, 130, 263, and 445 days following transplantation.

DISCUSSION

This study demonstrates that nonleukemic cryopreserved autologous peripheral stem cells collected while marrow function was in a relatively steady state durably restored hematopoiesis following marrow-ablative therapy. Because each patient received autologous cells, regeneration of marrow rather than engraftment by peripheral stem cells cannot be disproved, but no explanation other than engraftment of peripheral stem cells seems likely to account for peripheral evidence of hematopoiesis at 7 days following high-dose therapy. This technique may offer patients ineligible for autologous bone marrow transplantation, because of bone marrow metastases, prior pelvic radiation, or excessive anesthetic risks, the opportunity to receive marrow-ablative high-dose cytotoxic therapy.

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Autologous Transplantation Using Peripheral Blood Stem Cells: A Case Report and Review of the Literature

Kevin J. Cockerill and Axel R. Zander

High-dose chemotherapy followed by autologous stem cell rescue is an increasingly popular investigational treatment for hematologic malignancies and selected solid tumors. Encouraging results in certain tumors have been seen in patients whose first-line and subsequent salvage radiation and chemotherapy regimens have failed. The experience in Hodgkin's disease is a case in point. Jagannath *et al.* (1) reported the results of 30 patients treated at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston and the University of Nebraska using the CBV regimen (cyclophosphamide, BCNU [carmustine], and VP-16-213 [etoposide]) followed by autologous bone marrow stem cell rescue. In this population of patients who had been extensively pretreated, one-half achieved complete remission. One third of these patients continued in complete remission up to 44 months later and were possibly cured by this unique salvage therapy.

Hematologists and oncologists who treat Hodgkin's disease are increasingly aware of the availability of megadose chemotherapy with stem cell autografting as a treatment option and are tailoring their initial treatments to

facilitate bone marrow stem cell collections when feasible. In some cases, however, extensive marrow contamination by tumor or prior radiotherapy limits the availability of bone marrow stem cells. Also, in rare cases general or regional anesthesia necessary for bone marrow stem cell harvesting is associated with an unacceptable risk to the patient. Peripheral blood stem cells (PBSCs) obtained by multiple leukapheresis circumvents the problem of availability and may be associated with less frequent tumor cell contamination. The following case and review are presented to illustrate several points regarding this treatment.

MATERIALS AND METHODS

Using the IBM 2997 cell separator we collected stem cells eight separate times from a patient with relapsed Hodgkin's disease. The initial two procedures took 3 hours each, and the remaining six took a full 4 hours each. Most of the procedures were performed using a Vascath central venous catheter (Gambro, Lincolnshire, IL) as access for both the draw and return lines. Flow rates and centrifuge speeds were selected for optimum peripheral blood mononuclear cell (PBMC) collection. These were a flow rate of 40 ml/minute with a centrifuge speed of 938 rpm or a flow rate of 45 ml/minute with a centrifuge speed of 995 rpm. Collected volumes varied from 198 ml to 300 ml. The resulting cell suspensions were adjusted to 10 U/ml with heparin before further processing.

The buffy coat was isolated from the cell suspensions and cryopreserved at a concentration of 10^8/ml using 10% dimethyl sulfoxide and 20% autologous plasma from which cryoprecipitate was previously removed for freezing medium as described elsewhere (2). Samples from each separate batch were analyzed for granulocyte-macrophage colony-forming units (CFUs-GM) and erythroid burst-forming units (BFUs-E) immediately and after thawing.

RESULTS

The patient, a 20-year-old man, initially presented with Hodgkin's disease, stage IIIB. After initial complete remission after therapy with mechlorethamine, Oncovin (vincristine), procarbazine, and prednisone combined with Adriamycin (doxorubicin), bleomycin, vinblastine, and dacarbazine (MOPP-ABVD), he relapsed on two subsequent occasions. His subsequent treatment involved MOPP-ABVD, mantle radiation to 37 Gy, CCNU (lomustine), methotrexate, etoposide, another series of MOPP-ABVD, and most recently cytarabine, cisplatin, and dexamethasone.

In May 1986 high-dose chemotherapy with autologous bone marrow transplantation was planned. Results of bilateral bone marrow biopsies were negative for involvement with Hodgkin's disease, so multiple bone marrow aspirations were performed with the patient under general anesthesia. Cells were cryopreserved. In the course of this process, six separate iliac crest bone

marrow biopsies were done. All results were completely normal, except those from one biopsy specimen that showed Hodgkin's disease.

In September 1986, a full month after complete hematologic recovery from chemotherapy, a Vascath catheter was placed into the left subclavian vein through which eight leukaphereses were performed and cells were cryopreserved.

The patient was next admitted to the hospital in early October. On presentation he had hectic fever and chills believed to be owed to B symptoms from Hodgkin's disease. In addition, he had severe back pain owed to thoracic vertebral involvement with tumor and measurable disease in the thoracolumbar spine and bones of the right knee. Chest x-ray films demonstrated extensive bilateral parenchymal Hodgkin's disease involvement. Three days later he was treated with the intensively myelosuppressive CBV chemotherapy regimen (1.5 g/m^2 cyclophosphamide days 1-3, 450 mg/m^2 carmustine, day 1, 100 mg/m^2 etoposide every 12 hours for six total doses). Forty-eight hours after the last dose of chemotherapy his PBSCs (27×10^8 mononuclear cells/kg with 23×10^4 CFUs-GM/kg) were reinfused over 24 hours because of the large volume of cells involved. Within days of completion of chemotherapy, his fever, chills, and back pain resolved. He demonstrated generous trilineage engraftment on bone marrow biopsy by day 10, which was reflected in his peripheral counts by early reticulocyte, platelet, and granulocyte recovery (absolute granulocyte count 0.5×10^9/l by day 12, platelet count 50×10^9/l by day 14, and reticulocyte response by day 10). He required platelet support for only the first 10 days after transplantation. An excellent response to chemotherapy was documented by complete clearing of his pulmonary infiltrates by day 21.

DISCUSSION

The feasibility of using circulating stem cells for autologous hematopoietic reconstitution was initially apparent in work with chronic myelogenous leukemia (CML). Goldman *et al.* (3) demonstrated markedly increased CFUs-GM in the peripheral blood of patients with CML in chronic phase. Viability of these cells, cryopreserved as bone marrow stem cells, was maintained for up to 5 years. In light of the ability of cells obtained by leukapheresis to consistently restore chronic phase clones in patients who entered blast transformation, the authors recommended their routine use over bone marrow stem cells in this setting.

In later experiments using the dog model, Abrams *et al.* (4) demonstrated a marked proliferative overcapacity in in vitro circulating hematopoietic cells after count recovery from cyclophosphamide administration. These cells were superior to cells harvested in a steady state in producing hematopoietic restoration after marrow-ablative doses of total body irradiation (TBI). However, if a dose of steady-state PBMCs was given that was high enough, survival with hematopoietic recovery did ensue in some animals. In man a similar "overshoot"

phenomenon was observed in CFUs-GM of PBMCs in very early remission from acute nonlymphocytic leukemia (5). These cells remained viable after 2 years of storage in the liquid phase of liquid nitrogen, and there was speculation about their suitability for hematopoietic restoration in man after marrow-ablative chemoradiotherapy.

Some initial clinical results using PBMCs for restoration of bone marrow function were discouraging. Hershko *et al.* (6) reported engraftment failure in a patient with aplastic anemia after infusion of 7.1×10^{10} PBMCs from her identical twin sister over several days. Bone marrow stem cells from the same donor, on the other hand, resulted in bone marrow recovery. In this case no conditioning regimen was given. In another instance reported by Abrams *et al.* (7), failure of engraftment was likewise demonstrated by PBMCs from an identical twin donor. In this case 9.8×10^{10} PBMCs given over several days were ineffective in restoring hematopoiesis in a young patient with Ewing's sarcoma conditioned with chemotherapy and TBI. Three additional patients were reported by Juttner *et al.* (8) in whom PBSCs were harvested in very early remission from acute nonlymphocytic leukemia at which time an overshoot in circulating CFUs-GM was noted. In all three, prompt initial engraftment was followed by a fall in counts by day 16 and a secondary but incomplete rise.

Isolated case reports following these initial accounts described successful PBSC engraftments when the products of several leukaphereses collected in the bone marrow recovery phase from chemotherapy were reinfused at once following marrow-lethal doses of chemoradiotherapy (9-11). Timing of PBSC collection—taking advantage of the CFU-GM overshoot phenomenon—was believed to be important in these cases to ensure hematopoietic reconstitution.

Korbling *et al.* (12) described a case in which rapid hematopoietic reconstitution ensued using the product of seven successive leukaphereses in a patient with Burkitt's lymphoma treated with cyclophosphamide and TBI. The initial stem cell harvest in this case was performed 2 weeks after the completion of chemotherapy. However, a definite CFU-GM overshoot was not apparent from cells collected during this time. Kessinger *et al.* (13) also described two patients with advanced breast carcinoma treated with cyclophosphamide and TBI followed by infusion of the products of seven leukaphereses on day 0. In both patients prompt trilineage engraftment ensued.

We collected PBSCs from our patient in a fashion similar to that reported by Korbling *et al.* (12) and Kessinger *et al.* (13). Perhaps most important, all patients were transfused within a 24-hour period following high-dose chemotherapy. The CBV regimen is usually a profoundly myelosuppressive treatment program producing an absolute granulocyte count less than 500/μl for an average of 21 days (Table 1). In contrast, our patient recovered an absolute granulocyte count over 500/μl in 12 days. In addition, recovery of platelet and reticulocyte counts compared favorably to those previously reported using bone marrow stem cells.

In contrast to older case reports discouraging the use of PBSCs for

Table 1. Comparison of Bone Marrow Stem Cell Rescue and Peripheral Blood Stem Cell Rescue

Recovery Characteristic	Bone Marrow Stem Cell Rescue[a] (range)	Peripheral Blood Stem Cell Rescue[b]
Time to absolute granulocyte count > 100/μl (days)	15 (8–40)	11
Duration of absolute granulocyte count < 500/μl (days)	21 (7–40)	12
Time to platelet count > 20,000/μl (days)	21 (6–58)	9
Time to platelet count > 50,000/μl (days)	28 (10–65)	14

[a] *Data from Jagannath* et al. *(1) (n = 39).*

[b] *Data from case reported in text (n = 1).*

hematopoietic reconstitution after marrow-ablative doses of chemoradiotherapy, more recent accounts report cases of successful, durable engraftment. Many authors have speculated about the parameters of cell number and proliferative capacity in vitro as predictors of consistent engraftment (2,14). However, numbers of mononuclear cells collected, as well as CFUs-GM and granulocyte-erythrocyte-macrophage-megakaryocyte CFUs, have thus far failed to unequivocally predict consistent engraftment, whether by bone marrow or peripheral blood stem cells (15). Presently the most reproducible results appear to depend primarily on the parameters of total number and timing of individual cell collections. Transfusing all cells in a single time period instead of at times scattered over several days also appears to be vital in producing consistent engraftment.

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Bronchoscopic Examination and Bronchoalveolar Lavage Preceding High-Dose Therapy and Autologous Bone Marrow Transplantation

William Vaughan, Stephen Rennard, James Linder, Mohammed Ghafouri, Mark Arneson, Anne Kessinger, and James Armitage

In autologous hematopoietic stem cell support protocols, mortality during the period of aplasia induced by high-dose therapy usually results from infection, pneumonia being the most threatening complication. Early diagnosis of pneumonia in patients with immunosuppression and granulocytopenia is made more difficult by the common occurrence of fever in these patients and the uncommon occurrence of radiographic or physical indications specific to pneumonia. In fact, these patients often undergo significant pulmonary decompensation before findings specific to pneumonia are seen on chest x ray or in physical examination. By this time, even such aggressive diagnostic procedures as open-lung biopsy and empiric broad-spectrum anti-infection therapy may be too late to save the patient (1,2). Recently, the technique of bronchoscopy with bronchoalveolar lavage was shown to have a high diagnostic yield for some opportunistic pathogens in immunocompromised patients (3,4). A procedure with low morbidity, it can be performed at the time of earliest suspicion of pulmonary infection.

Patients referred for high-dose therapy with autologous hematopoietic stem cell rescue often are already immunocompromised as a result of disease involvement and previous therapy. Their chest x rays may be abnormal because of tumor involvement, previous radiation therapy, or infection. For these reasons, we began performing routine pretreatment bronchoscopic examination and bronchoalveolar lavage for patients referred for high-dose therapy with autologous hematopoietic stem cell support.

PATIENTS AND METHODS

During the first 9 months of our pulmonary surveillance program, 35 of 37 patients treated at the University of Nebraska Medical Center with high-dose therapy and autologous hematopoietic stem cell support underwent pretreatment bronchoscopy and bronchoalveolar lavage. Twenty-three of these patients (62%) had abnormal chest x-ray results on admission. Eight patients died during the period of marrow hypoplasia, pulmonary failure being a major contributor to their deaths.

For patients undergoing general anesthesia for marrow harvest before chemotherapy, the pretreatment bronchoscopic examination was performed through the endotracheal tube in place for general anesthesia. Previously, when marrow had been harvested, the bronchoscopic exam had been done transorally using topical anesthesia, with diazepam and meperidine intravenously for sedation. In the currently used procedure, all visible bronchial segments were inspected with a flexible fiber-optic bronchoscope, which was then inserted into a gently wedged position for the lavage procedure. Sterile saline was infused in 20-ml aliquots and immediately aspirated. Six such lavage specimens were obtained in each of three separate segments. The corresponding aliquots from each lavage site were pooled, the pooled first aliquots from each site considered bronchial and the next four alveolar (5); the sixth lavage specimen was sent for culture. Cytologic assay of the specimen was performed on cytocentrifuge and millipore filter after filtering the preparation through loose nylon mesh to remove mucus. Cell counts and WBC differentials were done on each specimen, and Wright's, Gomori's methenamine silver, and Papanicolaou stains were made.

RESULTS

In addition to the 23 patients (62% of 37 patients treated) who had abnormal chest x ray findings at admission, 28 of 35 (80%) of the patients who underwent pretreatment bronchoscopic examination and bronchoalveolar lavage had abnormal findings. There was no correlation between the two types of abnormalities, nor were abnormal findings on admission chest x ray, admission bronchoalveolar lavage, and early death correlated (Table 1). The specific findings from bronchoscopic examination and bronchoalveolar

Table 1. Frequency of Abnormal Findings on Admission Chest X Ray, Bronchoscopy, and Early Death in Patients Managed With High-Dose Therapy and Autologous Hematopoietic Support

Diagnosis Therapy	No. of Patients	Abnormal Chest x ray	Abnormal Findings on Bronchoscopy	Early Death (< 30 days)
Lymphoma/CBV	14	6	12	2
Lymphoma/ACT	12	10	11	5
Solid tumor/Misc.	11	7	6	1
Total	37	23 (62%)	28 (80%)[a]	8 (22%)[b]

Abbreviations: CBV, cyclophosphamide, BCNU (carmustine), VP-16-213 (etoposide) (6); ACT, arabinofuranosyl cytosine, cyclophosphamide, total body irradiation (7).

[a]No correlation between abnormal admission chest x ray and abnormal admission bronchoscopic findings.

[b]No correlation between abnormal admission chest x ray or bronchoscopic findings and early death.

lavage at admission were, however, clinically useful. Abnormal visual findings included clinically unsuspected upper-airway obstruction in three patients, endobronchial tumor in three, extrinsic lower-airway obstruction in one patient, and severe mucus plugging and bronchitis in one patient each.

The clinically significant findings in lavage fluid included abnormal cytologic findings in nine patients, one of whom also had a positive culture for *Candida albicans.* In addition, more than 15% neutrophils (indicating bronchitis) was identified in bronchial washing from 14 patients and more than 5% alveolar neutrophils (indicating alveolitis) in lavage fluid from 10 patients (Table 2).

All eight patients who died during the period of marrow hypoplasia died in pulmonary failure. The causes of death included *Aspergillus* in two patients, *Candida* pneumonia in two, acute respiratory disease syndrome without established etiology in two patients, and *Pneumocystis carinii* and combined

Table 2. Lavage Fluid Findings From Pretreatment Bronchoalveolar Lavage of Patients in High-Dose Therapy

Normal	12 (34%)
Abnormal cytology	9 (*P. carinii*, 2; yeast 4; bacteria, 3; tumor cells, 1)
Abnormal culture	1 (*C. albicans*)
Bronchitis	14
Alveolitis	10

Candida and *Aspergillus* pneumonia in one patient each. Only one of these diseases was seen in the initial bronchoscopic examination and bronchoalveolar lavage, in a patient who, at admission, had very low numbers of *P. carinii* that were not recognized initially but were identified upon review. In this patient and the others who died, the etiology of their pulmonary dysfunction was identified by subsequent bronchoscopy and bronchoalveolar lavage, and none required an open-lung biopsy. Autopsies performed on all eight patients confirmed that there was no additional pulmonary pathology.

DISCUSSION

Patients referred for high-dose therapy with autologous hematopoietic support, at least in the early days of this therapy, were patients in a significantly immunocompromised state and with altered intrathoracic anatomy before treatment. In this population, a high incidence of clinically significant findings was identified by admission bronchoscopic examination and bronchoalveolar lavage. Many of these findings mandated clinical interventions that undoubtedly decreased the ultimate morbidity and mortality of the high-dose therapy and subsequent marrow aplasia. However, pulmonary failure remains the major proximate cause of death in this patient population, and repeated bronchoscopic examination with bronchoalveolar lavage is necessary to determine the etiology of significant changes in the patient's clinical course. No significant morbidity and mortality were associated with the pretreatment bronchial examination methods used in this patient population. Subsequent bronchoscopic exam and bronchoalveolar lavage, performed when the patients' clinical situation has deteriorated, do carry a higher risk, which may be avoided in many cases by vigorous response to findings of the pretreatment studies.

Based on the results reported in this chapter, we have continued to perform routine pretreatment bronchoscopic examinations and bronchoalveolar lavage in patients being managed in our institution with high-dose therapy and autologous hematopoietic support.

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Effect of Interleukin 2 on T Lymphocyte Colony-Forming Units After Autologous Bone Marrow Transplantation

Andre Bosly and Michel Symann

Immunodeficiency with increased susceptibility to microbial, viral, and mycotic infections usually persists up to 1 year (1) and even longer after allogeneic bone marrow transplantation. Concomitant with this immunodeficiency is a prolonged imbalance of T lymphocytic subpopulations in favor of T suppressor/cytotoxic subsets (2), which may be related to chemoradiotherapy conditioning, to immunosuppressive treatment after transplantation, or to graft-versus-host disease (GVHD) (3).

After autologous bone marrow transplantation (ABMT), the pattern of immunologic restoration is very close to that observed after allogeneic transplantation. However, ABMT is not associated with a histocompatibility problem, immunosuppressive treatment, or GVHD. Therefore, autologous transplantation appears to be a good model for studying lymphocyte repopulation after intensive chemotherapy.

In this study we have investigated T lymphocyte-subset recovery and T lymphocyte-proliferation capacity in 20 patients following ABMT.

PATIENTS AND METHODS

Twenty patients were studied. Diagnoses were: 12 small cell lung cancer, 4 lymphomas (3 non-Hodgkin's, 1 Hodgkin's disease), 2 germinal cell can-

cers, 1 melanoma, and 1 acute myeloid leukemia. Nineteen patients received intensive chemotherapy (Table 1) and one patient (with acute myeloid leukemia) received chemotherapy followed by total body irradiation (80 Gy). Cryopreserved autologous bone marrow was reinfused following intensive therapy; no subsequent maintenance chemotherapy was administered. Characteristics of the 20 patients are summarized in Table 1.

Analysis of T-Cell Subpopulations

Peripheral blood T lymphocyte subsets were studied at regular intervals after ABMT. T lymphocytes and T cell subsets were determined by binding of the following monoclonal antibodies: T11 (pan T cell, cluster of differentiation CD2), T4 (T helper/inducer CD4) and T8 (T suppressor/cytotoxic CD8), B1 (B lineage), I2 (HLA D/DR-related Ia antigen), and IL2R1 (TAC antihuman IL2 receptor), obtained from Coulter Immunology (Hialeah, FL); OKT3 (CD3) and OKT6 (Thymocytes CD1), purchased from Ortho Pharmaceutical Laboratories (Raritan, NJ). T lymphocyte and T cell subsets were counted by indirect fluorescence and flow cytometry (Epics©, Coulter). Absolute CD4- and CD8-positive T cell counts from the leukocyte count, percentage of lymphocytes in the differential count, and the percentages of CD1, CD2, CD3, CD4, CD8, I^+, and TAC^+ cells were calculated. Control values were determined in 25 healthy subjects.

T-Cell Colony Assay

Peripheral blood from patients or control subjects was collected in anticoagulant solution. Mononuclear cells were isolated after Ficoll-Hypaque gradient centrifugation. Cells from the interface were collected and washed in modified Dulbecco's medium (Gibco, Paisley, Scotland). Cells (10^6) were seeded for colony formation in the modified Dulbecco's medium in 35-mm Petri dishes (Nunclon, Denmark) in 0.5 ml soft agar (0.33%) on a 2.5-ml harder agar base (0.5%); 0.03 ml PHA (phytohemagglutinin) (Wellcome, HA15 Dartford, England) per Petri dish was added to the lower agar layer. Autologous plasma (20%) and mercaptoethanol 5×10^{-5} M (Merck, Schuchlardt, West Germany) were added to both layers. Colonies of more than 50 cells were counted 7 days after seeding. Cultures were incubated at 37° C in a humidified incubator with 10% CO_2. Recombinant human interleukin 2 (rIL2), generously provided by Professor Fiers (University of Ghent, Belgium), was added to the underlayer at a total dose of either 25 or 50 U.

Cytomegalovirus

Cytomegalovirus (CMV) infection was investigated by assaying serum antibodies IgM and IgG with an Elisa method, using viral urine cultures and histologic samples when necessary.

Table 1. Characteristics of Study Patients

Age	Sex	Disease	Status Before ABMT	Conditioning Regimen	Result of HDCh + ABMT	Survival After ABMT (mo)	CMV Status	Cause of Death
57	M	SCLC	CR	CBV	CR	55	-	Acute leukemia
21	M	AML	CR (2d)	C-A + TBI	CR	16+	+	—
31	M	GC	Ev	PCVM	CR	1	-	Toxic reactions
63	M	SCLC	Ev	CBV	PR	6	-	Relapse
51	M	SCLC	Ev	CBV	PR	0		Relapse
23	F	HD	PR	CBV	CR	26	-	Relapse
57	M	SCLC	CR	CBV	CR	12+	-	—
52	M	NHL	CR (2d)	CVM	CR	11+	-	Infection (mycosis)
47	M	SCLC	CR	CBV	CR	6	0	Relapse
41	M	SCLC	PR	CBV	CR	5	0	Relapse
55	M	SCLC	PR	CBV	CR	7	0	Relapse
44	M	SCLC	PR	CBV	CR	10	0	Relapse
57	M	SCLC	CR	CBV	CR	16	0	Relapse
39	M	SCLC	PR	PCVM	CR	16	+	Relapse
60	M	SCLC	CR	CBV	CR	10	0	Relapse
46	M	SCLC	CR	CBV	CR	21	0	Relapse
44	M	NHL	PR	BACT	CR	41+	0	—
45	M	NHL	Ev	CVM	CR	2	+	Infection (CMV)
22	F	GC	Ev	CBM	PR	3+	-	—
44	M	MM	Ev	BM	MR	7+	-	—

Abbreviations: ABMT, autologous bone marrow transplantation; HDCh, high-dose chemotherapy; CMV, cytomegalovirus; SCLC, small cell lung cancer; AML, acute myeloid leukemia; GC, germinal cancer; HD, Hodgkin's disease; NHL, non-Hodgkin's lymphoma; MM, melanoma; CR, complete remission; PR, partial remission; MR, minimal response; Ev, evolutive disease; TBI, total body irradiation; 0, not done.

Note: Chemotherapeutic agents—C, cyclophosphamide; B, BCNU; V, VP-16-213 (etoposide); A, ara-C; P, cisplatin; M, melphalan; T, thioguanine.

RESULTS

Figure 1 shows $T4^+$ and $T8^+$ lymphocyte values in peripheral blood during the first year after transplantation. Very early (at month 2), values of $T8^+$ reached normal levels and subsequently increased above the normal range. In contrast, during the first year following ABMT, $T4^+$ lymphocytes (helper/inducer) remained far below the normal values, as did the $T4^+/T8^+$ ratio, which remained below 1.0.

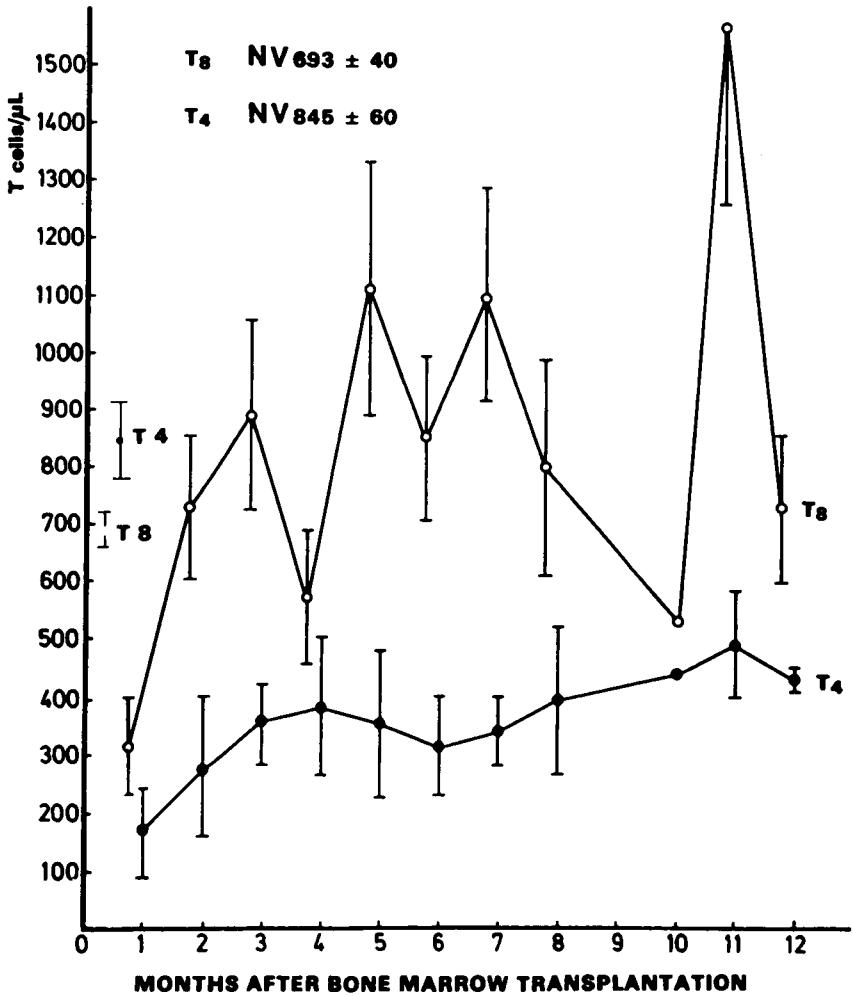

Figure 1. *Recovery of peripheral blood T cell subsets after autologous bone marrow transplantation (ABMT). Rapid recovery of $OKT8^+$ cells occurred within 2 to 3 months, but $OKT4^+$ T cells remained below normal for up to 12 months. Consequently, the T4:T8 ratio is reversed up to 1 year after ABMT.*

Sixty-nine T-lymphocyte colony cultures were performed in 20 patients from 1 to 51 months after autologous transplantation (Table 2). In 40 cultures no growth was observed. In 29 instances where growth occurred, the mean number of T colonies was lower than that observed for normal controls. These results are summarized in Table 2.

Seventeen cultures derived from eight different patients were analyzed in the absence or presence of rIl2. No difference was observed between cultures containing 25 or 50 U of rIL2. Results are summarized in Figure 2. In six cases where no growth was observed, adding rIL2 to the cultures resulted in five of six showing T colonies (424 ± 160; mean ± SEM). In 11 cases the T-colony number (783 ± 130) increased by 113% after the addition of rIL2 (1675 ± 175). These results are irrespective of CMV infection status (Fig 2).

DISCUSSION

Our data illustrate profound and prolonged abnormalities of T lymphocyte phenotypes and T lymphocyte-proliferation capacity after ABMT. In all patients, T4 lymphocyte values persist below normal up to 1 year after transplantation, while total lymphocyte, T lymphocyte, and T8 lymphocyte values quickly (after 2 months) recover to normal or even elevated values.

T lymphocyte-proliferation capacity, investigated by lymphocyte colony-formation technique, was decreased for prolonged periods, as long as 28 months. In vitro addition of rIL2 can restore T lymphocyte colony-formation capacity. Nevertheless, the values obtained after adding rIL2 remained below normal in most cases. Thus, IL2 restores T colony-formation capacity after ABMT as it has been reported to do after allogeneic transplantation (4). Therefore GVHD is not the explanation for this phenomenon. Viral infections,

Table 2. PHA-Stimulated T Cell Colony Formation

Months After AMBT	No. of ABMT Recipients Studied	No. of No Growth (%)	Mean ± SEM[a] of Colonies When Growth
1-3	26	16 (62)	311 ± 151
4-6	15	11 (73)	684 ± 257
7-12	13	5 (38)	764 ± 234
13-24	8	7 (87.5)	1024 ± 214
25-51	7	1 (14)	638 ± 275
	69	40 (58)	

[a] *Control colony values = 3493 ± 217.*

Abbreviations: PHA, phytohemagglutinin; ABMT, autologous bone marrow transplantation.

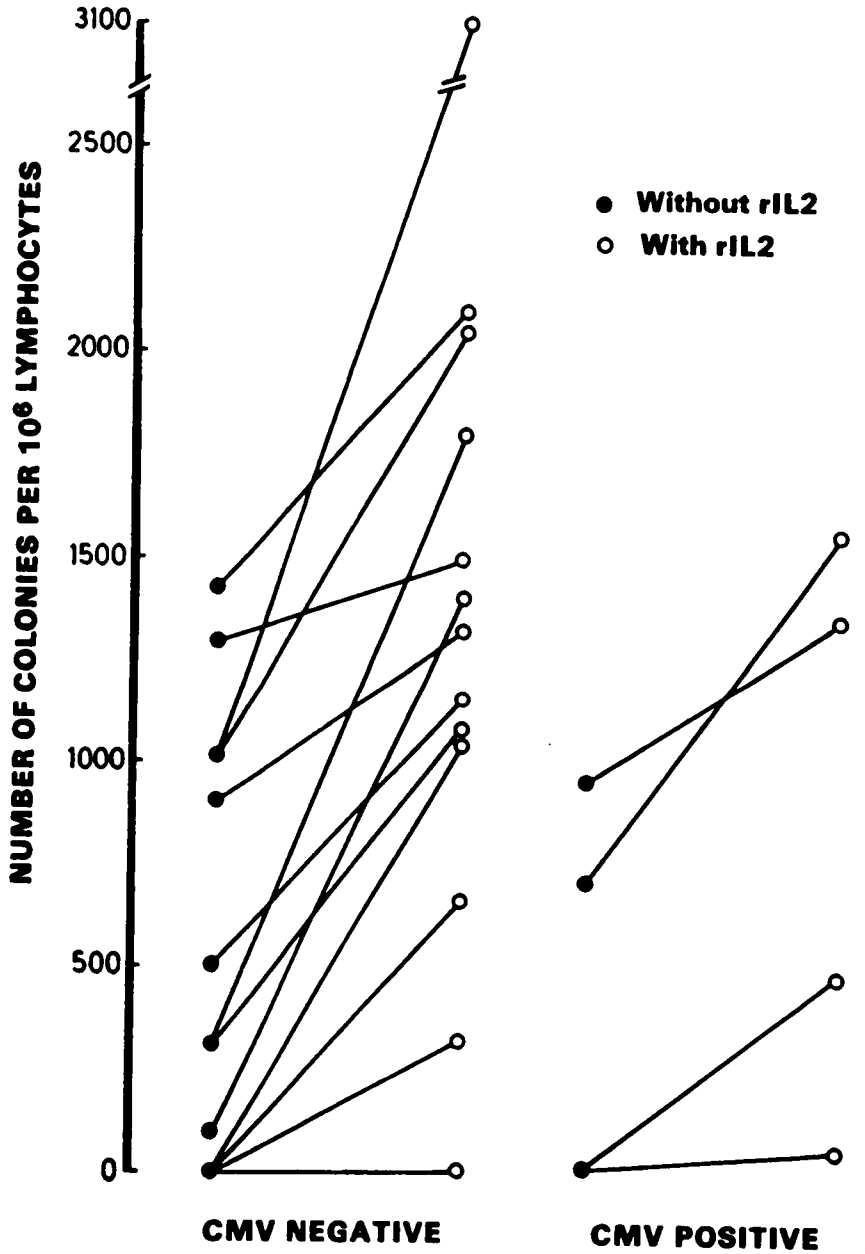

Figure 2. *T cell colony formation by peripheral blood lymphocytes from patients who have received autologous bone marrow with and without recombinant human interleukin 2 (rIL2). By adding 25 or 50 U/ml rIL2, T cell colonies were generated (426 ± 160) in five out of six cases where no growth had occurred. In 11 instances, the number of colonies was greatly increased with rIL2. No correlation exists between the effect of exogenous IL2 and CMV (cytomegalovirus) infection.*

notably CMV, are common after transplantation. They induce $T8^+$ proliferation and T4/T8 ratio inversion (5,6). Nevertheless, IL2 effects on T lymphocyte-colony formation were independent of CMV status, and thus viral infections cannot provide the sole explanation for the T lymphocyte abnormalities summarized previously. Conditioning chemotherapy with cyclophosphamide and melphalan used before transplantation can alter the lymphocytic microenvironment (7). Finally, transplanted T cells and their precursors may present quantitative or qualitative defects. It has been shown that after both allogeneic and autologous transplantation, T lymphocytes have a defect in IL2 production, but in liquid culture with mitogens (OKT3, PHA), T lymphocyte-proliferation capacity can be restored by exogenous IL2 (8).

This is consistent with our finding that, after ABMT, T lymphocyte colony-formation capacity was decreased but can be restored by adding rIL2. In light of these results, the clinical assessment of the effects IL2 administration has on immunologic reconstitution in autografted patients would appear logical.

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Supportive Therapy

H. Vriesendorp and J. Joshi, Chairmen

DR. H. VRIESENDORP: Are there any questions, quick and short please, for any of the speakers?

DR. K. DICKE: I have a question for the peripheral stem cell transplanters. Now that the growth factors are coming into place, first of all what will be the role of the peripheral stem cell transplants? Maybe Martin Korbling is willing to comment on that. Why don't we give bone marrow plus factors? I think that's much easier.

DR. M. KORBLING: The main question is: Is there any principal therapeutic advantage to using blood stem cells—let's say for patients with AML—and that means is the tumor cell contamination in those patients' blood less than in the bone marrow? We don't know, but we have to try it. We have to do clinical studies, and I think we in Heidelberg started that already. We can't at the moment do randomized studies, but that's a good question.

DR. VRIESENDORP: One more question, please.

DR. S. JAGANNATH: Yes, I just wanted to find out if you handled the stem cell cryopreservation any differently from the way bone marrow is handled.

DR. KORBLING: No, I think the cryopreservation technique is even easier. We don't do any manipulation on the mononuclear cell suspension, we don't do any gradient separation or Ficoll separation. We just take it out of the

separator and freeze it, because the red blood cell contamination of those mononuclear cells is pretty low.

DR. VRIESENDORP: Thank you very much, this closes this session.

X. Molecular Biology

Expression of Human Adenosine Deaminase in the Hematopoietic Cells of Monkeys

Martin A. Eglitis, Philip W. Kantoff, Al Gillio, Claudio Boridignon, Jeanne R. McLachlin, Robert C. Moen, Nancy A. Kernan, Donald B. Kohn, Sheau-Fung Yu, Stefan Karlsson, Evelyn M. Karson, James A. Zwiebel, Eli Gilboa, R. Michael Blaese, Arthur Nienhuis, Richard J. O'Reilly, and W. French Anderson

Recently developed retroviral vectors appear to be promising as vehicles for introducing genes into bone marrow cells. A number of qualities make them particularly attractive for gene transfer: 1) they have a wide host range, 2) a single copy can be inserted into the genomic DNA of the cell, and 3) they can be inserted with no apparent harm to the cell. Several laboratories have used such vectors successfully to transfer functioning genes into the hematopoietic cells of mice, both in vitro and in vivo (1-5).

Based on such early successes in mice, we have attempted to establish a model for autologous bone marrow transplant/gene transfer in nonhuman primates. These studies have been carried out in two locations with two different species of monkey. One was at the Memorial Sloan-Kettering Cancer Center using cynomolgus macaques, which have been used there for several

years in an active transplant program. The second was a more recently initiated program at the National Institutes of Health using rhesus monkeys.

Our goal has been to develop a protocol that could be applied toward the potential gene therapy of patients suffering from lethal genetic disease. The initial disease targeted for study is the severe combined immunodeficiency (SCID) caused by adenosine deaminase (ADA) deficiency. A number of technical and medical reasons have been enumerated as to why ADA-SCID is a likely candidate for gene therapy (6).

RESULTS

The vector used in the following experiments is one derived from the Moloney murine leukemia virus and is based on the parental vector N2 (5). A minigene consisting of a 1300-base pair (bp) sequence containing the full-length human adenosine deaminase-complementary DNA (ADA cDNA) (7) regulated by a 400-bp fragment of the early promoter of the virus SV40 early genes was inserted into the unique XhoI site of N2 (Fig 1) to create the vector SAX. Cells producing the SAX vector at viral titres of 2×10^6 were isolated and used to infect monkey bone marrow cells by one of the two protocols described below.

The initial protocol for primates was a modification of one that we had found to be capable of introducing the N2 vector into murine colony-forming units from the spleen (CFU-S) at an efficiency of over 85% (5). In this procedure, bone marrow cells were aspirated from the long bones of an anesthetized monkey and then nucleated cells were isolated by centrifugal sedimentation through a Ficoll-Hypaque gradient. These nucleated cells were then exposed

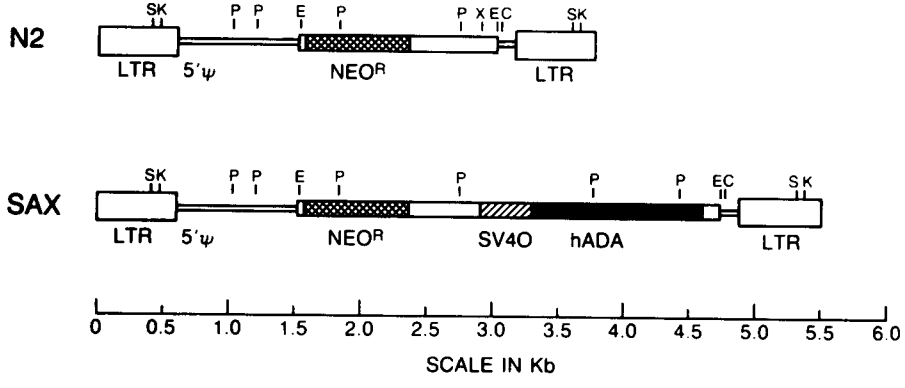

Figure 1. *Retroviral vectors N2 and SAX. LTR, long terminal repeat; 5', retroviral 5' splice donor site; P, packaging signal; Neo^R, neomycin-resistant gene (cross-hatched area is coding region); SV40, SV40 early promoter; hADA, human ADA cDNA. The symbols S, K, P, E, X, and C refer to restriction endonuclease sites SacI, KpnI, PstI, EcoRI, XhoI, and ClaI, respectively.*

to the SAX vector by plating into a dish containing a monolayer of irradiated vector-producing cells. After an overnight co-cultivation, during which time the donor monkey was lethally irradiated, the nonadherent bone marrow cells were recovered from culture by gentle aspiration, washed, and infused into the monkey through an indwelling catheter.

Despite close clinical coverage, the bone marrows of the first four animals listed in Table 1 failed to fully reconstitute and the animals had to be killed, either because they developed antibiotic-resistant sepsis or became refractory to platelet transfusions. It became clear that the co-cultivation protocol was not yielding satisfactory results, in part because of the limitations it placed on the total number of cells that could be treated with vector and in part because of excessive losses of bone marrow cells over the lengthy time of treatment during co-cultivation.

Because of the problems encountered with the co-cultivation protocol, we developed a procedure wherein bone marrow was no longer incubated together with vector-producing cells. Instead, the supernatant virus-containing medium from the producer cells was removed, filtered to remove any loose cells, and then used to infect bone marrow cells. Large volumes of viral supernatants could be collected and frozen while retaining their infective quality. Test infections to generate drug-resistant CFUs in vitro showed that bone marrow could be infected by a 2-hour incubation at 37° C with the viral

Table 1. Primate BMT/Gene Transfer: Summary for hADA Gene

No.	Name	Date	Method	Reconsti-tution	ADA Analysis			
					NPT	ADA	% A-I	% Endog
1	Bill (C)	7/12/85	C′	No	Pos	Pos	1	<0.01
2	Mork (R)	9/6/85	C′	No	—	—	—	—
3	Mindy (R)	9/6/85	C′	No	—	—	—	—
4	Kate (C)	10/29/85	C′	No	—	—	—	—
5	Ethel (R)	10/29/85	S	Yes	Neg	Pos	3	<0.01
6	Robert (C)	11/19/85	S	Yes	Pos	Pos	66	~0.5
7	Kyle (C)	11/19/85	S	Yes	Pos	Pos	17	~0.2
8	Venus (R)	11/20/85	C′	No	Pos	Pos	0.5	<0.01
9	George (C)	3/19/86	S	Yes	Neg	Neg	0	0
10	Ken (C)	3/19/86	S	Yes	Neg	Pos	2	<0.01
11	Oppie (C)	8/6/86	S	No	—	—	—	—
12	Barney (C)	8/6/86	S	Yes	Neg	Neg	0	0

Abbreviations: BMT, bone marrow transplantation; ADA, adenosine deaminase; NPT, neomycin phosphotransferase; C, cynomolgus macaques; R, rhesus monkeys; C′, co-cultivation; S, supernatant; Endog, endogenous.

supernatant. This "supernatant" procedure permitted us to treat a much larger quantity of bone marrow cells and also resulted in much better recoveries of treated cells after infection. Using this protocol, the animals' bone marrow was rapidly reconstituted (see animals 5-7 and 9-12, Table 1).

Animals with fully reconstituted marrows were analyzed by Southern blotting (8) to look for the presence of vector sequences integrated into the genome, by neomycin phosphotransferase (NPT) assays (9) for the presence of the bacterial neo^R marker gene, and by assays for human ADA gene expression. Human and endogenous primate ADAs were separated by ion-exchange chromatography, after which the ADA activity in the human and monkey regions was quantitated by thin-layer chromatography to measure conversion of radioactive substrate (Fig 2) (10).

Overall, of the 12 animals that underwent bone marrow transplant/gene transfer (Table 1), 8 could be analyzed as described above, but the marrows of

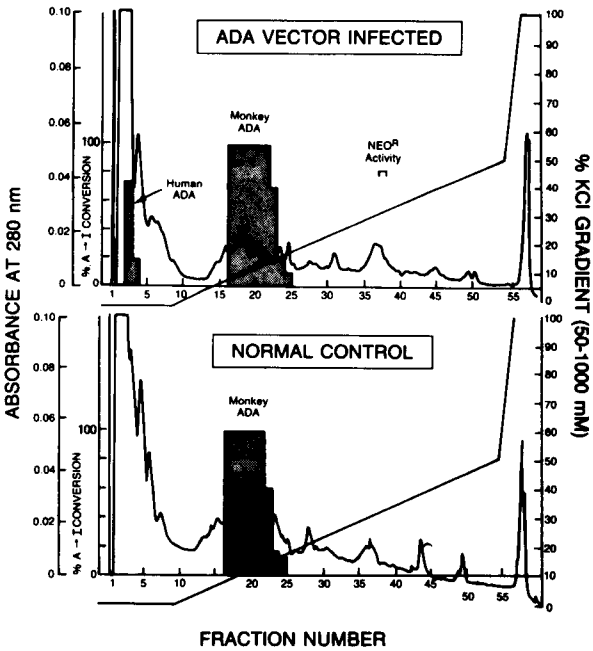

Figure 2. *Adenosine deaminase (ADA) and neomycin phosphotransferase (Neo^R) activity in primate bone marrow cells 10 weeks after transplantation—assay of monkey no. 6 versus untreated control. The graph represents the 280-nm absorbance of bone marrow lysate fractionated on a Pharmacia Fast Protein Liquid Chromatography Mono Q column using a KCl salt gradient. Stippled bars represent ADA activity expressed as the percent conversion of adenosine to inosine (%A→I). Fractions containing human ADA, monkey ADA, and Neo^R enzyme activity are denoted.*

4 were never fully reconstituted and the animals died before they could be analyzed. In six of the eight remaining animals, human ADA activity could be detected, ranging from less than 0.01% of endogenous monkey activity to roughly 0.5% of endogenous activity. In four of the six human ADA-positive animals, varying levels of NPT activity could also be detected. In only one animal (Bill, no. 1) could vector DNA (representing less than 1/10 single-gene dosage equivalents) be detected.

Such low levels of human ADA activity in these animals could represent either near-endogenous levels of expression in a very few cells or very low levels of expression in a large proportion of cells. Since the lower limit of sensitivity of our Southern analyses is 1/20 single-gene dosage equivalent, and since in five of six human ADA-positive animals nonvector DNA could be detected, it is possible that less than 5% of the hematopoietic cells in these animals carry the SAX vector. In an effort to confirm that the low enzyme levels observed were due to a small proportion of cells containing and expressing vector-delivered genes, we performed in situ hybridization of peripheral blood cells using a radioactively labeled probe specific for transcripts from the bacterial neo^R gene; results indicated that 28 of 3415 cells expressed the vector-derived gene. It must be noted, however, that expression of the neo^R gene does not necessarily mean that the accompanying human ADA gene is also expressed. Nonetheless, the sum of these data suggest that probably less than 5% of bone marrow cells were infected and that those infected were expressing human ADA at levels nearly equivalent to those of the endogenous monkey ADA.

The levels of human ADA described here always are the peak levels observed in any of the primates studied; in no animal were the peak levels maintained. For example, in animal no. 7 (Kyle), the maximal level of 0.2% was observed on day 104 posttransplant. Human ADA levels declined from this maximum until no more activity could be detected by day 160 posttransplant. Shortly thereafter (on day 181), a limiting dilution analysis (11) of peripheral T cells was performed to determine if any cells could be detected that had become resistant to the drug G418 as a result of the presence of the neo^R gene. In the absence of drug selection, 1/7 of peripheral T cells could be cloned (Fig 3[line A]). After selection in 100 μg/ml G418 (a concentration at which uninfected T cells were all killed), 1/93 of the T cells from animal no. 7 could still be cloned (Fig 3[line B]). Therefore, approximately 8% of the clonable T cells on day 181 posttransplant still carried expressing vector sequences. It must be emphasized that such a limiting dilution analysis really only accounts for a portion of all peripheral T cells. Therefore, the seemingly high percentage of infected cells should not be interpreted as meaning that an equally high number of total T cells carried functional vector. Rather, it may only mean that at the time of assay, vector-containing T cells still were present in the peripheral blood of the animal.

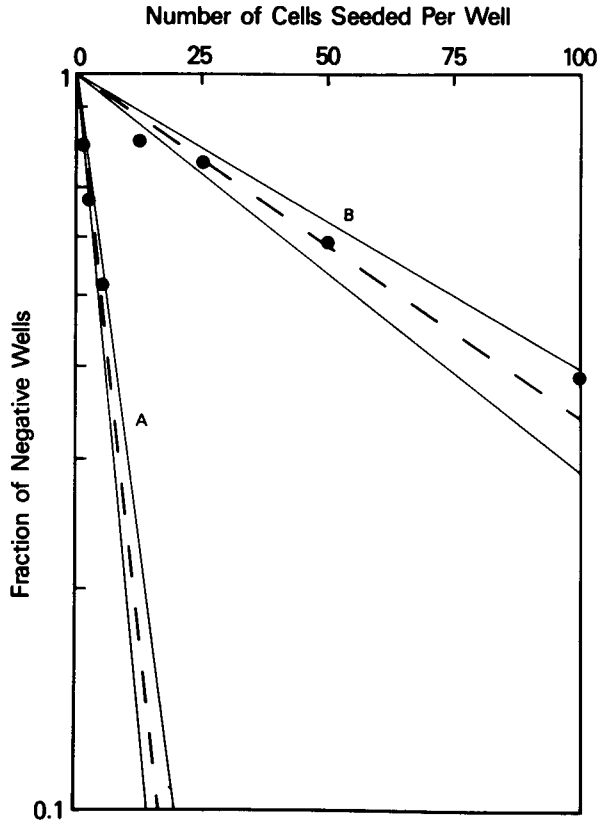

Figure 3. *Limiting dilution analysis of clonable G418-resistant T cells from peripheral blood mononuclear cells from monkey no. 7 collected on day 181 after autologous transplantation of SAX-infected marrow. Assays were performed utilizing the method of Kernan* et al. *(11). The fraction of negative wells was determined by microscopic examination on day 10 of culture. The slope of line A is the frequency (1/7) of clonable T cells in the absence of G148. The slope of line B is the frequency (1/93) of clonable T cells in the presence of 100 μg/ml of G418. This concentration of G418 completely inhibited growth of T cells isolated from noninfected monkeys (i.e., no positive wells in four separate control experiments).*

DISCUSSION

We have used the amphotropic retroviral vector SAX, with contains the cDNA for human ADA, along with the bacterial neo^R gene to develop a primate model for autologous bone marrow transplantation/gene transfer. These studies with cynomolgus macaques and rhesus monkeys have shown that adequate numbers of bone marrow cells will survive after in vitro culture and exposure to a retroviral vector to provide these animals with full hematopoietic reconstitution and long-term survival. In six of eight monkeys with reconsti-

tuted marrows, evidence has also been obtained indicating that gene transfer and expression is possible, albeit at low levels.

The expression of human ADA that was obtained was transitory, but this could be due to one of a number of possibilities. For example, infection might have been restricted to more differentiated hematopoietic progenitors that have a limited life span in vivo. Or, the overall infection efficiency might have been so low that, without some selective growth advantage, the infected population was progressively diluted from the whole hematopoietic system with time. Also, it may be possible that the infected cells express extraneous neoantigens or have undergone some other cell-membrane modification that elicits a response from the reconstituting immune system of the recipient. Still another possibility might be that the decline in human ADA activity is due to selective loss of vector sequences as a result of vector instability. Finally, the human ADA sequences might in some manner be specifically inactivated in the primate marrow cells over time. At the present time we have insufficient information to exclude any of these explanations.

The level of human ADA expression has also varied from animal to animal in these experiments. For example, the human ADA activity found in monkey no. 1 was 2% to 5% of that found in monkeys no. 6 and no. 7 despite the fact that vector sequences were detected in animal no. 1 but not in the other two. Thus, although monkey no. 1 apparently contained SAX in a greater proportion of its cells, the relative expression from each integrated vector was at a much lower level. The variables that may influence vector expression in vivo are not yet understood. It is clear, however, that vectors that do not express in one series may be capable of doing so in others. We have used the SAX vector efficiently to transfer the ADA gene into murine hematopoietic cells in vivo, yet have been unable to detect any human ADA expression in any of these mice.

Would gene therapy still be possible with such poor infection efficiencies and low levels of expression? In many cases gene therapy probably would not be possible, but in the case of the ADA gene it may be. Permanent engraftment of cells containing expressed genes would require successful infection of pluripotent stem cells and probably positive selective pressure in vivo. In ADA-deficient patients, unlike the monkeys used here, selective pressure for cells containing an expressed ADA gene might be present (6).

All of the eight animals with reconstituted marrows have been and continue to be monitored for ill effects after treatment of their marrow with the retroviral vector. None of the monkeys has shown any sign of retroviremia, marrow dysfunction, hematopoietic malignancies, solid tumors, or other signs of a pathologic condition. Despite the evidence for successful engraftment of cells containing expressing genes delivered by retroviruses, we believe that clinical application of this gene transfer protocol will require a higher and more stable level of expression of the inserted human ADA gene. We are currently investigating means by which to improve the infection efficiency of

primate marrow as well as to improve the consistency of expression of human ADA from animal to animal.

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Electric Field-Mediated DNA Transfer Into Human Hematopoietic Cells

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A number of techniques have been used to transfer DNA into mammalian, and in particular into human, cells (1). More recent studies indicate that transfection of hematopoietic progenitor cells may be best accomplished by means of retroviral vectors (2) or by electric field-mediated DNA transfer (3,4). We have elected to study the latter method in detail in order to develop gene therapy protocols for certain hematopoietic disorders.

ELECTRIC FIELD-MEDIATED DNA TRANSFER

The exposure of cells to a brief electric impulse results in the passive transfer across the cell membrane of molecules present in the surrounding medium (5). This technique, also termed electroporation, has been shown to be effective for the transfer of DNA into many cell types, including those refractory to traditional transfection procedures (6). DNA entry is believed to occur via local areas of membrane depolarization created by the external electric field (5,7-9).

Several devices designed to deliver brief electric impulses are now commercially available. We used an apparatus similar to one described by Potter *et al.* (6) comprising a power source (ISCO 494 electrophoresis power supply) and an electroporation chamber. The chamber consisted of a

disposable plastic cuvette (Sarstedt, Princeton, NJ) shortened at the top by 1 cm with platinum foil electrodes (0.025 mm x 50 mm x 9 mm: ALFA, Danvers, MA) mounted down the sides with epoxy glue. The distance between the electrodes was approximately 4.5 mm. The ISCO power supply emits a high-energy pulse that decays exponentially within 1 μsec to an oscillating tail of 20 μsec duration. Elimination of the tail abolished DNA transfer, suggesting that both the high-voltage pulse and the low-energy tail are required for electroporation. The electroporation of hematopoietic cells is performed with the power supply typically set to deliver a pulse of 1.9 kV, which is equivalent to a field strength of 3.8 kV/cm. Efficient DNA transfer into lymphoid cells has also been observed with a commercial device (BTX Transfector 800, Biotechnologies and Experimental Research Inc., San Diego, CA), which delivers a square pulse in nonconductive media.

CHARACTERIZATION OF DNA TRANSFER

The characterization of DNA transfer by electroporation was studied with a variety of lymphoid cell lines (3). We found that the level of transient gene expression, determined as chloramphenicol acetyltransferase (CAT) activity, as well as stable expression, expressed as the number of transfected cells, increased with increasing DNA concentration in the medium. The data suggested that the number of cells taking up DNA increased with increasing DNA concentration.

An analysis of pools of stable transfectants showed that the total amount of DNA introduced into cells increased with the DNA concentration, but when individual clones within the pool were analyzed, most were found to contain a low gene-copy number despite transfection with high DNA concentrations. These data may be explained by the presence of a subpopulation of cells taking up large amounts of DNA, perhaps because of an intrinsic biologic difference or as the result of heterogeneity in electric field strength.

Higher levels of gene expression were obtained when linear rather than supercoiled DNA was used for electroporation. Both transient and stable gene expression were similarly increased, suggesting that the mechanism of DNA transfer may be affected.

An analysis of isolated clones showed that the transferred sequence integrated at random sites in the DNA and that unintegrated DNA was not detected, even when electroporation was performed with high DNA concentrations. We also found that integration occurred exclusively at the site of linearization, so that the structure of the integrated DNA can be predicted.

We also observed that co-transfection of unlinked sequences proceeded efficiently, since 50% to 100% of transformants were co-transformants.

Our attempts to improve transfection frequency by treating cells with chloroquine or sodium butyrate, which have been reported to increase transfection frequency by the DEAE dextran and calcium phosphate

methods, respectively (10,11), were unsuccessful. The lack of success with chloroquine is consistent with the notion that DNA introduced by electroporation is not incorporated into lysosomes (12). Pretreatment with a mitotic-arrest agent such as Colcemid produced a twofold increase in transient gene expression but did not influence stable expression.

The influence of different regulatory sequences on the transient expression of DNA introduced by electroporation was also examined. In all the lymphoid lines tested, the simian virus 40 early region was a better promoter than was the Rous sarcoma virus long terminal repeat.

ELECTROPORATION OF HUMAN HEMATOPOIETIC PROGENITOR CELLS

We have applied the electroporation method to the transfer of DNA into human hematopoietic cells (4). The results of transient gene expression studies demonstrated that normal human nucleated marrow cells were successfully transfected by electroporation.

We next showed excellent human granulopoietic progenitor-cell recovery (as measured in the colony-forming unit granulocyte-macrophage [CFU-GM] assay) after exposure of nucleated marrow cells to the electric field alone (96 ± 3%, n = 3). The granulocyte-macrophage colonies obtained after electroporation were indistinguishable from control colonies. Electroporation per se, therefore, does not affect human CFU-GM viability and probably does not perturb hematopoiesis. We subsequently showed that selectable genes (neo^R, gtp) introduced into marrow cells by electroporation were expressed in the progeny of hematopoietic progenitor cells. We found that 0.8% to 2.7% of CFU-GM expressed the resistant phenotype. DNA transfer was confirmed by hybridization analysis. We further concluded that this transfection frequency may be a minimal value reflecting our highly stringent selection conditions and may reflect a subpopulation of cells expressing high levels of the transferred gene. The actual transfection frequency may be up to 15-fold higher.

Further improvements in transfection frequency may be achieved with refinements in the electroporation apparatus and by more detailed analysis of physical parameters, such as pulse shape, width, and frequency.

ADVANTAGES OF THE ELECTROPORATION TECHNIQUE

Our studies of electric field-mediated DNA transfer have enabled us to identify numerous advantages associated with the technique. They can be summarized as follows:

1. DNA introduced by electroporation does not appear to be mediated by the lysosomal compartment, so that the transferred sequence is not subjected to degradation or mutation.
2. The technique appears to work with most cell types tested, including cells refractory to traditional transfection procedures.
3. Manipulation of DNA into specialized vectors is unnecessary.
4. The DNA can be transferred in low copy number, a condition that may be important for the transfer of stringently regulated genes.
5. The structure of the integrated DNA can be predicted, since integration occurs at the linearized ends.
6. Both transient and stable gene expression can be monitored.
7. Unlinked sequences can be co-transferred efficiently. It may therefore be unnecessary to link genes to selectable markers in cases in which genetic manipulation is difficult or in which screening of large numbers of sequences is required.
8. Viral sequences are avoided for gene transfer and expression. The presence of viral sequences can have several potentially negative effects. By recombination with a provirus present in the host genome, they can produce an infective virus. They can also cause instability in the integrated sequences as a result of recombining viral sequence elements. And finally, they can cause gene expression to be reduced after cell differentiation, possibly as a result of the methylation of viral elements.

CONCLUSION

Although currently the electroporation technique is not as efficient as retrovirus-mediated DNA transfer, further studies to characterize and refine the method are likely to result in improvements in transfection frequency. If this can be accomplished, electroporation may prove to be the method of choice for therapeutic gene transfer.

ACKNOWLEDGMENT

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Aberrant Gene Expression in Acute Myelogenous Leukemia

Mary Jean Evinger-Hodges, Mark Blick, Joel Bresser, and Karel A. Dicke

Evidence is accumulating that cellular oncogenes (proto-oncogenes) are important in normal cellular proliferation and differentiation (1,2). At least three proto-oncogenes encode for proteins that are related to either known growth factors or their receptors: c-*sis*, c-*erb*B, and c-*fms* (3-5). In addition, a number of proto-oncogenes appear to be involved in cellular differentiation: c-*src*, c-*fos*, and c-*fms*.

Interest in the relationship between human cancer and oncogenes developed with the discovery that DNA from human and animal tumors contained activated oncogenes that, when transferred to tissue culture cells, induced malignant transformation (6-8). The expression of proto-oncogenes has also been examined in numerous human malignancies using DNA and RNA hybridization techniques. These studies have produced growing evidence that qualitative and quantitative abnormalities in cellular oncogene expression may be associated with neoplasia.

Chromosomal study of the hematopoietic system is attractive because the chromosomal locations of many cellular oncogenes are now known and because chromosomal rearrangements are known to exist widely in human leukemia (9). Several oncogenes (*myc, myb, abl, sis*) are located at the breakpoint of chromosomal translocations or inversions specifically asso-

ciated with particular leukemias; this may lead to deregulated expression of these oncogenes. Furthermore, structural abnormalities and amplifications associated with the enhanced expression of cellular oncogenes have been consistently observed in neoplastic hematopoietic cells.

At the moment, no monitor system exists for the presence of leukemic cells in remission. The leukemic colony-forming-cell (CFC-Le) assays are not suitable for detecting leukemic cells in remission, since the culture conditions used also give rise to colonies derived from normal cells. In addition, the frequency of CFC-Le is low, 0.01% to 0.1% of the leukemic-cell population, which makes detection in remission unlikely. Because of these limitations, we have begun to investigate the use of molecular hybridization technologies, in particular RNA in situ hybridization, as sensitive detection methods for leukemic cells.

RESULTS AND DISCUSSION

Northern Blot Analyses

Expression Levels

We have found that many of the proto-oncogenes studied were expressed in human normal bone marrow (Table 1). These data support the idea that these genes play a role in adult normal hematopoietic metabolism. Most of these genes (with the exception of c-*fos*) are expressed at very low levels in normal marrow samples. In contrast to the constant low-level expression of these genes in normal cells, their level of expression is high, but variable, in different forms of leukemia (Table 1). This high level of expression is particularly noticeable for c-*myc* in all leukemic samples studied. Under conditions where c-*myc* expression is barely detectable in normal bone marrow samples after northern blot hybridization to a ^{32}P-labeled probe, a strong signal is obtained for all acute leukemia samples tested. Although this increase in signal may simply be a result of an increase in the proportion of proliferating blast cells present in the cell population, it may also reflect an overexpression of c-*myc* in the leukemic cell population.

Transcript Size

There are data to suggest that the presence of an aberrantly sized transcript of certain proto-oncogenes is associated with leukemia (10). In all but two cases, c-*fes* and c-K-*ras*, the transcript size present in normal hematopoietic cells is the same as that detected in leukemia (Table 1). When probing with c-*fes*, we find a broad hybridization band probably representing two or three unresolved bands. In some samples the transcript size is slightly smaller than 2.7 kb and in other samples slightly larger. We can find no correlation between the varying transcript sizes and the samples tested. This leads us to believe that the ability to detect an aberrant transcript will be

Table 1. Level of Oncogene Expression in Human Normal Bone Marrow and Leukemia Samples

	c-*myc*	c-*myp*		c-*fos*	c-*fes*	c-N-*myc*	c-*erb*-Alc		c-K-*ras*		c-N-*ras*		c-H-*ras*	
Cell type	2.4 kb	3.7 kb	2.3 kb	2.2 kb	2.7 kb	4.0 kb	5.0 kb	2.0 kb	5.2 kb	1.2 kb	5.0 kb	1.6 kb	2.9 kb	1.4 kb
Normal cells														
Bone marrow														
Buffy coat	+	+		++++	+	+	+	+	+	++	+	+	+	+
Pellet	+	+		+	+	+	+	+	+	++	+	+	+	+
Peripheral blood														
Monocytes	+	−		++++	ND	ND								
Lymphocytes	+	−		ND	+	+								
Leukemias														
AML	+++	+++		+	+	ND	+	+	++	+	++	+	+	+
AMMol	+++++	+++		++++	++	ND	ND	ND	ND	ND	++	++	ND	ND
ALL	+++	+++		+	−	+	−	+	+	−	ND	ND	++	++
CML-BP	+++++	+++		+	++	ND	+	+	++	−	++	−	++	++
CML-CP	+	+++		+++	++	+	++	++	+	−	+	+	+	+

Abbreviations: AML, acute myelogenous leukemia; AMMol, acute myelomonocytic leukemia; ALL, acute lymphoblastic leukemia; CML-BP, chronic myelogenous leukemia—blast phase; CML-CP, chronic myelogenous leukemia—chronic phase; kb denotes transcript size.

Symbols: −, no expression detected; + to +++++, low to high levels of expression as determined by densitometry; ND, not done.

important in only a few select forms of leukemia such as Philadelphia chromosome-positive chronic myelogenous leukemia (Ph^1 + CML).

A second example of variability in transcript size is present with c-K-*ras*. In normal samples we find a small (1.2 kb) transcript predominantly expressed (Table 1). This transcript is either absent or expressed at only very low levels in leukemia samples with the exception of chronic lymphocytic leukemia (CLL) (Lee *et al.*, personal communication).

Limitations of Our Prior Studies

Since nearly all our studies were carried out on mixed populations of cells, it is impossible to say whether particular oncogenes are expressed in a certain subpopulation of cells or on all cells present in a sample. We are also unable to distinguish between a high level of gene expression in a few cells and a relatively low level of expression in a majority of the cells. Evaluation of the expression of proto-oncogenes at the level of the single cell, rather than in whole populations, will be more informative and specific in determining the proportion of cells within each population that expresses these genes. The study of proto-oncogene expression at the single-cell level is feasible by using RNA in situ hybridization techniques, which are currently being used in our laboratory.

In situ Hybridization

Untreated or Relapsed Acute Myelogenous Leukemia (AML)

At this time, we have studied the expression of three genes, c-*myc*, c-*sis*, and c-*abl*, in patients with untreated acute leukemia. Although we anticipated the overexpression of c-*myc* in these patients based on northern blot data, we were surprised at the very high level expression of c-*sis* that is often present (Fig 1). As expected, c-*abl* expression is at a level similar to that found in normal peripheral blood cells, although there occasionally appears to be a greater percentage of cells positive for c-*abl*.

By northern blot analysis we are rarely able to detect expression of either c-*sis* or c-*abl*. Yet the expression of these two proto-oncogenes, in addition to c-*myc*, was easily detectable at the single-cell level. If similar levels of expression are present in residual leukemic cells from remission marrow, the cells should be readily discernible.

Remission

Initial studies on unfractionated bone marrow samples obtained from patients with AML support the presence of a subpopulation of cells that overexpress c-*myc*, c-*sis*, or both at the RNA level (Fig 2). Although we have no conclusive evidence that this cell population is involved in the leukemic event, results suggest this possibility.

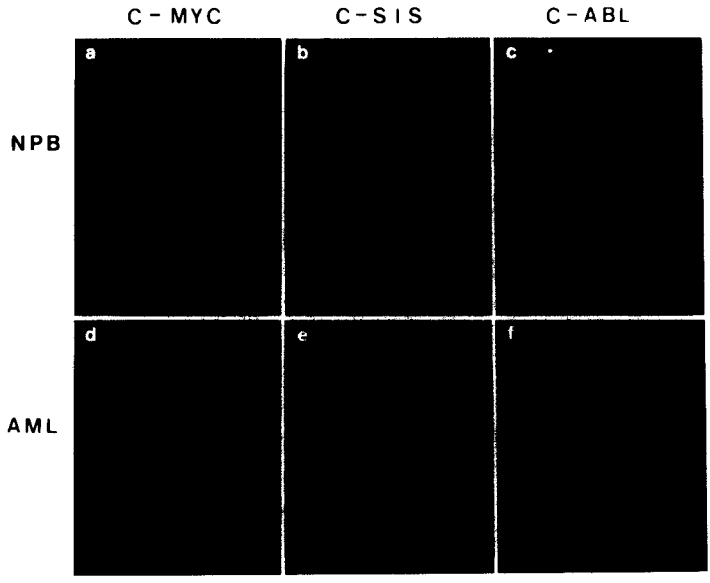

Figure 1. *Expression of* c-myc, c-sis, *and* c-abl *in normal peripheral blood and AML. Cytospin preparations of peripheral blood samples were fixed in 75% ethanol/20% acetic acid before hybridization with single-standard antisense RNA probes labeled with biotin. Hybridization was detected by fluorescein isothiocyanate linked to streptavidin and was photographed through a fluorescence microscope.*

CONCLUSIONS

In relapse or in the untreated patient, detection of leukemic cells is relatively simple (e.g., by morphology, electron microscopy, cytochemistry, cytogenetics, and in vitro CFC assays).

Detection of leukemic cells in remission is much more difficult. We estimate the number of leukemic cells to be 10^8 in remission. Given this estimate, approximately 10^6 leukemic cells are present in the marrow cell suspension to be used for transplantation, which is 1% of the total bone marrow pool. The frequency of the leukemic cell population, therefore, is one leukemic cell in 20,000 cells. None of the techniques for detecting leukemic cells in relapse is either sensitive or specific enough to trace this population in remission. Therefore, these assays cannot be used as monitor systems of the separation techniques used to eliminate leukemic cells from remission marrow-cell suspensions. For these reasons we have begun to investigate the use of molecular hybridization techniques as possible sensitive detection methods—such as detecting aberrant proto-oncogene expression—for identifying leukemic cells.

Recently, we and other investigators have studied oncogene expression

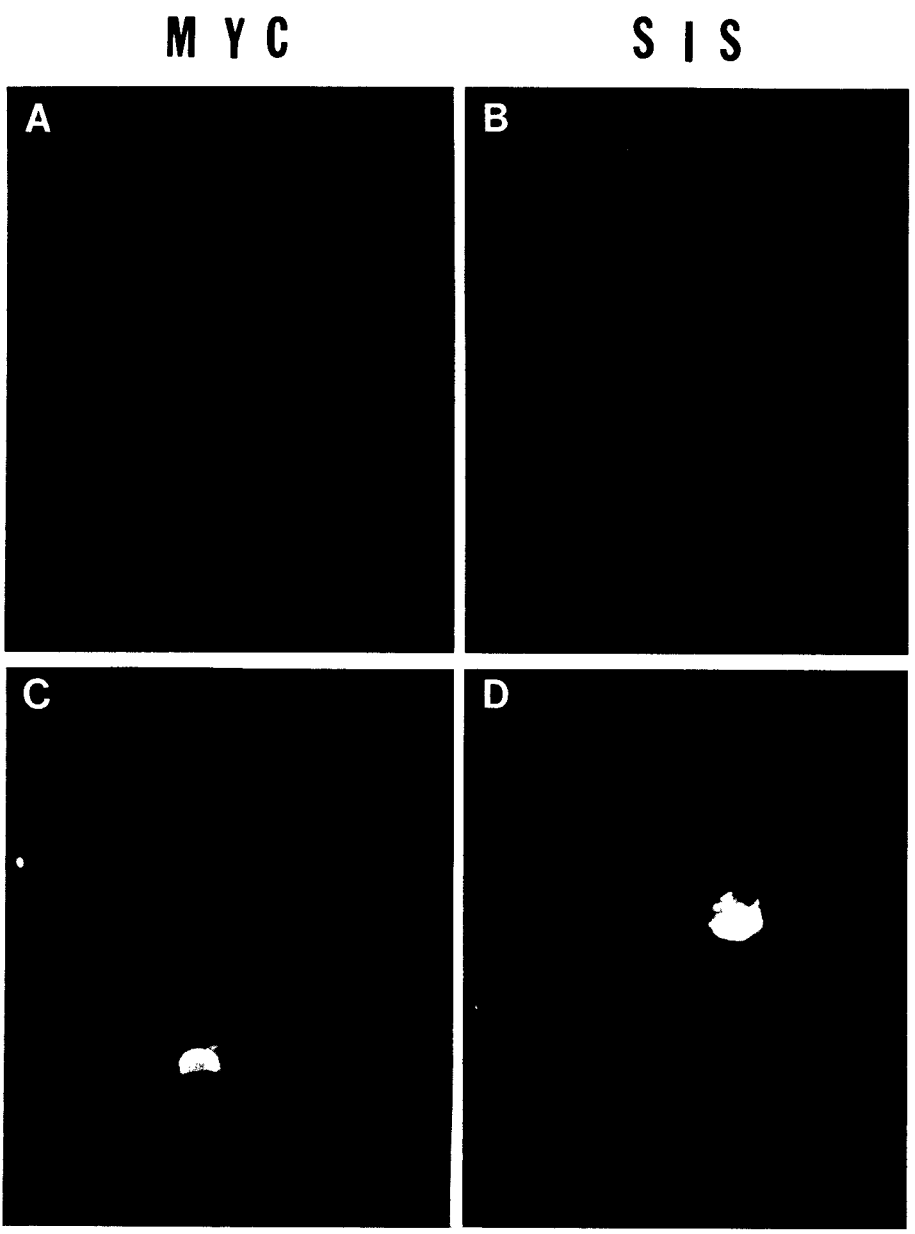

Figure 2. *Aberrant* myc *and* sis *expression in remission marrow. Slides were prepared of bone marrow obtained from normal donors and from patients with acute myelogenous leukemia (AML) in remission. The slide preparations were fixed and hybridized as described under Figure 1. Panels A and B show hybridization signals found in normal bone marrow. Panels C and D, taken from a patient with AML in remission, show a small number (approx. 0.1%) of cells that express high levels of* myc, sis, *or both in comparison to normal-level expression.*

in human leukemia. We found that the expression levels of a number of proto-oncogenes vary greatly between normal hematopoietic cells and leukemic cells. From evidence in the literature and from our own studies, we think that the detection of inappropriate proto-oncogene expression in hematopoietic cells by RNA in situ hybridization technologies is potentially sensitive enough to detect residual leukemic cells in remission marrow.

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Clonal Detection of Remission by Restriction-Fragment Length Polymorphism and Methylation Analysis

Kerry Taylor, Sushant Hardikar, Craig Chinault, Ken McCredie, and Gary Spitzer

With current intensive chemotherapy, morphologic remission in acute nonlymphocytic leukemia (ANLL) can be attained in 65% to 70% of patients. However, a patient who achieves complete remission has only a 20% to 25% chance of remaining free of leukemia with the various chemotherapy programs administered after remission (1). Possible reasons for the high risk of recurrence include: 1) persistence of undetectable chemotherapy-resistant leukemic cells; 2) de novo reinduction of leukemia in normal hematopoietic stem cells; and 3) the persistence in remission of a preleukemic population capable of differentiating into mature elements after frankly leukemic cells have been killed by chemotherapy (2). This population may be susceptible to development of overt leukemia with ensuing relapse. This latter hypothesis envisages ANLL as arising from two or more genetic events: the first enables emergence of a clone of hematopoietic preleukemic precursors that are capable of normal differentiation but have a proliferative advantage over normal precursors; the second, a successive event or events, allows overt leukemia to supervene.

According to the somatic mutation theories of carcinogenesis, neo-

plasms result from the selective growth advantage of a single mutated cell (3). Elegant studies by Fialkow and coworkers, using isoenzymes of the X-linked gene glucose-6-phosphate dehydrogenase (G6PD) as markers, have accumulated evidence for a clonal origin of leukemia (4,5). In heterogeneous women, exclusive expression of the same member of the X chromosome pair in each tumor cell, rather than the normal mosaic pattern of expression expected as a result of the random nature of X-chromosome inactivation, provides strong evidence for single-cell origin. Such work has left little doubt that many malignant hematologic disorders (e.g., ANLL, chronic myelogenous leukemia [CML], myelofibrosis) arise from the clonal expansion of a hematopoietic cell that often has multipotent developmental capabilities.

In 1984, Jacobson and colleagues described a case of ANLL with abnormal cytogenetic and enzymatic evidence of clonality of the leukemic blasts wherein morphologic remission was characterized by a normal karyotype, but enzyme analysis presented persistent evidence for a clonal origin of the repopulating normal blood cells (6). The same group earlier (7) reported a patient with ANLL who had a predominance of leukemic clonal-type G6PD genes in buffy coat cells, platelets, and granulocyte and erythroid colonies after she entered remission. Despite the interest of these observations, this analysis technique does not allow one to gauge the true frequency of such a clonal remission, because heterozygous black women are the only analyzable group.

A new approach to clonality developed by Vogelstein *et al.* allows a larger population of women to be examined (8). The method uses a BamHI restriction fragment-length polymorphism (RFLP) within the first intron of the hypoxanthine guanine phosphoribosyl transferase (HPRT) locus. In women with polymorphic karyotypes, who constitute 25% of the population, BamHI digestion and Southern blot analysis of DNA show a heterozygous pattern of 22-kb and 9.5-kb pair fragments after hybridization with probes from the first intron of the gene. The population of informative women (i.e., heterozygotes) is thus considerably larger than the G6PD heterozygous population.

Subsequent DNA analysis is based on the interpretation of different methylation patterns on active and inactive X chromosomes at the HPRT locus. Tumors originating from a single cell in a polymorphic woman show an association of particular methylation patterns with either the maternal or the paternal allele, whereas normal cells or other polyclonal populations would be expected to show approximately equal distribution of each pattern with each allele. The different methylation patterns are easily detected by Southern blot analysis of DNA digested with BamHI plus a methylation-sensitive restriction enzyme such as HpaII or HhaI. These enzymes have the capacity to recognize methylated cytosine (C) residues, a critical feature, as activation of many genes (including those on the X chromosome) is accompanied by changes in methylation of C residues. Although these changes do not affect all sites that can potentially be methylated, they occur

consistently at some sites and can be monitored there by such restriction endonucleases.

There are at least six HhaI sites within the first intron of the HPRT gene (Fig 1). HhaI cleaves at the sequence CCG:C (G-guanine), but does not cleave at this sequence when either C is methylated. HhaI site 1 is unmethylated in active chromosomes. Cleavage at that site changes the mobility of the BamHI fragment by less than 3%, and this small change in mobility can barely be detected on agarose gels. Sites H2 to H6 are each methylated in over 95% of active X chromosomes, but at least one of sites H2 to H6 is unmethylated in most normal inactive X chromosomes. The use of the second enzyme HpaII allows interpretation when tumors are resistant to HhaI digestion (25-30% of cases). HpaII, like HhaI, is sensitive to changes in methylation and cleaves at the sequence G:CGG except when the C is methylated.

There are at least nine HpaII sites within the HPRT locus (Fig 2). HpaII sites 1 to 3 are unmethylated in the active chromosome and methylated in the inactive chromosome. Sites 4 to 9 are methylated in the active chromosome, and at least one is unmethylated in most inactive X chromosomes. Applications of this approach have been reported using a single enzyme and HPRT probe to study clonality of a variety of tumors (8) and to detect a clonal population of granulocytes in patients with ANLL at presentation, in remission, and in relapse (2). In the latter group, Fearon *et al.* documented 3 of 13 clonal remissions by examining the DNA of remission granulocytes. This suggested that the persistence of a single dominant hematopoietic clone in patients in clinical remission is not rare.

We have refined the methodology of Vogelstein and colleagues by always using two enzymes (HhaI and HpaII) and by using a second probe (9-11). The use of two enzymes allows for interpretation in cases in which the inactive chromosome is resistant to digestion by one enzyme. Probe I is a 1.4-kb EcoRIXhoI fragment isolated from a plasmid subclone of the human HPRT clone Hh13, and probe II is a 0.65-kb HpaII fragment derived from probe I. In situations where the tumor is resistant to both HpaII and HhaI digestion, the use of the 0.65-kb probe enables recognition of the active chromosome. HpaII cleaves a 0.65-kb fragment from the active X chromosome. The remainder of

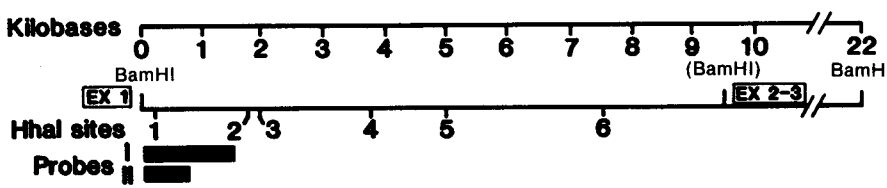

Figure 1. *Diagram of the 5'-half of the human HPRT gene. The polymorphic BamHI site is shown in parentheses, and positions of exons relative to the polymorphic fragments are indicated. HhaI cleavage sites and hybridization probes referred to in the text are also denoted.*

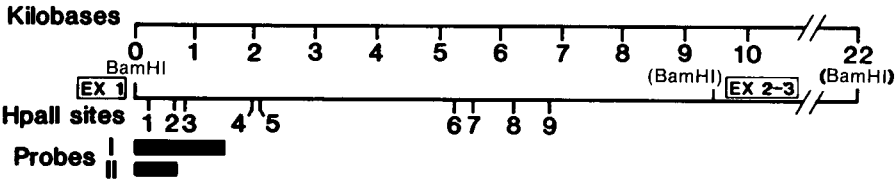

Figure 2. *5'-half of the HPRT gene, this time showing HpaII cleavage sites.*

the active fragment no longer has homology to the probe and thus is no longer seen. Therefore, the use of two enzymes and two probes enables one to establish clonality in all polymorphic women.

The following case illustrates the advantage of this methodology. A 21-year-old white woman presented to our institution in first hematologic remission of acute monocytic leukemia. Cytogenetic analysis revealed a normal female karyotype. Three months later, leukemic relapse occurred with 90% blasts in marrow and blood, and the patient underwent further chemotherapy. Allogeneic bone marrow transplantation from an HLA-identical brother was subsequently performed in second remission; engraftment was noted, and the donor male karyotype (46XY) was documented in 14 metaphases on day 19 posttransplant. Bone marrow aspirate on day 71 revealed leukemic relapse with 96% blast cells. Cytogenetics revealed a female karyotype with multiple abnormalities. The patient died on day 91 with resistant leukemia.

DNA from the patient was analyzed during two relapse phases, one preceding bone marrow transplantation and one 71 days after transplantation. As shown in Figure 3, in each case digestion of the two BamHI alleles with either HpaII or HhaI produced little or no shift in fragment mobilities when probe I was used. The failure to detect hybridization in the 22-kb region with probe II in the BamHI, HpaII digests indicates that all active alleles were associated with the 22-kb fragment. The conclusion of leukemic monoclonality is thus supported by results with this probe.

We are currently involved in examining two major groups of patients for clonality during remission. The first group comprises patients with acute myeloid leukemia in remission. We are monitoring 94 patients in complete remission at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston and are currently analyzing their granulocyte DNA for clonality. In the second group are patients with chronic myeloid leukemia, chronic phase, who are receiving interferon therapy. Many of these patients

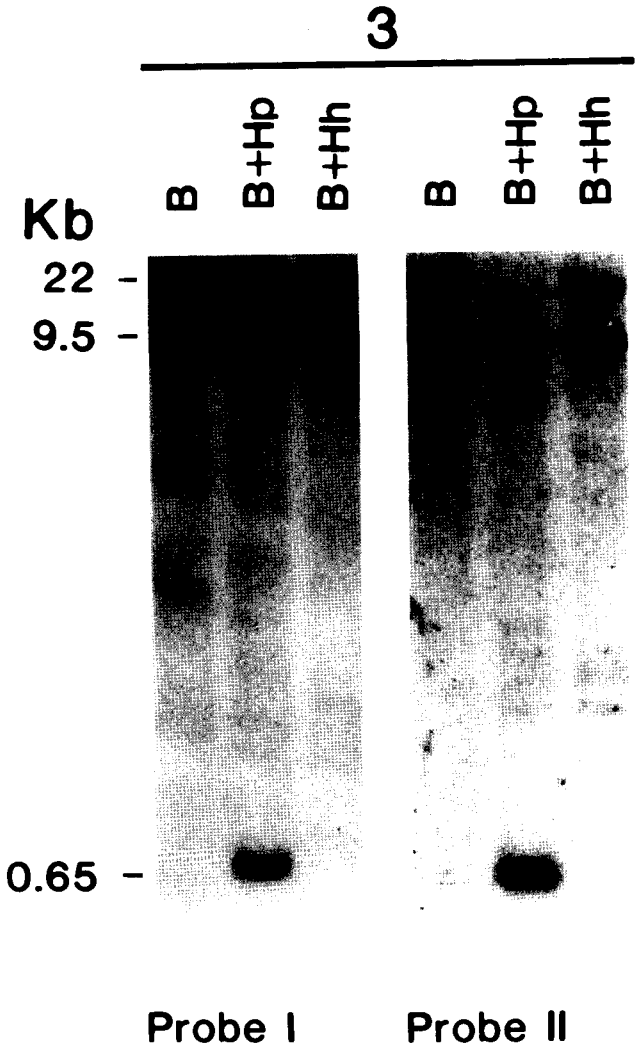

Figure 3. *Two analyses of the DNA of a patient with acute monocytic leukemia. Changes evident in probe II prove monoclonality.*

are achieving hematologic remission and cytogenetic improvement (12). Our methodology permits us to evaluate the clonality of their granulocytes, which may increase our understanding of a Philadelphia chromosome-negative hematologic remission.

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XI. Summary

Summary

D. W. van Bekkum

Attempts to employ high-dose chemotherapy or total body irradiation (TBI) followed by autologous bone marrow rescue for the treatment of advanced disseminated cancer and leukemia were started as long ago as 1959. Three children suffering from terminal relapsed leukemia received treatments of TBI and reinfusion of autologous bone marrow that had been collected during remission and stored for 5 months at −70°C (1). In the same year, Black *et al.* (2) reported their findings on 10 patients with disseminated cancer who received treatments of a high dose of nitrogen mustard and shortly afterward received reinfusions of their own bone marrow, which had been collected just before the treatment.

These studies evoked great enthusiasm. It is amusing to read my comments in the introduction of a symposium on bone marrow therapy and protection in irradiated primates held 25 years ago in Rijswijk. Referring to studies just completed at that time in monkeys and in mice, I said ". . . no particular hazard can be attributed to the use of autologous marrow and this treatment is becoming rapidly introduced as a method permitting the administration of otherwise dangerous amounts of chemotherapeutics and irradiation in cancer therapy" (3). Until 1965, at least 14 other reports appeared, most of which were on the combination of high-dose chemotherapy and autologous bone marrow transplantation (ABMT) (4).

In none of these studies could it be convincingly demonstrated that the

administration of the autologous bone marrow was effective in enhancing hematopoietic reconstitution. The technique of cryopreservation, if employed, was probably inadequate and could not be evaluated because suitable assays were not available.

In the early 1970's, in vitro cultures for the quantitative measurement of viable hematopoietic precursor cells became available, and these were used to monitor the development of reliable cryopreservation techniques. Confirmation was obtained from in vivo experiments with monkeys using the preservation of the protective capacity of the frozen bone marrow cells in lethally irradiated animals (5).

At about the same time, the Seattle group began to treat blastic crisis of chronic granulocytic leukemia with cyclophosphamide and TBI followed by reinfusion of cryopreserved autologous bone marrow collected during the chronic phase of the disease (6). In 1975, Karel Dicke started to explore, at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, the possibility of using autologous bone marrow to treat acute leukemia, because this offered an obvious possibility to avoid graft-versus-host disease (GVHD), which at that time was a very troublesome complication of human leukocyte antigen-identical allogeneic bone marrow transplantation (BMT). In addition, the use of autologous bone marrow would allow treatment for patients with leukemia who did not have a matched donor. Others began pursuing the same approach. The patients of these early trials were all in relapse, but during the past few years centers have increasingly used ABMT as treatment for patients in first remission.

There is no doubt that Karel Dicke deserves credit for the sustained promotion of the use of ABMT as a treatment for patients with leukemia as well as malignant lymphoma and other tumors. The use of ABMT as a rescue for high-dose cytoreductive treatment shows an exponential increase, which can be seen from the data recently published by an international cooperative study group (7) and presented at the meeting by Armitage.

It is appropriate that the meeting (in 1984) and now the third meeting on ABMT were organized at M. D. Anderson Hospital, where much of the pioneering clinical work is being performed. Many of the animal and in vitro experiments, which formed the basis for ABMT, were carried out during the years Karel Dicke spent with me in Rijswijk, and I feel honored and pleased that Karel recalled our profitable collaboration today.

LEUKEMIA

The first four sessions of the meeting were programmed to discuss ABMT in the treatment of leukemias.

Major questions that remain unanswered are: 1) whether purging of the bone marrow graft collected during first remission is effective, and 2) how the results of ABMT compare with those of chemotherapy alone or with allogeneic

T cell-depleted bone marrow grafts. The analysis of the European Bone Marrow Transplantation Group (EBMTG) did not indicate a difference between purging and no purging (8), but this study is a retrospective one and not a controlled clinical trial.

The general impression is that ABMT in first-remission patients with acute nonlymphocytic leukemia (ANLL) may result in similar survival rates as are being obtained with allogeneic BMT, but the follow-up periods are not yet long enough to provide insight into late relapse rate.

Several speakers suggested that TBI is not necessary in the conditioning for ABMT, which might be an advantage in the long run. As alternative cytotoxic treatment, they employed high-dose combination chemotherapy, such as BCNU (carmustine), *m*-AMSA, cyclophosphamide, VP-16-213 (etoposide), or carmustine, Adriamycin (doxorubicin), ara-C (cytarabine), cyclophosphamide, and thioguanine.

Goldstone showed that the latter regimen could be repeated followed by a second ABMT (so-called double autografting) in some patients. The basic idea underlying double grafting is to accumulate the cytoreductive (antileukemic) effect and to affect an "in vivo purging" of the bone marrow. Many patients, however, cannot tolerate double grafting so that a selected subgroup of only 40% proceeded to the second graft.

Both Lowenberg and Gorin observed a slower recovery of platelets and granulocytes in their autografted patients than is seen after allografting. Although this difference was particularly apparent in patients with ANLL, it was not in patients with acute lymphocytic leukemia (ALL). Both ALL and ANLL patients have received similarly severe chemotherapy before their marrow was harvested, so it is unlikely that this factor accounts for this phenomenon. The delayed hematopoietic regeneration after ABMT might be disease related, although the underlying mechanism remains obscure at present. One explanation that came to my mind was based on a difference Prins and I observed in spatial distribution of cells in rat models of acute myelogenous leukemia (AML) and ALL (9). With the progression of AML in the bone marrow we found that AML blasts tend to occupy the sites that are preferred by stem cells (i.e., the endosteal surfaces), while ALL blasts are distributed randomly over the bone marrow. This has led to a more rapid replacement of stem cells by AML blasts with a resulting steeper decrease in stem cell numbers than in ALL.

There is general agreement that questions on the value of ABMT can only be solved by appropriate prospective randomized trials, such as were started in 1984 by a Dutch ABMT study group and reported by Lowenberg. So far, 29 patients have been entered in the autologous marrow arm of this study, and eight of these patients have relapsed. The study is continuing.

Results of ABMT in leukemia patients during second or subsequent remissions were reported by several speakers. In general, survival is less than that observed in patients treated in first remission and that difference is

particularly apparent in patients with ANLL. Yeager purged ABMT pharmacologically with 4-HC (4-hydroperoxycyclophosphamide) and reported that disease-free survival in this group of patients compared favorably with that of patients treated with allogeneic BMTs. Ramsay used purging with monoclonal antibodies and found the overall relapse rate to be greater following ABMT than with allogeneic BMT.

It was suggested that early relapses following ABMT may be ascribed to failure of in vitro purging (i.e., reinfusion of leukemic clonogenic cells), while late relapses would be due to inadequate eradication of the leukemia in the host. If that were indeed true, future efforts would have to be directed at more effective treatment of the patients, as well as at more effective purging.

Employing the brown Norway rat myelocytic leukemia model of AML, Hagenbeek showed that relatively simple modifications of the conditioning regimen may result in increased eradication of leukemic cells. For instance, reversal of the order of the two-drug regimen, cyclophosphamide and busulfan to busulfan and cyclophosphamide, allowed for a significant reduction of the relapse rate in rats rescued with syngeneic bone marrow.

There is agreement that, in effect, ABMT is not profitable in curing chronic myelogenous leukemia (CML), because this disease is a stem cell disease, meaning that when CML becomes clinically manifest pluripotent stem cells have been transformed. It implies, therefore, that CML provides a clear-cut indication for allografting.

Purging

The variety of methods that are being explored seems to reflect the wealth of means we have to inactivate, label, and separate hematopoietic cells, both normal and malignant. The bottleneck for the evaluation of in vitro purging remains that none of the clonogenic assays presently available for human stem cells seems to be a correct measure of the cell that effectuates hematopoietic repopulation.

A number of groups are now using clonogenic assays for primary leukemic cells (instead of leukemic cell lines) to evaluate the antileukemic efficacy of their purging agents. In this way 4-HC, Asta Z, and a new promising derivative, aldophosphamide, were studied. Others employ mixtures of monoclonal antibodies directed against leukemic cell antigens, or ricin-antibody conjugates. Combinations of these various agents are also under study.

The final criterion for effective purging is the absence of relapses and timely recovery of hematopoiesis after ABMT. However, even with adequate purging, a relapse can originate from residual leukemic cells in the host. We have here a problem that can only be solved with sensitive and specific detection methods for leukemic cells.

Several speakers approached this issue by describing novel markers for leukemic cells, among them some fascinating new applications of in situ

hybridization techniques. The lack of proper means of evaluation in patients again underlined the need for realistic animal models of residual disease, such as the brown Norway rat myelocytic leukemia model.

LYMPHOMAS

The second large session of the meeting was devoted to the use of ABMT in the treatment of non-Hodgkin's lymphoma (NHL). This session reflected one of the statements recently made by the organizers, "The exact role of high-dose chemotherapy in salvaging relapsing or resistant patients, as well as its role in intensification therapy for patients who have a high risk of relapse, is difficult to define. One of the main reasons for this difficulty is that the treatment of lymphoma is everchanging" (10). Therefore, patient selection and prognostic indicators were main topics for the discussions.

Buckner presented results of more than 100 resistant patients with NHL who were treated with ablative cytoreduction and BMT. The overall disease-free survival rate was approximately 20%. Interestingly, these results were not different from those in patients treated with syngeneic or allogeneic bone marrow grafts. This would suggest that therapeutic failures are mainly due to inadequate tumor eradication in the host. The Seattle group, in an attempt to improve these results, has recently replaced the conditioning of cyclophosphamide and TBI with a regimen of high-dose cytarabine and TBI, but this has not produced better results.

Philip (from Lyons) announced one of the most important developments, which was an international randomized study in relapsed adult intermediate and high-grade NHL. This trial is to be initiated in January 1987. The autologous bone marrow arm of the study uses involved-field irradiation and intensive chemotherapy (BEAC regimen: carmustine, etoposide, cytarabine, and cyclophosphamide). This study will enroll 300 patients and has disease-free survival as its end point. The importance of such trials cannot be overestimated, because proper animal models for the study of therapeutic regimens for malignant lymphomas are not available. Other speakers described their pioneering work on the application of ABMT in patients with Burkitt's lymphoma and multiple myeloma, which involved purging of the bone marrow graft. In the case of multiple myelomas, there may be a place for allogeneic BMT. When ABMT is used, it might be profitable to concentrate on the use of purified stem cells instead of on the selective elimination of myeloma cells.

SOLID TUMORS

The treatment of neuroblastomas was dealt with in a separate session. Because of the special characteristics of neuroblastoma cells, this disease offers good prospects for successful purging of the marrow. Because of the

limited occurrence of this disease, there is a strong need for cooperative studies. Attempts to proceed in this way have stimulated the discussions on improvements in the staging of the disease and on the identification of prognostic factors.

Among the other solid tumors discussed during this symposium were breast cancer, small cell cancer of the lung, melanoma, soft tissue sarcoma, and glioblastoma.

It is apparent that for the disseminated solid tumors, the situation at present is comparable to that in leukemia 15 to 20 years ago. Most of the studies are of the phase I and II type, and the patient populations are those with a very poor short-term prognosis (i.e., they have extensive and resistant disease). The emphasis has to be entirely on the design of more effective antineoplastic regimens. Purging of the bone marrow is at this time of secondary importance only. If at all necessary, purging is not expected to provide insurmountable problems in view of easily utilizable characteristics of most solid tumor cells. Several speakers emphasized the essential role of deliberate phase I studies for selecting the feasibility and tolerability of supra-dose chemotherapy regimens with ABMT support. Since the rescue technology with autologous bone marrow is now available, the maximally tolerated doses of drugs with critical toxicity for the bone marrow have to be established. This has to be done in patients with advanced disease, but once tolerable dosages have been defined yielding significant response rates, a shift to application in cases during an earlier phase of the disease will automatically take place.

One such attempt was reported by Miser (from the National Cancer Institute) who treated various sarcomas (Ewing, rhabdomyosarcomas, and primitive neuroectodermal tumors) in 68 patients in first remission with five cycles of vincristine, doxorubicin, and cyclophosphamide, followed by intensification with TBI and ABMT.

The overall event-free survival rate of 30% at 48 months was not different from that observed following conventional treatment of these poor-prognosis patients. The conclusion was that the efficacy of TBI as adjuvant ABMT therapy was not clear.

In view of the difficulties involved in seeking effective ablative regimens for these various disseminated solid tumors, the need for realistic animal models at this stage of the work was also stressed. Now that the specific biological behavior of some human tumor types has become better known, it is indeed necessary that transplantable animal tumors of the same histological type be selected on the basis of closer resemblance to the corresponding human tumors. The laboratory work required for such a selection is considerable, but seems to be fully justified as the interest of the clinicians in this area is currently growing.

GENETIC THERAPY

The last session of the meeting was organized around recent developments in genetic engineering of somatic cells, in particular cells of the hematopoietic system. The main thrust in this area is on efforts to insert the adenosine deaminase gene into adenosine deaminase-deficient bone marrow cells and to bring it to expression. This approach would be of interest to hematologists and immunologists, but perhaps not so clearly to oncologists. However, the organizers quite rightly envisage that successful genetic manipulation of any somatic cells may have attractive implications for the treatment of cancer cells. When it was feasible to equip tumor cells with genetic information that would counteract or compensate the genes responsible for malignant behavior, an entirely new treatment modality could easily develop.

In this way, an excellent balance is achieved between work performed at the bedside, applied research in the laboratory, and fundamental investigations, the applications of which are still entirely speculative. The organizers deserve our gratitude for such an achievement.

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Concluding Remarks

Karel A. Dicke

I am impressed by the recent advances made in acute leukemia, Hodgkin's disease, neuroblastoma, and breast cancer. In leukemia, the results of autologous bone marrow transplantation (ABMT) in first complete remission (CR1) are very promising; however, patient selection might be a problem. Randomized studies need to be done, and the role of purging needs to be established.

Significant progress has been made in the treatment of Hodgkin's disease patients who are in relapse. It is also evident that, in Hodgkin's disease, the same rules hold (i.e., the more responsive the disease is to normal-dose chemotherapy, the higher the cure rate).

In neuroblastoma, poor prognosis grade III and good prognosis grade IV, high-dose cytoreduction is clearly the therapy of choice.

Interesting studies are going on in breast cancer, one of which is the in vivo test of normal-dose chemotherapy response, identifying the patient population which may benefit from high-dose chemotherapy. Hopefully, randomized studies will be organized in stage IV disease and poor prognosis stage II disease.

Last but not least, credit needs to be given to Drs. G. Spitzer, T. Philip, and J. Armitage, who organized the first randomized study of ABMT in responsive relapsed non-Hodgkin's lymphoma, the Parma study. This study is worldwide and will attract many participants. Hopefully, it will be well under way at the

time of the Fourth International Autologous Bone Marrow Transplantation Symposium to be held here in Houston in August 1988, which is also the time of the Annual Meeting of the International Society of Experimental Hematology.

I am impressed by the recent advances in the field over the past few years and, hopefully, this trend will continue. Study design and the choice of the appropriate drugs are of crucial importance.

Thank you for your attendance and active participation. We will see you again in 1988.

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