Autologous Bone Marrow Transplantation

Proceedings of the First International Symposium

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To all the patients
who have had autologous bone marrow transplantations.
Without their courage, suffering, endurance, and stimulation,
we never would have attained this level of achievement.

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Preface

It is no wonder that the revival of autologous bone marrow transplantation started in acute leukemia. We, the participants and contributors to this symposium, did not forget the classic paper of McGovern et al in the *New England Journal of Medicine* in 1957 describing relapsed patients with acute lymphoblastic leukemia treated with total body irradiation and stored autologous marrow cells collected in remission.

Since that time, fortunately, things have changed; the quality of complete remission, the quality of marrow storage, and the quality of high-dose cyto-reduction. Beyond this, in vitro methods to grow normal progenitor cells and leukemic cells have been developed in efforts initiated by our Israeli and Australian colleagues. These in vitro methods gave us the tools to monitor any transformation of techniques aimed at separating normal cells from tumor cells, and so another area started to blossom.

Still, this is the tip of the iceberg. We participants in this symposium realize that the most effective treatment of cancer is to touch the roots of the disease through growth regulation and genetic manipulation. Although this sounds farfetched and commonplace at the same time, the solution will be found by illuminating these areas. Autologous bone marrow transplantation may provide us with these tools, and methodologies available today have already made it possible to treat a significant number of patients.

The purpose of the meeting was to compare results, discuss data, and plan new strategies. The application of autologous bone marrow transplantation goes far beyond leukemia. Several sections of the proceedings cover data obtained from treatment of solid tumors, and although definite conclusions cannot yet be drawn, promising leads are visible.

The enthusiasm of the participants gathered from all over the world contributed to the success of this symposium. This meeting would not have reached that quality without discussing not only successes but also failures.

The authors are fully responsible not only for what has been written in the manuscripts and what has been discussed in the panel discussions but also for the treatment of the patients.

Based on the results, we think that autologous bone marrow transplantation has conquered a place in the arsenal of cancer therapy. Many unknowns have yet to be disclosed, but the first steps have been taken. We hope this symposium has sparked the initiative to focus our efforts on achieving a better understanding of the biology of tumor growth and treatment. At the very least it has enhanced collaboration between the various teams, increasing the chances of success so needed for our patients.

Karel A. Dicke

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Our special thanks are owed to Joanne Taylor and Debbie Rees who devoted a tremendous amount of time to organizing the symposium and preparing the manuscripts of the proceedings. The final editing has been done by The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston Department of Scientific Publications under the direction of Walter J. Pagel.

I. Clinical Autologous Bone Marrow Transplantation Studies in Leukemia

Autologous Bone Marrow Transplantation for CALLA-Positive Acute Lymphoblastic Leukemia: An Update

R. C. Bast, Jr., S. E. Sallan, C. Reynolds, J. Lipton, and J. Ritz

INTRODUCTION

In the last 3 years we have undertaken a phase I clinical trial of autologous bone marrow transplantation (ABMT) in children and adults in second or third remission of CALLA-positive acute lymphoblastic leukemia (ALL) (1). Earlier studies in animal systems have demonstrated that 2-3 logs of malignant cells could be eliminated from an excess of syngeneic bone marrow without destroying the ability of normal precursors to reconstitute lethally irradiated recipients (2-5). Preclinical studies indicated that the J5 monoclonal antibody (MAb) against the common ALL antigen (6) would bind to the malignant cells of more than 70% of patients with ALL. Treatment with the J5 antibody and complement destroyed greater than 99% of ^{51}Cr-labeled CALLA-positive cells (7,8), but did not inhibit the growth of granulocyte-macrophage colony-forming cells (CFC-GM), erythroid burst-forming units (BFU-E), or bipotent granulo-erythropoietic precursors (9). In more recent studies, treatment with a combination of J5 anti-gp26 CALLA and J2 anti-gp26 has proved more effective than either single reagent for eliminating clonogenic Burkitt's lymphoma cells from an excess of human bone marrow (10). The combination of reagents did not inhibit growth of normal hematopoietic precursors that could be measured in cell culture. Since there is no satisfactory assay for the pluripotent human bone marrow progenitors in vitro, the present study was undertaken to determine whether reconstitution would occur after ablative chemoradiotherapy in which autologous bone marrow was reinfused following treatment with MAb and complement. The following is a brief update of our experience, which will be reported in greater detail elsewhere (SE Sallan, JM Lipton, J Ritz et al., manuscript in preparation).

PATIENTS AND METHODS

ABMT was undertaken in 24 ALL patients, ages 3-41 years. Of the 24 patients, 1 was in first remission, 15 were in second remission, 7 were in third remission, and 1 was in fourth remission. Twenty-three had had CALLA-positive leukemic cells detected in peripheral blood or bone marrow during pretransplantation relapse and one had CALLA-negative blasts detected at posttransplantation relapse. Patients who were in second or third remission had a variety of regimens, including vincristine, prednisone, and L-asparaginase. Remissions were consolidated with VM-26 and cytarabine (ara-C). Intrathecal prophylaxis was delivered with ara-C hydrocortisone. Bone marrow was harvested and mononuclear cells were separated on gradients of Ficoll-diatrizoate. Approximately one-third of the bone marrow was cryopreserved in 90% autologous serum and 10% dimethyl sulfoxide (DMSO) as a "backup." The other two-thirds of the marrow were treated three times with rabbit complement in combination with either J2 or J5 antibody, or both (1). After being treated with antibody and complement, the bone marrow was similarly cryopreserved and stored in the vapor phase of liquid nitrogen. Ablative chemoradiotherapy was administered with VM-26, ara-C, cyclophosphamide, and total body irradiation (8.5 Gy or 2 Gy x 6). Autologous antibody-treated bone marrow was then thawed and reinfused. Patients were treated in a clean environment with oral antibiotics and observed for hematopoietic and lymphoreticular reconstitution.

Table 1. Survival of Patients Following Autologous Bone Marrow Transplantation for CALLA-Positive ALL

Current Status	No. of Patients	Survival Duration (mo)							
Alive and well	8	44+	42+	29+	28+	16+	12+	6+	4+
Relapsed	8	8	7	3	3	3	2	2	2
Toxic deaths	8	3	3	2	1	1	1	1	1

Note: *CALLA, common acute lymphoblastic leukemia antigen; ALL, acute lymphoblastic leukemia.*

RESULTS

Among the 24 patients, 8 relapsed with CALLA-positive leukemia (6 at <3 months, 2 at >3 months); 8 died while in remission (3 with hemorrhages, 3 with fungal infections, 1 with toxoplasmosis, 1 with an acute respiratory distress system); and 8 remain in remission from 4+ to 44+ months (Table 1). Five patients survived more than 12 months without disease recurrence. All five had had a first remission for more than 2 years. Only one patient had a disease-free interval following transplant (42 months), which exceeded the last previous remission (31 months). Four of the five patients received transplants during a second complete remission. None of the long-term survivors relapsed while receiving chemotherapy.

Survival of patients following ABMT was compared to survival of 18 patients from Boston who received allogeneic transplants. Of the 18 patients, 11 received their transplants from New York, 5 from Seattle, and 2 from Boston. Although the median survival for the autologous group was less than that for the allogeneic graft recipients, survival appeared to plateau in both groups at approximately 30% (Fig 1).

Evidence of myeloid recovery was obtained in all autologous graft recipients who survived more than 20 days. Granulocytes (>1000) were observed in peripheral blood between 16 and 78 (median 44) days; reticulocytes (>0.9%) between 15 and 76 (median 34) days; and platelets (>25,000) between 16 and 103 (median 50) days. Platelet recovery was not obtained in nine patients in remission prior to relapse or to death. T cells, observed in the peripheral blood between 9 and 19 (median 11) days, exhibited a distinct T3+T8+T10+ phenotype. Up to 1 year was required for a normal T4/T8 ratio to be achieved. B cells were observed in peripheral blood between 31 and 128 (median 57) days.

DISCUSSION

Reconstitution and long-term survival following chemoradiotherapy suggest that neither CALLA nor gp26 is expressed on pluripotent stem cells. Similar long-term survival in autologous and allogeneic transplant recipients further suggests that relapse may relate to a failure of ablative therapy to eliminate leukemic cells in situ, rather than to cleanse bone marrow in vitro. Whether removal of leukemic cells in vitro is essential for long-term survival remains to be established. The impact of bone marrow cleansing will be seen more clearly in patients whose marrow is contaminated with clonogenic tumor cells, as well as in diseases in which long-term survival is substantially increased when syngeneic transplantation is compared to conventional treatment. Given the relatively modest fraction of long-term survivors following syngeneic transplantation, it may be difficult to establish a significant difference

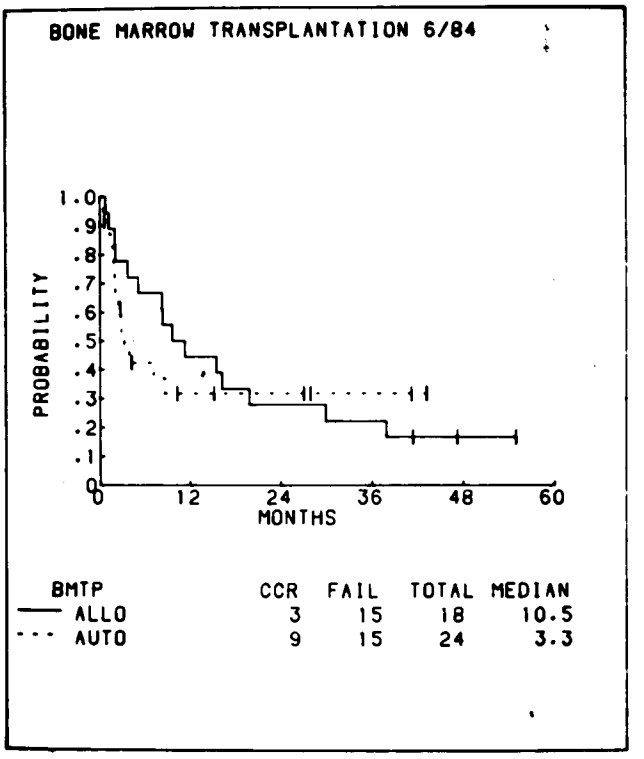

Figure 1. Comparison of survival of allogeneic graft recipients and autologous graft recipients.

between transplantation with marrow that has or has not been treated with MAb and complement--and given the extraordinarily good prognosis for most pediatric patients with CALLA-positive ALL, it will also be difficult to justify a trial to test the impact of transplantation in patients who are in first remission. The experience with CALLA-positive ALL that has been gained to date may, however, provide a prototype for studies of ABMT in other lymphoreticular malignancies (LM Nadler, T Tadvorian, L Botnick et al., unpublished observations).

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Prolonged Survival in Patients with Acute Myeloid Leukemia in First Remission Following Autologous Bone Marrow Transplantation

A. K. Burnett

INTRODUCTION

Allogeneic bone marrow transplantation for acute myeloid leukemia (AML) currently represents the best chance of long-term leukemia-free survival (1-4). The morbidity and mortality of the procedure owing to the immunobiological complications of graft-versus-host disease (GVHD), pneumonitis, and immunosuppression limit its success. Since these problems increase with age, patients older than 35 years as well as those for whom no suitable donors are available are ineligible for allograft. Autologous bone marrow obtained during remission represents an alternative source of hematological stem cells to effect hematological reconstitution following elective ablative treatment with cyclophosphamide and total body irradiation (TBI). Experience with syngeneic transplantation indicates that, in addition to avoiding GVHD, problems associated with immunosuppression and pneumonitis may be diminished (5). It therefore may permit safe application of the technique to an older population of patients with AML and hence a larger proportion of those with the disease.

Conceptual objections to autologous transplantation for patients with leukemia are the possibility of reinfusion of residual leukemia cells and the loss of a graft-versus-leukemia effect. At present, studies of autografts with unmanipulated marrow are insufficient to permit evaluation of purging techniques in vivo. Statistical evidence has been presented to suggest that an antileukemic effect of GVHD exists (6), but this effect is not apparent in patients with AML in remission.

The timing of autograft is likely to be important. Leukemia relapse is a major reason for lack of success in allografts done in relapse (7) or in second or subsequent remission (8). For these reasons, we have undertaken elective autologous bone marrow transplantation (ABMT) in first remission achieved by cyclophosphamide and TBI. In these studies, we stored bone marrow at $4^{o}C$ for up to 54 h.

PATIENTS AND METHODS

Twelve consecutive patients, age 18-53 years, were entered on the protocol and had a minimum follow-up of 65 weeks. They received induction and consolidation therapy with the 'DAT' protocol (9) (Table 1).

Bone marrow was harvested and cryopreserved 6-8 weeks before autograft. A second harvest was stored at $4^{o}C$ as previously described (10); 0.98-3.8 x 10^{8} cells/kg were collected in 710-1390 ml of bone marrow. The patients received two doses of cyclophosphamide at 60 mg/kg and TBI (single fraction) at 9.5 Gy delivered at 5.5 cGy/min. Following irradiation, bone marrow was reinfused. The patients were then nursed by reverse barrier techniques in laminar air-flow rooms, where they received irradiated cytomegalovirus-free blood products, prophylactic infusions of platelets, gut decontamination with oral antibiotics, sterile food, and in some cases, total parenteral nutrition (11).

At hospital discharge, usually dependent on adequate hematological reconstitution, the patients received prophylactic Septrim (sulfamethoxazole-trimethoprim mixture). No further chemotherapy was given.

RESULTS

The 12 patients were induced into initial remission with 2-4 pulses of chemotherapy. They received 3-14 consolidation/maintenance pulses before autograft.

Table 1. Autologous Bone Marrow Transplantation in First Remission AML

Patient No.	Age/Sex	Chemotherapy Pulses		Remission Duration (weeks)			Current Status
		Induction	Consolidation/ Maintenance	Pre-ABMT	Post-ABMT	Total	
1	41/M	2	12	48	148+	196+	A & W
2	33/M	3	14	55	133+	188+	A & W
3	34/M	3	7	90	120+	210+	A & W
4	53/M	3	9	42	20	62	Dead-pneumonitis and relapse
5	53/M	2	8	31	79+	110+	A & W
6	33/M	2	6	20	66+	86+	A & W
7	28/F	3	5	18	32	50	Dead-relapse
8	44/F	3	11	48	65+	113+	A & W
9	27/F	3	5	19	44	63	Dead-relapse
10	53/F	3	4	21	15	36	Dead-relapse
11	18/F	3	4	18	15	33	Dead-relapse
12	48/F	4	3	16	29	45	Dead-relapse

Note: *AML, acute myeloid leukemia; A & W, alive and well.*

The protocol was well tolerated. The defects of pulmonary function and the patterns of immune reconstitution and viral infection have been reported elsewhere (11). One patient (no. 11) had severe hemorrhagic cystitis despite the use of 2-mercaptoethanol to prevent urotoxicity (12); total cystectomy was well tolerated.

Hematological reconstitution was satisfactory, neutrophil counts of 0.5 x 10^9/l being obtained on median day 26 (range, 11-32 days) and 1.0 x 10^9/l on median day 30 (range, 23-50 days). Platelet regeneration followed two patterns. In six patients (nos. 2-5, 10, and 12) platelet levels recovered to greater than 5 x 10^{10}/l on median day 38 (range, 27-40 days), whereas the others did not reach this level before 40 weeks. Platelet antibodies were not demonstrated in these patients, but megakaryocytes were present in marrow biopsy specimens. No patient had hemorrhagic problems that required platelet transfusion, nor did thrombocytopenia prolong any hospital admission.

Six patients relapsed at 15, 15, 20, 29, 32, and 44 weeks after autograft. Six patients remain in continuous unmaintained remission 65+ to 148+ weeks, producing total first remissions of 86+ to 210+ weeks.

DISCUSSION

Cyclophosphamide and TBI therapy even for older patients is well tolerated. Half of the patients remained in remissions of very encouraging duration. It is interesting to note that the six relapses occurred within 1 year of autograft. This is similar to the pattern of relapse noted after syngeneic (13) and allogeneic transplantation, whereas in chemotherapy protocols, the risk of relapse is probably a continuous 25% per year of remission.

A legitimate criticism of this study is that a number of patients were well into first remission at the time of autograft and were therefore a selected group. But even patients in remission for 12 months face a probability of relapse in the subsequent 2 years. In addition, these patients were consecutive patients and were not selected for the autograft protocol. Only one patient who received six or more pulses of consolidation/maintenance therapy has relapsed. As we moved the autograft earlier into remission, relapses did occur. Thus, successful autograft may be dependent on sufficient cytoreduction's being given beforehand. It is our current policy to delay autograft until the completion of six consolidation pulses, which is usually about 6 months into remission.

Sufficient studies involving autografting in first remission with adequate cytoreduction using unpurged marrow will provide a valuable baseline for the evaluation of techniques of purging in vitro. In addition, they will indirectly assist in the elucidation of the postulated graft-versus-leukemia effect. Our experience suggests that the protocol will be well tolerated even by older patients and that a proportion will achieve useful disease-free survival.

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Autografting for Patients with Chronic Granulocytic Leukemia: Current Status and Future Possibilities

R. E. Marcus and J. M. Goldman

INTRODUCTION

Results of Autografting in Transformation

Patients in blast-cell transformation (BT) of chronic granulocytic leukemia (CGL) may be treated with high-dose chemotherapy or chemoradiotherapy followed by hematologic reconstitution with blood- or marrow-derived stem cells (1-5; HD Preisler, A Raza, DJ Higby et al., unpublished observations). Almost all patients are thereby restored to hematopoiesis that is characteristic of the chronic phase of CGL, but such pseudoremissions are usually short-lived. The median survival for autografted patients is approximately 25 weeks. A minority of patients survive more than 1 year and a very small number of patients may survive 2 or more years in second chronic phase with no further treatment.

In almost every case, BT recurs (1,3,5). Morphologic and cytogenetic evidence suggests that recurrent BT is due to reemergence of the same cells involved in the original BT and not to the development de novo of transformation in the autografted cells. Further confirmation of this extreme resistance of CGL blasts to chemotherapy comes from the frequency of recurrent leukemia after allografting in blast crisis. For this reason, over the last 7 years we have attempted to intensify the chemotherapy administered to patients before autografting (3). Our attempts to prolong the duration of the second chronic phase have not, in general, been successful; drug toxicity is greatly increased in the more intensive protocols, especially when these include total body irradiation (TBI) (6). Consolidation therapy without autografting, similar to that used in acute myeloid leukemia (AML), has been used in second chronic phase and preliminary results suggest that such treatment may be of some benefit (HD Preisler, A Raza, DJ Higby et al., unpublished observations). Alternatively, autografting in second chronic phase may itself be used as consolidation therapy.

Second Autografts

Between June 1978 and July 1983, we treated 26 patients with autografts on more than one occasion (3). In the majority of cases, the second treatment was administered when BT recurred; the overall median survival from the date of the first autograft for these patients was 52 weeks (range, 30-80 weeks). This was a selected group of patients since those whose BT recurred relatively rapidly after the initial autograft were judged less suitable for a further autograft than those whose second chronic phase lasted more than 3 months after the first procedure. Of the patients who required autografts in the last 3 years and on more than one occasion, five remain alive at 40-80-week intervals after their initial presentation in BT (Table 1); all, however, have some evidence of recurrent BT.

Over the last year, we adopted a policy of treating patients in BT with two successive courses of identical chemotherapy each followed by autografting. The chemotherapy regimen consisted of cytarabine (1 g/m^2) twice daily on days 1-3 followed by melphalan (140 or 160 mg/m^2) on day 4, and the autografts each consisted of 10 x 10^8 nucleated blood cells/kg on day 5. Of the seven patients who entered the protocol, two completed the treatment (patients 1 and 5; Table 2), three died early, and two (patients 6 and 7) were not in the protocol long enough for evaluation.

Table 1. Multiple Autografts for CGL

Patient	Sex/Age	Type of Transformation	No. of Autografts	Survival (wks)[a]	Karnofsky Status
1	F/39	L	4	80+	80%, asymptomatic CNS disease
2	M/24	MK	2	55+	90%, in BT
3	M/24	MYL	4	48+	90%, in BT
4	M/52	MYL	4	45+	20%, pancytopenic
5	F/35	MF	4	40+	90%, CP

[a]*Survival is calculated from date of first autograft. Date of analysis, 20/07/84.*

Note: *CGL, chronic granulocytic leukemia; F, female; L, lymphoid; CNS, central nervous system; M, male; MK, megakaryoblastic; BT, blast-cell transformation; MYL, myeloid; MF, myelofibrotic; CP, chronic phase.*

Restoration of Philadelphia Chromosome (Ph^1) Negativity

It is well known that high-dose chemotherapy can restore some patients to partial or completely Ph^1-negative status (7-11). Similarly, there are some tantalizing reports of patients with Ph^1 negativity after autografting (2,3,12). Possible explanations for this observation are as follows: 1) The Ph^1-negative metaphases in the recovering bone marrow could be of nonmyeloid origin. 2) The stored hematopoietic cells may comprise a mixture of Ph^1-positive and Ph^1-negative cells (13,14), but the former are selectively damaged by cryopreservation. 3) The stored cells are a mixture of Ph^1-positive and Ph^1-negative cells, but the latter have a proliferative advantage, at least in the short term following reinfusion (Fig 1). There is some in vitro evidence that confirms that some Ph^1-negative progenitor cells are present at diagnosis in the peripheral blood of patients with CGL (14).

Unfortunately, these episodes of "induced" Ph^1 negativity have been relatively short in most cases. Furthermore, it is possible that these Ph^1-negative cells are part of the malignant clone. Even then, the deliberate induction of a period of Ph^1 negativity could prolong the duration of chronic phase since these Ph^1-negative cells might constitute a less evolved cohort of the leukemic population and thereby benefit patients by restoring the disease to an earlier phase. Blood or marrow stem cells harvested during the phase of Ph^1 negativity have been used successfully for subsequent autograft procedures (8,11), and some of these patients have been long-term survivors.

Autografting in First Chronic Phase

Because survival after the onset of BT is usually short, it is worth considering the possibility of autografting in chronic phase. Possible rationales for the procedure are as follows: (a) It is possible that busulphan or hydroxyurea administered at dosage affects the stem cell compartment and the total granulocyte mass (as reflected in the blood count) differently. Thus, the leukemic stem cell compartment may enlarge progressively during the course of the disease, even when the blood count is adequately controlled. Autografting with high doses of alkylating agents such as busulphan may, however, have the capacity to reduce the size of the stem cell compartment and thereby also reduce the chance of

Table 2. Patients with CGL Scheduled to Receive Elective Double Autografts

Patient	Sex/Age	Type of Transformation	Chemotherapy	Actual No. of Autografts	Survival (wks)[a]	Karnofsky Status or Cause of Death
1	F/38	MYL	Ara-C x 2 melphalan	2	15+	100%, CP
2	M/38	MYL	Ara-C melphalan	1	4	Died of infection
3	F/45	MYL	Ara-C x 2 melphalan	2	8	Rapid recurrence of BT after first autograft, died of renal failure
4	M/25	MYL	Ara-C melphalan	1	2	Died of fungal pneumonia
5	M/36	MYL	(1) Melphalan (2) Ara-C, melphalan	2	16+	100%, CP
6	F/30	MYL	Ara-C melphalan	1	4+	100%, CP
7	M/61	MYL	Ara-C melphalan	1	2+	60%, not yet evaluable

[a]*Survival is calculated from date of first autograft. Date of analysis, 20/07/84.*

Note: *CGL, chronic granulocytic leukemia; F, female; MYL, myeloid; Ara-C, cytarabine; CP, chronic phase; M, male; BT, blast-cell transformation.*

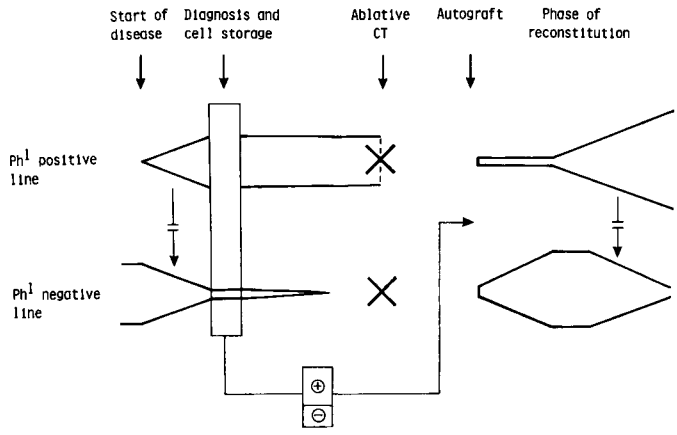

Figure 1. Speculative schematic representation of the progression of Ph¹-positive (above) and Ph¹-negative (below) hematopoiesis in a patient with CGL. Cryopreserved buffy-coat cells contain a mixture of Ph¹-positive and Ph¹-negative stem cells. After autografting, Ph¹-negative cells proliferate more rapidly at first than do Ph¹-positive cells; later, the Ph¹-positive population regains its intrinsic proliferative advantage. The vertical arrows (with interrupted shafts) reflect the direction of probable inhibitory forces.

transformation in a leukemic stem cell. (b) If the duration of chronic phase were genetically programmed, albeit in a manner that cannot be predicted with precision in any individual patient, the capacity to restore hematopoiesis that is characteristic of an earlier phase of the disease should prolong the duration of the chronic phase. (c) If an adequate proportion of patients could be restored to Ph¹-negative status, and if such restoration reflected normal hematopoiesis, then the duration of survival after diagnosis might again be prolonged.

Postulates (a) and (c) provide a speculative rationale for performing an autograft procedure soon after diagnosis and for repeating it at 1- or 2-year intervals, perhaps with reharvesting bone marrow after each autograft. Postulate (b) provides a rationale for autografting 2 or 3 years after diagnosis and for repeating it at intervals thereafter. It would be extremely difficult to evaluate possible clinical benefits that may accrue from such autografting. The appearance of Ph¹-negative marrow metaphases may give some information, but a randomized comparison of survival for autografted and nonautografted patients would ultimately be required.

We treated two patients with autograft procedures while they remained in the first chronic phase. One patient was a 34-year-old woman who was treated with cyclophosphamide (60 mg/kg x 2) and TBI (2 Gy x 5) and in whom subsequent transfusion of HLA-identical marrow cells, depleted of T-lymphocytes, failed to engraft. A second transfusion of untreated marrow from the same sibling donor also failed to engraft. The patient then received 20 x 10.8/kg autologous blood-derived buffy-coat cells, cryopreserved at the time of diagnosis. Engraftment ensued rapidly. Thirty-three percent of her marrow metaphases were Ph¹-negative 4 and 8 weeks after autografting (7/21 and 10/30 metaphases, respectively).

The second procedure was electively performed on a 34-year-old man who had had Ph¹-positive CGL for 30 months and who lacked HLA-identical siblings. The nature and risks of the procedure were first discussed in detail with the patient. He received a dosage of busulphan (4 mg/kg orally for 4 consecutive days), followed by melphalan (60 mg/m² on day 5) and 10 x 10.8/kg reconstituted blood-derived buffy-coat cells on day 7. He was discharged from the hospital 16 days after autografting with the following hematology values: (rising) WBC count of 1.6 x 10.9/l and platelet count of 75 x 10.9/l.

CONCLUSIONS

Autografting for patients in BT of CGL is technically easy, but prolongs life only marginally in the majority of cases. There are a number of possible reasons why autografting for patients in the first chronic phase of CGL could prolong life; and the time for undertaking a suitably designed pilot study, perhaps on a multicenter basis, has now arrived.

ACKNOWLEDGMENTS

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Autologous Bone Marrow Transplantation for Acute Leukemia in Europe

N. C. Gorin

A registry has been opened to collect European data on autologous bone marrow transplantation (ABMT) therapy in patients with acute leukemia (AL). Results of the first European survey (updated to January 1984) are presented. Fifteen centers have reported that ABMTs have been performed on a total of 141 patients with AL. Results of geographic distribution of 135 patients (46 acute lymphocytic leukemia [ALL], 83 acute nonlymphocytic leukemia [ANLL], and 6 unclassified AL) are presented in Table 1.

Of the 46 ABMTs performed in ALL patients, 19 were performed while the patients were in relapse (first relapse, 7; subsequent relapses, 12) and 27 ABMTs were performed while the patients were in complete remission (CR) after high-dose therapy as consolidation (in first remission, 16; in subsequent remissions, 11) (Table 2). In 17 patients, the reinfused marrow had been previously purged with the following: cyclophosphamide derivatives, 10; monoclonal antibodies, 6; and deoxycoformycin, 1. For ALL patients who were in relapse, the probability of being in remission and of surviving 20 months post-ABMT was approximately 10%. Patients who have been consolidated by ABMT and are in their first CR have a 65% probability of remaining in CR 12 months post-ABMT without maintenance therapy (Fig 1). The actuarial survival rate at 12 months is 60%.

Overall, results from purged marrow appear to be better than results from untreated marrow; however, purged marrow has been used to consolidate patients in CR, whereas untreated marrows have essentially been reinfused in patients who are in relapse. Therefore, no conclusive evaluation of the purging procedures can be done. It must be emphasized that of eight ALL patients in first CR consolidated by total body irradiation (TBI) (7) or 6-thioguanine, aracytine, CCNU, cyclophosphamide (TACC) (1), and autografted with marrow purged by cyclophosphamide derivatives (6), monoclonal antibodies (1), or deoxycoformycin (1), all are currently in unmaintained CR (1+ to 11+ months). Of the 83 ABMTs performed in ANLL patients, 37 were done in patients in relapse (31 in first, 6 in subsequent remissions), and 46 were done to consolidate patients in CR (39 in first, and 7 in subsequent remissions) (Table 3).

In the group of patients autografted in relapse, there is no reported case of cure. The patient with the longest disease-free survival (44 months following first ABMT + 24 months following second ABMT) developed a central nervous system (CNS) leukemic infiltration despite previous prophylaxis and died while in persistent systemic CR, which suggested that an occasional cure can probably be achieved as a rare event (Fig 2). On the other hand, high-dose consolidation therapy and ABMT in first CR is associated with high rates of persistent remissions and survival (Fig 3).

As of January 1984, 28 patients autografted in first CR with untreated marrow, 7 relapsed, 1 died, and 20 (71%) remained in unmaintained CR, from 3+ to 35+ months (Table 4). Nine patients were still in CR more than 1 year post-ABMT. Eleven additional patients were autografted in similar condition, but with marrow purged by cyclophosphamide derivatives; 2 patients died too early for evaluation, 3 relapsed, and 6 (55%) remained in unmaintained CR from 3 to 15+ months.

As mentioned earlier, there is, at present, no indication that using purged marrow is superior to transplantation of untreated marrow. However, the follow-up is too short to draw any conclusion, since it may be postulated that patients receiving nonpurged marrow will relapse earlier. Results of ABMT in first CR for patients with ANLL appear to be

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Table 1. Geographic Distribution of Patients with ALL and ANLL

	Distribution of patients		
		ALL	ANLL
Barcelona			1
Besançon		9	19
Bologna			1
Bordeaux			1
Geneva			1
Genova	5		
Glasgow			9
London	1	11	7
Marseille		7	16
Nice		1	
Paris (St Antoine)		12	10
Parma		3	
Pesaro		6	6
Roma		2	7
Rotterdam			3
Utrecht			3
Total		**51**	**84**
Excluded for analysis		5	1
Analyzed		46	83

Total acute leukemias : 135

Table 2. ABMT--Status of Patients with ALL

STATUS OF THE PATIENT			MARROW	DFS	(%)
Total: 46	CR : 27	First : 16	treated : 9	8	(89)
			untreated : 7	4	(57)
		Subsequent : 11	treated : 4	1	
			untreated : 7	3	
	Relapse : 19	First : 7	treated : 1	1	
			untreated : 6	1	
		Subsequent : 12	treated : 3	1	
			untreated : 9	1	(11)
			Total: treated : 17	**11**	**(64)**
			untreated : 29	**9**	**(31)**

similar to results of allogeneic transplantation at 12 months. Obviously, a longer follow-up is necessary to draw any conclusions in this regard.

Pooling all the data together has yielded additional information. For instance, TBI is associated with better results than all combination chemotherapy regimens combined in a single group: of 21 receiving TBI, there were five relapses and deaths; of 31 receiving combination chemotherapy, there were 16 relapses and deaths.

The kinetics of recovery of hematopoiesis are related to the number of bone marrow cells infused in a nonlinear model. In patients receiving doses of greater than 5 x 10^4 CFU-C/kg, the median days for recovery to 10^9 leukocytes/l and 50 x 10^9 platelets/l have been 14 and 25, respectively, compared to 22 and 35 in the group of patients receiving less than 3 x 10^4 CFU-C/kg doses of bone marrow.

Also of interest is the possibility of performing ABMT with nonfrozen marrow, which is under investigation by A. Burnett et al. The nonfrozen marrow is stored at 4°C for 54 h, during which period a high-dose regimen with cyclophosphamide (60 mg/kg/day x 2 + TBI) can be administered.

Table 3. ABMT--Status of Patients with ANLL
(European Survey: January 1984)

Status of the Patient			Marrow		DFS	(%)
Total: 83	CR: 46	First: 39	treated:	11	6	(55)
			untreated:	34	21	(62)
		Subsequent: 7	treated:	0	0	
			untreated:	7	2	
	Relapse: 37	First: 31	treated:	1	0	
			untreated:	24	3	(13)
		Subsequent: 6	treated:	1	1	
			untreated:	5	1	
		Total:	treated:	13	7	(54)
			untreated:	70	27	(39)

Note: ABMT, autologous bone marrow transplantation; ANLL, acute nonlymphocytic leukemia; DFS, disease-free survival; CR, complete remission.

One hundred thirty-five AL patients treated with ABMT have been reported to the registry since January 1984. More recently, ABMT has been performed after high-dose therapy to consolidate patients in remission. Most of the ABMTs were done while the 55 recipients were in first CR.

Although reinfusing remission-cryopreserved marrow in patients in relapse produces a high rate of subsequent CRs, it results in a very small long-term survival rate (<10%). Interestingly, the patient surviving the longest, a 20-year-old man with acute myelocytic leukemia (AML), had a disease-free survival of 6 years following two ABMTs after the first relapse. He eventually died of leukemic infiltration in the CNS, despite previous prophylaxis and persistent systemic remission.

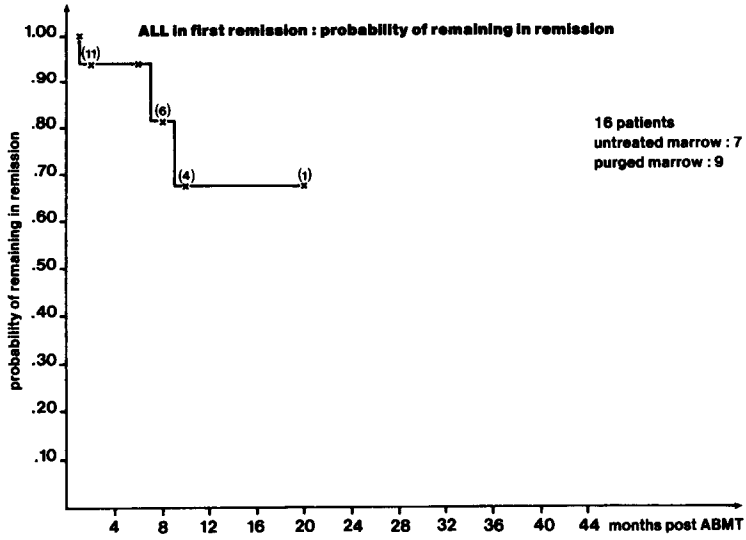

Figure 1. ALL patients in first remission and the probability of their remaining in remission.

Table 4. ABMT for ANLL in First Complete Remission: Untreated Marrow
(European Survey: January 1984)

Team	No. of Patients	Cytoreductive Regimen	Relapses (duration : months)	Results Deaths	DFS (duration : months)
Besancon	3	TACC		1	2 (32+, 32+)
Geneva	1	HDM			1 (3+)
Glasgow	9	TBI	1(5)		8 (4+, 24+)
London	3[a]	modified BACT + ADR			3 (3+, 34+)
Marseille	4[a]	HDM	3(4, 7)		1 (8+)
Paris (St. Antoine)	1	TACC	1(5)		0
Pesaro	2	TBI	1(9)		1 (20+)
Rotterdam	3	TBI	1(13)		2 (8+, 35+)
Utrecht	2	TBI			2 (2+, 3+)
Total	28		7	1	20 (71%)[b]

Note: *ABMT, autologous bone marrow transplantation; ANLL, acute nonlymphocytic leukemia; DFS, disease-free survival; TACC, 6-thioguanine, aracytine (cytarabine), CCNU, cyclophosphamide; HDM, high-dose melphalan; TBI, total body irradiation; BACT, carmustine, cytarabine, cyclophosphamide, thioguanine; ADR, Adriamycin.*

[a] *Double ABMT in two patients for each center.*

[b] *Includes 10 long-term (>1 year).*

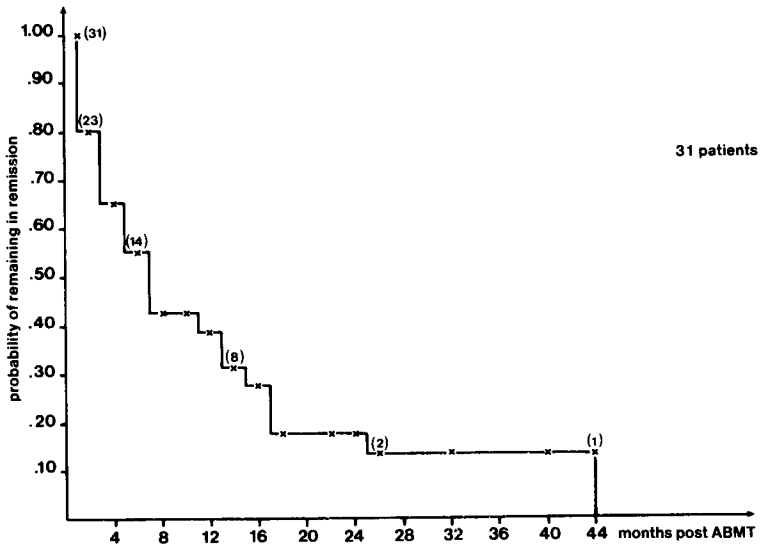

Figure 2. ANLL patients in relapse and the probability of their being in remission.

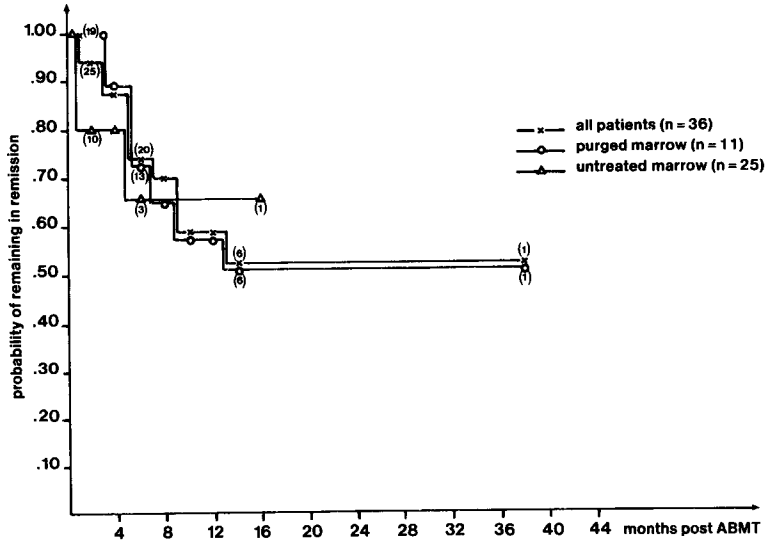

Figure 3. ANLL patients in first remission and the probability of their remaining in remission.

Although the follow-up has not been long (at most 35 months), it appears that high-dose therapy and ABMT, delivered in the consolidation mode during the first CR, may be associated with a high rate of unmaintained disease-free survival.

At present, there is no valuable indication that ABMT with purged marrow is superior to ABMT with crude marrow. However, a longer follow-up is necessary to detect the possible improvement by the purging procedures. Will patients engrafted with unpurged marrow relapse earlier? There is some indication that recovery from hematopoiesis is faster in patients receiving higher doses of marrow (>5 x 10^4 CFU-C/kg) and that TBI may be superior to chemotherapy.

In conclusion, two suggestions are proposed. One is to consolidate patients with AL early in their first CR, by TBI + ABMT, with purged marrow whenever possible, and with crude marrow otherwise. In any case, the quality of the remission at the time of bone marrow collection may be the most critical factor for success. Therefore, additional consolidation courses following remission induction should be given before bone marrow collection--and a careful analysis of the marrow by appropriate means is mandatory. The other suggestion is that although maintenance chemotherapy following ABMT may prove to be useful in the future, it should not be used at the present time so that a conclusive analysis of the data can be made.

Autologous Bone Marrow Transplantation in Acute Leukemia: A French Review of 120 Patients

P. Herve and the French Study Group on Autologous Bone Marrow Transplantation in Acute Leukemia

INTRODUCTION

The results of massive cytoreductive therapy followed by autologous bone marrow transplantation (ABMT) in acute leukemia are not conclusive (1-4). Although high-dose cytoreductive regimens with ABMT appeared to be very effective in obtaining complete remissions (CRs) in leukemia patients in relapse (1,5), prolonged remissions were achieved in only a few cases. The actual step is to evaluate ABMT in well-defined groups of acute leukemia patients who are in remission.

A potentially serious drawback to the use of autologous marrow for transplantation is that leukemic cells contaminate the bone marrow at the time of harvesting so that leukemic cells will be reinfected with bone marrow cells at the time of transplantation. At the present time, most of the ABMT protocols include in vitro manipulation of the bone marrow before freezing, in an attempt to eliminate the residual leukemic cells. Among the various methods of "purging" bone marrow cell suspensions collected from patients in remission (6), in vitro incubation with chemical agents or with monoclonal antibodies (MAbs) plus complement or with MAb conjugated to ricin has been used. In this chapter, the results of 120 leukemic patients undergoing ABMT in seven French centers between January 1980 and June 1984 have been recorded.

PATIENTS AND METHODS

Patients

Since 1980, 120 patients (74 males and 46 females) with acute leukemia were entered into ABMT programs. The median age of the 82 adult patients was 30.4 years (range, 15-55 years), and the median age of the 38 children was 8.8 years (range, 2-15 years).

Of the 67 ABMTs performed in acute nonlymphoblastic leukemia (ANLL) patients, 30 were done in relapsed patients and 37 in CR patients (28 in first CR and 9 in second CR).

Of the 53 ABMTs performed in patients with acute lymphoblastic leukemia (ALL), 12 were in relapse and 41 were in CR (12 in first CR, 25 in second CR, 4 in third CR). There were 31 patients with non-T cell non-B cell ALL, 6 patients with T-cell ALL, and 4 patients with B-cell ALL (Table 1).

Conditioning Treatment before ABMT

Twenty-five ANLL patients (18/25 in relapse) received high-dose melphalan (HDM) (140-240 mg/m^2) given as an intravenous bolus through a central venous catheter (7). Thirty-one patients (most ANLL) received the high-dose chemotherapy combination TACC, which contained 6-thioguanine (200-400 mg/m^2 daily over 4 days), cytarabine (in continuous infusion, 200-400 mg/m^2 over 1 day), and cyclophosphamide (45 mg/kg daily over 4 days or 60 mg/kg over 2 days). Seventeen patients (most ALL) received the BACT regimen, modified by the Institut Gustave Roussy, which contained 6-thioguanine (200 mg/m^2 daily over 4 days), cytarabine (200 mg/m^2 daily over 4 days), BCNU (200 mg/m^2 daily over 3 days), and cyclophosphamide (50 mg/kg daily over 4 days). Forty-seven patients (most ALL) received cyclophosphamide (60 mg/kg daily over 2 days) and 10-12 Gy total body irradiation (TBI) in single dose or fractionated doses.

Table 1. Intensive Therapy and Autologous Bone Marrow Transplantation in Acute Leukemia[a]

Teams	ANLL Relapse	ANLL CR	ALL Relapse	ALL CR
Besancon (Eastern Coop. Group)	11	15	2	16
IGR/CRETEIL	2	1	3	8
Marseille	10	7	2	3
Nantes	-	2	-	1
Nice	-	-	-	3
Paris St. Antoine	5	9	5	9
Paris Hotel Dieu	2	3	-	1
Total	30	37	12	41

[a]*Taken from the French Survey (June 1984) - Distribution of patients.*

Note: *ANLL, acute nonlymphoblastic leukemia; ALL, acute lymphoblastic leukemia; CR, complete remission.*

Methods of Purging Remission Bone Marrow

Bone marrow cells were chemically purged with the following agents (8-10): a) 4-hydroperoxycyclophosphamide (4-HC), b) a metabolite of the oxazaphosphorines (Asta Z 7557, INN mafosfamide), and c) deoxycoformycin. According to the cell concentration per milliliter, the Asta Z doses varied from one team to another, i.e., 30-50 µg/ml/1 x 10^7 cells or 50-120 µg/ml/2 x 10^7 cells. The final cell concentration depended on the technique for bone marrow cell separation available in each group (Haemonetics technique or Ficoll-Hypaque separation on IBM 2991). Forty-eight patients (25 ANLL, 23 ALL) received purged bone marrow transplants. Of the 48 patients, one had received deoxycoformycin-treated bone marrow.

Bone marrow cells were purged with anti-CALLA MAb and complement. In a cooperative study (4 French teams), we have been investigating the use of three anti-CALLA MAbs: J-5 (11), ALB-2 (12), and BA-3 (13). One course of baby rabbit complement was used. Thirteen ALL patients received MAb-pretreated bone marrow. One T-cell ALL patient received bone marrow incubated with immunotoxin (T-101) coupled with a disulfide bond to the A-chain of the ricin molecule (14).

Autografting Conditions

In all, patients' bone marrows were collected during first or second remission. All patients received cryopreserved marrow. In the HDM-treated patient groups, bone marrow was harvested a second time and a second course of HDM was given, as a form of remission consolidation, to eight patients after an average of 5.3 months (range, 2-10 months) after the first ABMT.

The median interval between initiation of remission and bone marrow collection was 3.2 $\pm$ 2.5 months in ANLL and 4.8 $\pm$ 4 months in ALL. The median interval between bone marrow collection and ABMT was 2.05 $\pm$ 1.9 months in ANLL and 2.3 $\pm$ 1.7 months in ALL. After bone marrow grafting, no maintenance treatment was given.

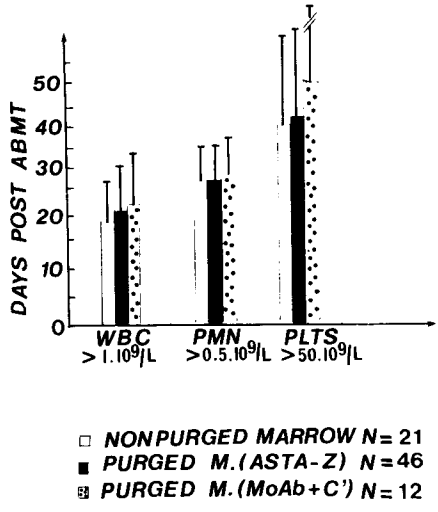

Figure 1. Influence of two different purging methods on hematopoiesis.

RESULTS

Engraftment and Graft Function

Sustained engraftment occurred in 104 of 107 evaluable patients. One patient developed late graft failure 25 days after ABMT. Following nonpurged marrow reinfusion (21 patients), peripheral blood neutrophils attained a level of greater than 0.5 x 10^9/l at a median of 25 days (range, 15-35 days). Platelet recovery to a level greater than 50 x 10^9/l was reached at a median of 40.5 days (range, 20-60 days).

In the patients receiving bone marrow purged either with cyclophosphamide derivatives (N = 46) or with MAbs plus complement (N = 12), the hematopoietic recovery was not significantly delayed in comparison with nonpurged ABMT (Fig 1).

Platelet recovery took more than 90 days in 8/65 patients (12.3%) undergoing engraftment while in remission (seven received purged and one received nonpurged bone marrow).

Survival in Patients Treated while in Relapse

CR was achieved by 26 of the 30 (86%) ANLL patients. The median remission duration was 11 months (range, 2.5-48 months). Four patients (13%) had unmaintained disease-free survival longer than 2 years (e.g., 24+, 32+, 33+, 48 months). Of the four patients, two received second courses of HDM.

CR was achieved in 9 of 12 (75%) ALL patients. The median remission duration was 4.2 months (range, 2-8 months). No patient achieved a long-term CR.

Survival in Patients Treated while in Remission

ANLL

A summary of the results is shown in Table 2. In the French experience, only 11 patients received nonpurged marrow. Among them, five patients engrafted while in CR and conditioned with high-dose chemotherapy are still in remission 13-41 months after ABMT. Thirteen of the 26 patients who received chemopurified bone marrow remain in continuing

Table 2. ABMT for 37 ANLL Patients[a] in Complete Remission

Preparative Regimen	Purging Method	Patients Transplanted	Early Death	Time of Relapse after Transplant (mo)	DFS Duration (mo)
	No	11	1	5 3.6 (2-5)	5 25.2+ (13+ - 40+)
Chemotherapy (TACC, HDM)	Yes	14	4	3 6.3 (5-8)	5 11+ (3+ - 24+)
TBI + CY	Yes	14	1	5 3.4 (2-6)	8 7.1+ (3+ - 14+)
Total		37	6	13	18 (48.6%) 10 > 12 mo (27%)

[a]*Twenty-eight patients in first CR; nine in second CR.*

Note: *ABMT, autologous bone marrow transplantation; ANLL, acute non-lymphoblastic leukemia; DFS, disease-free survival; TACC, 6-thioguanine, aracytine (cytarabine), CCNU, cyclophosphamide; HDM, high-dose melphalan; TBI, total body irradiation; CY, cyclophosphamide.*

complete remission (CCR), lasting from 3 to 24 months. Five of them have been in unmaintained CCR for more than 12 months. There was no significant difference between the relapse rates of the combination chemotherapy-treated group (3/8 evaluable patients) and the cyclophosphamide and TBI-treated group (5/13 evaluable patients).

ALL

Among the 41 patients who underwent ABMT, 28 were children and 13 were adults. All but 3 children were treated during second CR, and 9 of 13 adults were treated during first CR. A summary of the results is shown in Table 3. Twenty-eight patients received cyclophosphamide plus TBI as the pretransplantation regimen; 13 patients received chemotherapy alone.

Among the 26 patients who remain in CCR, from 3 to 40 months, 15 are children and 11 are adults.

Overall, eight patients had recurrence of leukemia within 8 months (range, 2-8 months) after ABMT. Three (25%) of the recurrences were among the 12 patients who underwent engraftment while in first remission, and 5 (22%) were among the 22 patients who underwent engraftment in subsequent remissions. It must be emphasized that of the 27 patients conditioned with cyclophosphamide plus TBI and who underwent autoengraftment with purged marrow while in CR, 20 are currently in unmaintained CCR, median duration 7 months (range, 3+ to 21+ months) from ABMT.

Transplantation-Related Deaths

The accurate causes of early deaths are often difficult to establish, since there have been several potentially lethal events. In 78 patients given autologous bone marrow while in CR, 13 (16.6%) died within 4 weeks from the conditioning therapy. Among the fatal complications were cardiac failure (four patients), interstitial pneumonia (three patients), severe infection (three patients), and central nervous system hemorrhage (one patient). Fatal veno-occlusive disease was observed in only two patients in this series.

Table 3. ABMT for 41 ALL Patients[a] in Complete Remission

Preparative Regimen	Purging Method	Patients Transplanted	Early Death	Time of Relapse after Transplant (mo)	DFS Duration (mo)
	No	3	3	-	-
Chemotherapy (BACT)	Yes	10	2	3 4.3 (2-8)	5 7+ (2+ - 15+)
TBI + CY	No	1	0	0	1 (40+)
	Yes	27	2	5 5.6 (4-8)	20 7+ (2+ - 21+)
Total		41	7	8	26 (63.4%) 5 > 12 mo

[a]*Twelve patients in first CR.*

Note: *ABMT, autologous bone marrow transplantation; ALL, acute lymphoblastic leukemia; DFS, disease-free survival; BACT, 6-thioguanine, cytarabine, BCNU, cyclophosphamide; TBI, total body irradiation; CY, cyclophosphamide.*

With cyclophosphamide plus TBI as the conditioning regimen, only 3 (7.1%) early deaths were noted; 10 (27%) early deaths were noted with high-dose combination chemotherapy.

DISCUSSION

The population in this study of 120 patients with acute leukemia who underwent ABMT is heterogeneous in terms of previous chemotherapy, age, number of remissions, and details of the high-dose preparative regimen employed.

This review of the French experience raises a number of remarks. At the time of relapse, high-dose cytoreductive therapy followed by ABMT remains an effective treatment to achieve CR only when initiated early. However, in most cases, the duration of remission obtained appeared to be short. Few patients with long-term disease-free survival have been reported. These data indicate that ABMT must be done while the patient is in CR. The quality of the remission at the time of bone marrow harvesting may be a crucial factor for success (15). An effective preparative regimen to eliminate residual leukemic cells needs to be better defined. The toxicity of the intensive therapy must be taken into account before a patient in CR is entered into an ABMT protocol. The toxicity of the combination chemotherapeutic regimen as used in our studies is unacceptable for patients treated in first CR. It is beyond any doubt that cryopreserved remission bone marrow is capable of engraftment and subsequent hematopoietic reconstitution. The role of purging in ABMT is not yet established, more than one method of purging is available (6).

In France, a cooperative clinical trial is under study in both ANLL and ALL using bone marrow purified with Asta Z (mafosfamide). Forty-eight patients have undergone transplantation of autologous bone marrow that has been treated by this chemical agent. It is well established that hematopoietic engraftment can be obtained even though the growth of granulocyte-macrophage colony-forming cells in the chemopurified bone marrow was totally suppressed; the pluripotent stem cells survive the high doses of Asta Z.

The observation time has not been long enough to allow definite conclusions about the effect of Asta Z treatment of bone marrow on the relapse rate. Controlled studies must be done in order to evaluate the role of purging in ABMT for acute leukemia.

In France, the use of immunologic methods for purging is in its infancy; the clinical data are not yet sufficient for evaluation (8,9).

The combination of two purging methods may be more effective than a single method and could lead to complete elimination of leukemic cells from the harvested bone marrow. This approach is under investigation by our group. The effects of the hematopoietic stem cell population in terms of recovery and viability are under study.

In spite of the short follow-up, the low incidence of relapse appears to be very encouraging in this cooperative study. ABMT has proven to be an attractive alternative to patients who lack MHC-identical donors in the management of ANLL or ALL in first or second remission.

A national protocol is under study to compare ABMT with purged bone marrow for patients lacking an identical donor versus the allogeneic transplant in childhood ALL in second CR.

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Autologous Bone Marrow Transplantation in Children with Recurrent Acute Lymphoblastic Leukemia: Summary of Clinical Results of the Munich Cooperative Group

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Autologous bone marrow transplantation (ABMT) has been used as treatment for patients with malignancies in an advanced stage. The patient's own marrow is reinfused after chemo- and radiotherapy at high dosage in order to rescue the patient from irreversible bone marrow damage. A major problem of ABMT with acute leukemia is the contamination of marrow with residual leukemic cells (1). The treatment of bone marrow with heterologous antisera in vitro has prevented leukemia after transfer of a mixture of syngeneic marrow and leukemic cells in AKR mice (1). Similarly, heterologous antisera have been produced against acute lymphoblastic leukemia (ALL) of common type (c-ALL) and T-lymphocytes (2-4,5). These antisera were multiply absorbed and rendered nontoxic against hematopoietic precursor cells cultured in semisolid medium (2-4). This report gives a brief summary of the clinical outcome in six patients studied.

Marrow was obtained during the first remission, separated with a Haemonetics model 30 cell separator, and treated with absorbed rabbit-anti-c-ALL globulin in a concentration of 10 mg/200 ml marrow before freezing as previously described (2). After rapid thawing of the marrow, pH was adjusted to 6.9 using ACD plasma, and the marrow was reinfused without further washing. The antileukemic conditioning regimen had been well tolerated in allogeneic transplantations (6) and consisted of 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU) (200 mg/m^2 on days -12 and -11), a continuous infusion of cytosine arabinoside for 5 days (200 mg/m^2 daily from day -11 until -7), cyclophosphamide (60 mg/kg on days -6 and -5), and total body irradiation (TBI) with 9.4 Gy the day before marrow infusion. Radiation was administered from two opposing ^{60}Co sources at a dose rate of 5.5 R/min. Patients received oral nonabsorbable antibiotics and were kept in a laminar air flow unit.

Three patients (M.F., S.D., U.P.) were in third relapse and three in third remission (I.S., T.Z., M.F.) at the time of ABMT. Marrow was stored during first remission between 9 and 15 months after diagnosis (Table 1). The first relapses occurred between 15 and 43 months; the second relapses between 20 and 50 months after diagnosis. The first relapse of patient T.Z. was in the central nervous system (CNS), and the second relapse of patient M.F. occurred simultaneously in the marrow and CNS. Two patients grafted in relapse (M.F. and S.D.) died early after transplantation with septicemia and one patient on day 56 with hemorrhage. One patient died with relapse of leukemia five and one-half months after transplantation, and two patients are alive. One of these is being treated for relapse, and the other patient suffers from leuko-encephalopathy. The degree of hematopoietic restitution is summarized in Table 2. There were signs of restitution in all patients, and a few islands of hematopoiesis were found in all, even in patient M.F. who died 7 days after grafting. In general the recovery of blood counts was slower than that after allogeneic transplantation. In two patients (I.S., U.P.), the recovery of platelet counts was delayed (see Table 2).

The results indicate that prolonged remissions can be induced with intensive chemotherapy, TBI, and reinfusion of autologous marrow. Furthermore, treatment of the marrow with absorbed anti-c-ALL globulin does not prevent hematopoietic restitution. At present, it cannot be distinguished whether relapse of leukemia is due to residual leukemic cells in the marrow graft or in the patient. The risk of relapse from residual leukemia in the patient may be lower when ABMT is performed in second remission (7). Various methods have been studied for eliminating

Table 1. Summary of Autologous Marrow Grafts in Acute Lymphoblastic Leukemia (Kinderklinik Univ. Munchen, AG-KMT Munchen, 7/84)

PATIENT	TIME IN MONTHS FROM DIAGNOSIS TO MARROW STORAGE	RELAPSE FIRST/SECOND	AUTOLOGOUS MARROW GRAFT	REMISSION DURATION	SURVIVAL TIME	COMMENT
M.F.8,M.	15	30 / 35	39	N.E.	7 DAYS	SEPTICEMIA NO BLASTS
S.D.8,F.	9	15 / 20	24	N.E.	23 DAYS	SEPTICEMIA NO BLASTS
I.S.8,F.	13	39 / 57	63	56 DAYS	56 DAYS	NO RECOVERY OF PLATELETS
U.P.7,F.	12	32 / 38	42	4 1/2 MO.	5 1/2 MO.	RELAPSE
T.Z.13,M.	14	22(CNS)/30	36	3 MO.	>9 MO.	RELAPSE
M.F.8,M.	14	43 / 50	53	27 MO.	>33 MO.	LEUKOENCEPHALOPATHY

Note: *Marrow was obtained in first remission and treated with absorbed, polyclonal anti-C-ALL globulin; marrow was infused after preparation with 1,3-bis-(2 chloroethyl)-1-nitrosourea, cytosine arabinoside, cyclophosphamide, and 9.4 Gy total body irradiation in second relapse or third remission.*

Table 2. Restitution of Hematopoiesis Following Autologous Marrow Grafts in Acute Lymphoblastic Leukemia (Kinderklinik Univ. Munchen, AG-KMT Munchen, 7/84)

PATIENT	SURVIVAL TIME	MARROW INFUSED PER KG BODY WEIGHT		RETIC.	DAYS UNTIL RECOVERY OF LEUKOCYTES /µL				PLATELETS/ µL	
		MNC x 10^8	CFU-C x 10^3	>2 ‰	>350	>700	>1400	>2800	>40 000	>80 000
M.F.8,M.	7 DAYS	3.7	N.E.	-	-	-	-	-	-	-
S.D.8,F.	23 DAYS	3.3	313	19	-	-	-	-	-	-
I.S.8,F.	56 DAYS	2.2	169	15	16	17	19	21	-	-
U.P.7,F.	5 1/2 MO.	2.0	87	43	20	21	24	-	68	-
T.Z.13,M.	>9 MO.	3.5	378	17	10	10	28	<43	19	29
M.F.8,M.	>33 MO.	2.5	185	19	16	18	22	N.E.	22	27

Note: *M, male; F, female; MNC, mononucleocytes; CFU-C, colony-forming units in culture.*

leukemic cells in vitro (2,7-9); their value cannot be easily assessed. However, long-term remission for patients with relapses of acute leukemia have been reported by several groups (2,7-10), stimulating further studies of ABMT.

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Rotterdam Results with Autologous Bone Marrow Transplantation for Patients with Acute Myelogenous Leukemia in First Remission

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INTRODUCTION

The success of autologous bone marrow transplantation (ABMT) in the management of acute myelogenous leukemia (AML) does not necessarily depend on tumor cell purging only. The procedure of autologous reinfusion of a cell suspension per se, even if contaminated with a small number of tumor cells, may lead to a significant tumor reduction. Animal studies have shown that the establishment of tumors in a host following intravenous transfer requires a cell dose above a critical threshold. This threshold is thought to be determined by 1) the number of clonogenic cells in the tumor and 2) the seeding efficiency of the cells, which indicates the capacity of the cells to reach an appropriate tissue environment for extended proliferation.

In AML, an increasing number of extensive studies indicate the value of allogeneic bone marrow transplantation (BMT) from fully human leukocyte antigen (HLA)-identical donors for prolonging remission and improving disease-free survival (1-4). Allogeneic BMT encounters a considerable risk of graft-versus-host disease, interstitial pneumonia, and complications related to the severe immunodeficiency state during several months after engraftment. ABMT has the obvious advantage of being associated with significantly reduced incidences of these immunobiologically determined complications. This advantage, however, should be weighed against ABMT's price of a higher rate of AML relapse.

In this chapter, we report the results in five consecutive patients with AML in first remission who were transplanted with their own bone marrow.

METHODS

Bone marrow was collected from the iliac crest and pelvic spine in 2-4-ml aspirates and collected in bottles containing heparinized Hanks'. balanced salt solution (BSS). The buffy coat (prepared by centrifugation at 2000 x g) was filtered through a nylon gauze and then through a glass filter. Nucleated cells were counted in Turck's solution. Samples from the graft were cultured for bacteriological and granulocyte-macrophage colony-forming unit (CFU-GM) determinations. Cells were frozen in 10% dimethyl sulfoxide and 20% calf serum using a controlled rate freezer (Cryoson, Beemster, Holland) at $1^{\circ}C$/min and stored at $-196^{\circ}C$ in liquid nitrogen (5). Cyclophosphamide (60 mg/kg) was administered in saline during a 1-h infusion on 2 subsequent days under conditions of forced diuresis (>125 ml/h) and alkalinization of the urine (pH>6.0). In patients 2, 3, 4, and 5, mesna was given in divided portions at -10 min, +4 h, +8 h, and +12 h following the start of the cyclophosphamide infusion, up to a total dose of 48 mg/kg body weight on each of 2 days. Total body irradiation (25 MV photon-beam; average treatment distance, 420 cm) was administered on day -1 with two horizontal beams (anteroposterior and posteroanterior with the patient lying on either side) and delivered in one session at an average dose rate of 15.0 cGy/min (patients 1 and 2) or 5.5 cGy/min (patients 3, 4, and 5). The total dose was 8.0 Gy to the midline of the body with partial lung shielding, resulting in a dose of 7.0 Gy to the lungs.

The marrow graft was thawed on day 0 and step-wise diluted with Hanks' BSS according to previously described methods (5) and then reinfused during 30-45 min. CFU-GM cultures were done by a double agar-layer technique with a leukocyte feeder as previously described (6).

Table 1. Clinical Characteristics of 5 AML Patients undergoing ABMT during First Remission

	Patient				
Patient Characteristics	1	2	3	4	5
Age (yr)	30	48	15	35	44
Sex	F	F	M	F	F
Diagnosis (FAB type)	M4	M4	M3	M2	M1
Duration of CR until transplant (mo)	7	7	10	4	5
Transplant					
Cell doses/kg b.w. (frozen)	$2.5x10^8$	$1.5x10^8$	$1.9x10^8$	$3.8x10^8$	$2.8x10^8$
CFU-GM/kg b.w.	$3.1x10^4$	$2.1x10^4$	$1.0x10^4$	$8.2x10^4$	$6.2x10^4$

Note: *AML, acute myelogenous leukemia; ABMT, autologous bone marrow transplantation; CR, complete remission; b.w., body weight; CFU-GM, granulocyte-macrophage colony-forming units.*

Table 2. Results of Bone Marrow Transplantation

		Patient				
Characteristics		1	2[a]	3	4[b]	5
Granulocytes ($500/mm^3$)	(day)	36	23	27	24	24
Platelets ($50,000/mm^3$)	(day)	67	39	39	40	48
Posttransplant survival	(mo)	55+	14	28+	9	7+
Remission duration	(mo)	62+	21	38+	12	

[a] *Marrow relapse of AML at 13 mo.*
[b] *Marrow relapse of AML at 8 mo.*

Blood products for transfusion were irradiated (15 Gy); leukocyte-poor cotton-wool-filtrated red cells were given; platelet and granulocyte transfusions were always prepared from single donors using an Aminco continuous flow cell separator.

All patients were nursed in reverse isolation in a room (patients 1, 4, and 5) or a laminar air-flow unit (patients 2 and 3) from about day -10 until the granulocyte count had reached a value of $0.5 \times 10^9/l$. No ABMT for patients with AML performed in our institution were excluded from the analysis. Clinical data were evaluated as of August 1, 1984.

RESULTS

The pretransplantation characteristics of the patients are summarized in Table 1. All had been induced into remission following chemotherapy according to the AML-5 protocol of the European Organization on Research and Treatment of Cancer Leukemic Group. The outcome of transplantation is indicated in Table 2.

DISCUSSION

Current research on ABMT for patients with AML focuses on developing methods of tumor cell purging. The approaches at present suffer from two weaknesses. First, usually only a subpopulation of the tumor with a selected cellular property is removed, and it may be the relatively mature neoplastic subset (7). Effective separation can only be realized if the clonogenic tumor cells are included in purging, but most approaches are not founded on proper assays for monitoring their removal. Second, the clinical value of purging marrow grafts can only be established in direct comparison with transplantation of autologous, unseparated grafts. Our initial experiences indicate that the employment of unmodified whole autologous bone marrow--obtained during full remission, temporarily cryopreserved, then reinfused--does allow for excellent hematopoietic recovery and stable remission of promising duration (8). This is the case in spite of the fact that the bone marrow specimens utilized for grafting had previously been exposed to heavy induction therapy.

Two patients showed recurrences of leukemia following transplantation; the other three patients remain in their first remission for 62+, 38+, and 12+ months. These initial results do not permit any conclusions yet on the value of the therapeutic modality but emphasize the necessity to consider tumor cell purging results with reference to control data. Patients with AML in remission most probably hide small, though varying, numbers of tumor cells (subclinical leukemia), which at present cannot be quantified. On the basis of canine experiments (9), we suggest that the invisible tumor load during remission may be further reduced in an ABMT program. The black box of applying purging procedures to autologous grafts and the absence of the significant posttransplantation problems common in allogeneic BMT demand extended clinical trials of transplantation of *unseparated* autologous bone marrow.

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Single or Repeated High-Dose Chemotherapy with Melphalan and Autologous Marrow Transplantation in Acute Leukemias: The Marseilles Experience with 31 Patients

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INTRODUCTION

Marrow transplantation has provided the opportunity to use high-dose radiotherapy and chemotherapy regimens in cancer patients with poor prognoses. Such regimens, if they are efficient in relapsed patients, can be given as a superconsolidation therapy to patients in remission who have poor prognoses. These approaches have been clearly successful in allogeneic marrow transplantation of acute leukemias (1).

We investigated antileukemic effects of a single high dose of melphalan (HDM) followed by autologous bone marrow transplantation (ABMT) and found, like others, high response rates and low morbidity in heavily treated patients (2,3). Duration of response is usually poor (4,5). Frequent relapses occur because of contamination of marrow by leukemic cells, limited antileukemic effects of the preparative regimen, or both.

We review here our experience using HDM followed by ABMT in 31 patients with acute leukemias. The first group of patients did not receive any therapy after marrow transplantation; however, considering the high relapse rate in these patients, we had subsequent patients undergo a second marrow aspiration after the first graft, a second round of high-dose chemotherapy, and a second autologous marrow graft. The objectives were to decrease marrow contamination using a marrow with fewer leukemic cells and to reinforce antileukemic therapy in the recipient.

PATIENTS AND METHODS

Between June 1981 and June 1984, 31 patients with acute leukemia received HDM followed by ABMT in our institution. Median age was 27 years (range, 6-66 years). There were 17 males and 14 females. Twenty-two patients had acute myeloid leukemia (AML); FAB subclassification was M0 = 1, M1 = 4, M2 = 6, M3 = 4, M4 = 2, M5 = 5.

At the time of marrow transplantation, 11 AML patients were in relapse; 11 were in complete remission (CR) (two were in their second CR; nine in their first). Out of these 22 AML patients, 9 did not receive further therapy after graft (four in relapse, five in CR); 13 were scheduled for a new marrow transplantation, and 8 received a second graft (six in relapse, and two in CR).

Nine patients had acute lymphoblastic leukemia (ALL). Two were adults and seven, children; median age was 8.5 years (range, 6-27 years); there were five males and four females. At the time of marrow transplantation, five were in relapse and four were in CR (three in second CR, one in first CR). All ALL patients were at least scheduled for a second graft, and at this writing four have received two ABMTs.

Melphalan was given by intravenous bolus with hydration (3 l/m^2/24 h) through a central venous line. Thirty patients received 140 mg/m^2; one received 240 mg/m^2.

Of the 12 patients who received 2 courses of high-dose chemotherapy, 8 had AML and were treated by melphalan at the same dosage. Four had ALL: one received a second course of melphalan; three received a second non-cross-resistant high-dose chemotherapy regimen--CBV (cyclophosphamide; 1,3, bis-(2 chloroethyl)-1-nitrosourea [BCNU]; and VP-16-213) (6). Doses were 1.5 g/m^2 cyclophosphamide for 4 days, 300 mg/m^2/day BCNU, and 125 mg/m^2 VP-16-213 for 4 days.

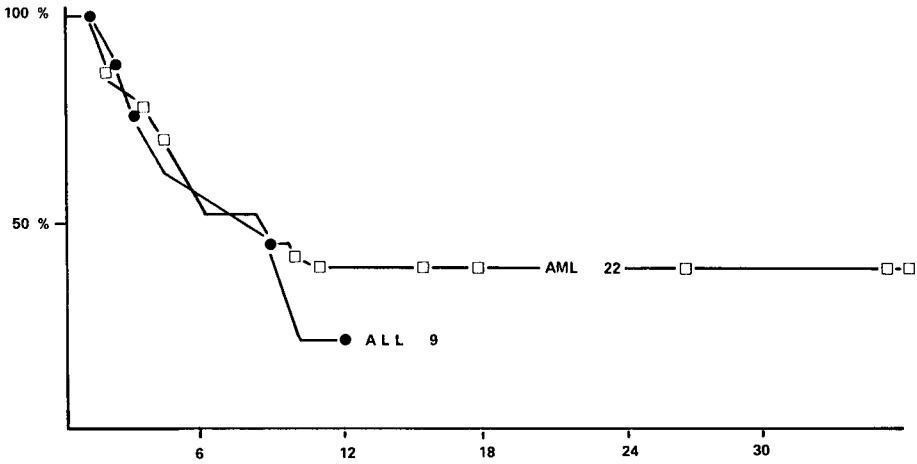

Figure 1. Disease-free survival (in months) of 31 patients after autologous marrow transplantation.

Eight ALL patients received intravenous methotrexate after melphalan administration and their first graft, the schedule designed in Seattle to prevent graft-versus-host disease (GVHD) (7). The aim of this regimen was to try to purge in vivo the transplanted marrow.

During CR, marrow was aspirated from iliac crests, processed on an Haemonetics 30, and cryopreserved in dimethyl sulfoxide (DMSO) and plasma as previously described (8). In five patients grafted in remission, "fresh" ABMT was done after 24-h storage at room temperature (9). Cryopreserved marrow was rapidly thawed and reinfused 12-24 h after HDM without washing. No in vitro marrow purging was used in any case. The second marrow harvest was scheduled when platelets reached at least 100,000/μl, with a median of 2.5 months (range, 2-6 months) after the first graft. Colony-forming cell (CFC) viability of all cryopreserved marrow was checked prior to transplantation (8).

All patients had right atrial catheters of large diameter. Six patients were treated in laminar air-flow rooms; other patients were treated in conventional rooms with reverse isolation barrier nursing. All blood products were irradiated with 15 Gy prior to transfusion.

RESULTS

Of 11 AML patients receiving HDM in relapse, 10 obtained a CR after their first HDM and ABMT. Duration of remission has been evaluated in these 10 patients and in the 11 other patients having transplantations during remission. Eight patients did not receive any therapy after the first HDM and ABMT: seven relapsed by a median of 4 months (five of them received transplants during their first CR). Five patients were scheduled for a second graft and one (initially grafted during third relapse) relapsed 2 months after the first transplantation. Eight patients received a second course of HDM and ABMT: one relapsed (at 9 months), one died in CR from pneumonia (at 5 months), six are in continuous complete remission (CCR), ranging from more than 10 months to more than 36 months (four of these six patients received their first course of HDM and first ABMT during a marrow relapse). Overall, disease-free survival is 41% in these 22 AML patients (Fig 1).

Five ALL patients received HDM and ABMT during a relapse, and all achieved a CR. Five patients were scheduled for a second graft: one died in CR (at 1 month) from pneumonia, two relapsed during the methotrexate regimen (at 2 and 3 months). Four received a second autologous graft, one after a new preparation with HDM and three after the CBV regimen: two are in CCR. Overall, disease-free survival of these ALL patients is 26% with a maximum follow-up of 12 months.

Table 1. Patient Treatment and Response

Treatment and Response[a]	Acute Myeloid Leukemia (N = 22)			Acute Lymphoid Leukemia (N = 9)		Total
	One Transplant	Scheduled for Second Transplant	Two Transplants	Scheduled for Second Transplant	Two Transplants	
Transplanted in relapse	4	1	6	2	3	16
Transplanted in CR	4	4	2	3	1	15
Total						31
Achieved CR when transplanted in relapse	3/4	1/1	6/6	2/2	3/3	15/16
Transplant-related deaths	-	-	1	1	-	2
Relapses after transplantation	7	1	1	2	2	13
Patients in continuous CR	1	4	6	2	2	15
Follow-up (mo)	27+	1+,2+,3+,4+ 18+,35+,36+	10+,11+,16+	2+,3+	9+,12+	9.5[b]

[a] *CR, complete response.*

[b] *Median.*

The main toxicity was hematologic, shortened by ABMT. After the first course of HDM, neutrophils were less than or equal to 500/µl for 16 days and platelets were less than or equal to 50,000/µl for 28 days in AML patients. In ALL patients, who received intravenous methotrexate, neutropenia less than or equal to 500/µl lasted 18 days and thrombocytopenia (≤50,000/µl) lasted 21 days. Morbidity was generally increased after the second marrow transplantation when compared with morbidity after the first, as previously reported (10). With only two transplant-related deaths (in relapsed patients, from pneumonia), the overall tolerance of this regimen of transplantation is acceptable (Table 1).

DISCUSSION

We confirm with a large series of patients the antileukemic activity of melphalan when given in huge doses (15 CRs/16 patients) (2,3). The duration of CR was, however, disappointing when no therapy was given after graft, even if patients underwent transplantation during CR. The feasibility of multiple autologous transplantation was suggested both by the small amount of marrow (1-2% of the total pool) needed to perform allogeneic or autologous marrow transplantation and by the repletion of stem cell pools after one marrow transplantation (8,11). If after high-dose chemotherapy and ABMT there is at least a 2-log leukemic cell kill and a 2-log normal cell amplification coming from the transplanted marrow, the final marrow collected should be markedly free of "contaminating" leukemic cells. Thus, the better results obtained with our patients when they received two grafts could be attributed to less contamination of the marrow transplanted second. However, one cannot exclude that repeated high doses of chemotherapy followed by the transplantation of "cleaned" marrow could be more efficient than one. The relatively high frequency of relapses after twin transplantation following treatment with Cytoxan (cyclophosphamide) and total body irradiation suggest that leukemic contamination is probably not the main explanation of relapse after ABMT (12).

Our preliminary results show that repeated transplantations after repeated courses of high-dose chemotherapy give longer durations of remission than one course and that some patients, even those who are grafted in relapse, can have relatively long disease-free survival. A prospective randomized trial is now under way in AML patients in first CR to compare remission duration of patients receiving repeated HDM and ABMT with those being treated with an intensive maintenance chemotherapy regimen.

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Autologous Bone Marrow Transplantation in Acute Leukemia and Lymphoma

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INTRODUCTION

Chemotherapeutic and radiotherapeutic treatments of malignancies have rapidly advanced, rendering the prognosis of some tumors more favorable. From data available from studies of in vivo systems, it has been hypothesized that a positive correlation exists between the possibility of complete destruction of the tumor burden (1,2) and the use of high doses of cytostatic drugs. However, these doses completely destroy hematopoietic precursors also, producing a condition in which bone marrow hypoplasia would be induced. This effect represents a serious limiting factor in possibly using such agents and doses in vivo (3,4). To obviate this risk, physicians have proposed that autologous bone marrow transplantation (ABMT) may provide the possibility of hematopoietic rescue following supralethal doses of chemotherapy or radiotherapy (1,5-7). In the therapy of leukemias and lymphomas ABMT has been proposed as a possible alternative to allogeneic transplantation in patients lacking a sibling with identical human leukocyte antigen (HLA). Autologous marrow is harvested from the patients after remission has been established. It is cryopreserved in liquid nitrogen and then reinfused in the same donor after the patient receives a conditioning regimen of supralethal doses to destroy the resistant malignant cells in the host. The major limit of ABMT, however, is the possible persistence in remission marrow of contaminating malignant cells that are able to cause a relapse. To prevent this situation, investigators have developed in vitro techniques to deplete malignant cells from bone marrow suspension, sparing the normal stem cell compartment, before the reinfusion into the patients.

At present, pharmacologic, physical, and immunologic methods have been proposed for purging bone marrow in vitro (8-11). The aim of the clinical studies reported here was to develop an in vitro system of purging marrow cells using Asta Z 7557, a new metabolite of cyclophosphamide, the cytotoxic effect of which nearly corresponds to that of 4-hydroperoxycyclophosphamide (4-HC).

MATERIALS AND METHODS

Patients with a diagnosis of acute lymphoblastic leukemia (ALL), acute nonlymphoid leukemia (ANLL), Hodgkin's disease, and non-Hodgkin's lymphoma (NHL) were included in the study. The criteria for selection of the patients were: 1) age of less than 40 years; 2) absence of significant liver, renal, and heart dysfunctions; 3) poor prognosis; and 4) evidence of remission marrow (number of blasts, 5%) as evaluated in multiple sequential aspirates. Moreover, three patients with ALL and two with NHL had been treated in the past with ABMT. Details about the patients are summarized in Table 1. The patients received high doses of chemotherapy and total body irradiation (TBI) at a single dose. Few patients, as indicated in Table 1, were treated with chemotherapy alone.

Bone marrow was collected at least 3 weeks after the end of the chemotherapy. Cells were aspirated from multiple sites on the posterior iliac crest from patients who were totally anesthetized. The amount of bone marrow collected was variable, but enough was collected to obtain 4 x 10^8 cells/kg of body weight. The cell suspension was filtered through screens of 50-μm pores, placed in 600-ml transfer bags, and then centrifuged at 2500 rpm for 15 min. The buffy coat was removed and the cells were counted and resuspended (2 x 10^7/ml) in 20% autologous plasma and 80% TC 199 tissue culture medium. A sample of the total suspension was kept for the evaluation of in vitro colony growth.

Table 1 - Patients' Clinical Data

Number of Patients	Age (years)	Diagnosis	Remission	Preparative Regimen	Remission (months)
4	15 - 28	ALL	1st CR[a]	Cy[b]-TBI	27+, 18+, 16+, 3+
1	24	ALL	2nd CR	Cy -TBI	4, relapse
1	27	ANLL	1st CR	BU[c]-Cy	2+
1	32	ANLL	2nd CR	BU	0.5, sepsis & renal failure
3	22 - 30	LYMPHOMA	2 PR, 1CR	BAVC[d]	15+, 3+ ∂, relapse
1	21	HODGKIN'S DISEASE	PR	Cy-TBI	3+

[a] complete remission
[b] cyclophosphamide: 50 mg/kg/4 days
[c] busulfan: 4 mg/kg/4 days
[d] BCNU: 200 mg/M^2 - day -4
ARA-C: 1200 mg/M^2 q 12H, days -5, -2
VP-16 1200 mg/M^2 q 12H, days -5, -2
Cyclophosphamide: 180 mg/kg, q 24H, days -5, -2

Asta Z 7557 is a stabilized metabolite of 4-HC, the cytotoxic efficacy of which corresponds to the activity of 4-HC. The compound is stable in the crystalline form, and in aqueous solution it produces the metabolite 4-HC. Asta Z was reconstituted from the powder in phosphate-buffered saline at a concentration of 10 mg/ml immediately before use.

Asta Z (80-100 mg/ml cell suspension) was added to the bone marrow cells, and the cell suspension was incubated for 30 min at 37°C in a water bath; the cells were cooled to 4°C for 5 min and then centrifuged at 3000 rpm for 10 min. A sample of the suspension was kept for the evaluation of in vitro colony growth. The pellet was gently resuspended, and cells were counted and resuspended at a final concentration of 4 x 10^7/ml in 55% autologous plasma, 35% TC 199 medium, and 10% dimethyl sulfoxide for cryopreservation.

Aliquots (50-100 ml) of bone marrow cell suspensions were transferred to Gambro Hemofreeze bags for cryopreservation and frozen in an automatic controlled freezer (Nicool) in which the temperature drops 1°C/min until -4°C, 2°C/min until -40°C, and finally 5°C/min until -140°C is reached. The bags were stored in the liquid phase of a liquid nitrogen freezer.

Prior to reinfusion into the patients, after the end of the chemotherapy and radiotherapy conditioning regimen, the cells were thawed by rapid immersion into a water bath 37°C. Cell viability was evaluated using the trypan blue dye exclusion test, and cells were infused into patients immediately. The cells (0.9-2 x 10^8/kg) were then reinjected, and the engraftment documented on the basis of recovery of normal hematologic parameters. None of the patients received chemotherapy after ABMT.

All patients were hospitalized in a laminar air-flow room. During the period of posttransplantation aplasia, patients were supported with previously irradiated (15-30 Gy) platelets and packed red blood cell transfusions. Antibiotics and antimycotic drugs with a broad antibacterial spectrum were administered until the patients were apyretic and the total leukocyte count rose to more than 1000/mm^3 with absolute granulocyte number more than 500/mm^3.

The hematologic recovery was evaluated by counting total leukocytes, platelets, and reticulocytes and by determining the hemoglobin level daily.

RESULTS

The clinical parameters of the 11 patients studied, including age, diagnosis, and duration of the remission after ABMT are listed in Table 1. The posttransplantation clinical course of the patients was characterized by severe hematologic toxicity, fever, infections, and alopecia in all cases. Gastrointestinal complications in seven cases included nausea, vomiting, and diarrhea. Two patients with progressive gastric or duodenal ulcers had moderate hemorrhagic symptoms of the gastrointestinal tract. In eight cases oral mucositis of variable duration and severity occurred. Three patients had liver dysfunction and moderate renal dysfunction, probably owed to toxicity. One patient died from polymicrobial sepsis and renal failure. The nadir of bone marrow aplasia occurred within 3-6 days after the end of the conditioning regimen. Eight of the 11 patients transplanted with purged bone marrow cells are in complete remission after various periods of time, as shown in Table 1. Two patients relapsed after 128 and 245 days, but both are still alive and receiving additional chemotherapy treatments. Despite the severity and multiplicity of the complications, only one of the 11 patients died because of sepsis and renal failure before hematologic recovery could be documented. Autopsy could not be performed. At the time of discharge, all patients were in good clinical condition. Neither consolidation nor maintenance therapy was given after ABMT.

DISCUSSION

The poor prognosis of most hematologic malignancies and the successful treatment with aggressive regimens lead to considering ABMT as the treatment of choice. Since allogeneic bone marrow transplantation is not a possibility for the majority of patients because of the lack of a matched sibling donor, the use of ABMT seems to represent an attractive therapeutic alternative. The major disadvantage in using autologous remission marrow is the possibility that the bone marrow is contaminated by undetectable tumor cells, thus possibly inducing relapse of disease in the host. Clearly, if the use of such marrow is to be optimized, the remaining malignant cells must be eliminated. Until now, physical, immunologic, and pharmacologic methods (9-12) have been proposed to purge in vitro the remission marrow cells. In our study of bone marrow purging, we used a new drug, Asta Z 7557, with the same in vitro cytotoxic effect of 4-HC. To evaluate whether this drug may be used, we had to consider several factors. First, Is the in vitro hematopoietic recovery delayed, inhibited, or both since the drug is toxic to committed stem cells in vitro? Second, Is it possible to demonstrate the therapeutic efficacy of the in vivo crytoreductive conditioning regimen to eliminate the malignant residual cells before the autograft? No significant differences have been demonstrated in in vivo hematopoietic recovery following the Asta Z purging of ABMT and that obtained following autologous transplantation of untreated marrow, according to data available in the literature (13).

Our data clearly demonstrate that in most of the cases, after intensive conditioning regimens, hematopoietic recovery by autologous bone marrow treated with Asta Z 7557 at doses ranging from 80-100 mg/ml is possible. In all cases no complications caused by the pharmacologic treatment in vitro were observed. It appears that Asta Z spares the stem cell compartment able to reconstitute the hematopoietic function in the host without any secondary effect strictly related to the drug.

The clinical data show that 10 of 11 patients have been surviving for 2-27 months, and eight of them are still free of clinical and hematologic evidence of recurrent disease. These data seem promising, though the short term of our trial and the heterogeneity of the cases evaluated do not allow us to draw statistically significant conclusions. However, the good recovery of hematopoiesis and the lack of clinical and hematologic complications related to Asta Z treatment encourage the use of this in vitro technique for ABMT.

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Panel Discussion: Session I

R. P. Gale, M. Keating, and K. A. Dicke, Moderators

Dr. Robert Gale: I thought what I might do is to make two points and then proceed to the series of questions Karel Dicke has formulated (see p. 383) and ask specific individuals on the panel for their comments. It is clear that one can do autologous bone marrow transplantation (ABMT); it is also clear that one can treat the marrow in vitro in order to remove leukemia cells, but how can we know if these manipulations are successful? The idea that 20% or 30% of patients with acute myelogenous leukemia (AML) might achieve long-term disease-free survival with modern intensive chemotherapy is probably acceptable to most investigators. The point I wish to make is that if one performs ABMT in patients who have been in remission for several months, it will prejudice the results. If the median duration of complete remission is 4 months before the transplant is performed, one must add 20%-25% to these long-term results such that the outcome of chemotherapy alone in this highly selective group is approximately 45%-50% long-term disease-free survival. It is important to bear this in mind, since we have not seen any convincing data as yet that the results achieved in AML in first remission with autotransplantations, with or without marrow purging, are superior. Nor have we seen any trial design that would answer this important question.

The second point relates to the question of what constituted an appropriate control group. One possibility is a randomized trial and the other is the use of identical twins. The latter data have been published and I will not go into detail. The relapse curve of 31 patients with AML in first remission engrafted from a genetically identical twin is about 60%. The reason for discussing the identical twins is that, in some regards, one may consider twins the ideal control group. Relapses occurring in 60% of those patients (95% confidence interval: 40%-80%) and are presumably due to persistence of leukemia in the patient. This is the level of relapse one would expect if one transplanted autologous bone marrow cells completely free of leukemia.

The question we should now address is the phase of leukemia in which an ABMT should be performed. Perhaps we should go through our panelists. We have heard Dr. Dicke's opinion that in AML, first remission is not a good time to do ABMT because it would be extraordinarily difficult to analyze the results of the intervention. Bob Bast, do you want to comment on this?

Dr. Robert Bast: Certainly. For the phase I studies I think it is appropriate to use the second and third remission patients. I might take at least mild exception to Karel in terms of first remission data. With acute lymphocytic leukemia (ALL), lymphomas, and even AML in adults, you might have an appropriate group to analyze in a randomized study. First remission might be the only place where you would see a large enough difference to demonstrate the efficacy or lack of efficacy of marrow purging.

Dr. Gale: One other point I wish to emphasize is that it is not sensible in today's presentations to lump together patients with AML in first, second, third, and fourth remission because it has been shown in large studies that the break point in relapse after allogeneic transplantation in AML occurs between patients in first remission and those in greater than first remission. In contrast, in ALL the break point occurs between those in first and second remission versus those in greater than second remission.

Dr. Emil Frei: I think that, aside from the phase I studies that Bob Bast mentioned, which probably could be done in second or third remission, once you have a program that produces a plateau anywhere above 20%, or 30%, if you are going to ask the question you really ought to do it in

patients in first remission. If you have got reasonable evidence that you can achieve any cure rate whatsoever, then I would think those with expertise in transplantation should probably ask the quantitative question in first remission, because the variables as you move out get enormous and you end up with a qualitative answer. Now that is not to say that I believe that transplantation is better than chemotherapy. I think for those of us who feel that chemotherapy is moving in parallel with transplantation we ought to use our resources in the best setting for asking a quantitative question and that again is in first remission. I think that for any question you ask after that you have variability that gets you into the qualitative arena.

Dr. Bob Lowenberg: This is very much in support of the statement by Dr. Frei. I think it would not be a good position to perform ABMT for AML in second or later remissions. First of all, the odds are that first remission data are going to be reasonably good. Generally it is very unlikely, and I think this is a major point, that there will ever be an answer from second remission ABMT for AML. Even allogeneic transplants have a high rate of relapse with a high rate of variability as far as the time of relapse is concerned. Furthermore, if one keeps in mind that after isologous grafting in first remission the relapse rate is 50%, in second remission it is even higher. In other words, one would need very large numbers of patients.

Dr. Gary Spitzer: Dr. Frei, why would you recommend going into first remission if nobody has shown a plateau with this modality of therapy in relapse or second remission.

Dr. Frei: Well, I thought I saw several plateaus there. You do not accept the evidence that the autologous marrow is curative in some patients with AML? Is that what I heard you say?

Dr. Spitzer: I thought that so far we have only about a 5% long-term, disease-free survivorship, in all the data I heard on relapse and the occasional patient in second remission. That is within the biological variation of chemotherapy, because you would have an equivalent number of patients in long-term, disease-free survivorship if you treated X number of patients with chemotherapy.

Dr. Gale: I would like to add an additional point. The question is not whether a plateau of survival exists following an ABMT, but whether this plateau has any relationship to the bone marrow autotransplant.

Dr. George Santos: In 60 patients with allogeneic transplants done by us, in first remission, second remission, third remission, and early relapse, we have had one relapse. Median follow-up of these patients is 2.75 years, the longest being 6 years. At least half of them are in second and subsequent remission. The data from Sloan-Kettering looks similar. So don't assume that second remission patients will have a high relapse rate unless you are going to stick to a protocol that is failing. Now let me comment about the first and second remission. We are performing ABMT in second remission to try to see if we can get something that would be better than expected. So far, the data are holding up. I think it could be done in first remission; the reason we won't do it is probably a practical one. I would like to see it done, with somebody who really knows their chemotherapy and has enough patients. If they are going to do a late intensification, then randomize to bone marrow transplantation or chemotherapy alone.

Dr. Martin Korbling: In our opinion, the major reason for doing ABMT in second remission, and I agree with George and Karel, is to be able to compare the remission duration after ABMT with the duration of first remission. I think that is still the major point of evaluation of therapeutic efficiency.

Dr. Michael Keating: I think the problem here is the either/or question. Is the best chemotherapy or ABMT equivalent or superior? But I think we need to address the end question. Take our best chemotherapy, which as maintenance is relatively nontoxic, and perhaps add an autologous program at a particular time. We have selected a time point of about 6 months, as a late intensification point to see if we can raise the plateau of the 20% to 25% long-term continuous remissions up to about a 30% to 35% complete remissions rather than using the exclusive. To me, it is a much more important question to see if we can improve our cure rate with combination therapy--chemotherapy and autologous transplant--rather than just seeing if autologous can match a 20% remission rate.

Dr. Axel Zander: I would briefly like to comment on our strategy in first remission. We use the same approach in AML and ALL. We felt we had to initiate BMT in first remission even though purging is not a question we will be able to address right now since the purging method will change over the next 1, 2, or 3 years. So we decided we will engraft a well-defined patient population about 6 months after diagnosis. The cyto-reductive regimen that we have chosen is not completely ablative; rather, it is a combination chemotherapy of cyclophosphamide, 1,3-bis-(2 chloroethyl)-1-nitrosourea, and vincristine (CBV) without total body irradiation (TBI) that allows allogeneic engraftment. It allows us to give more chemotherapy after the transplant, and therefore we might have an optimal combination of BMT followed by further aggressive chemotherapy to take care of possibly contaminating leukemic cells. This study is randomized.

Dr. Gale: I think we can conclude the AML session by saying that either nature or God has contrived to work against us. That is, in AML in first remission, where we believe we have the greatest chance of eradicating leukemia within the body, we have considerable difficulty in proving a benefit because we have such good results with chemotherapy. Statistically, the situation is much more favorable in second remission AML because most patients will relapse and therefore, any improvement in survival could be ascribed to an intervention such as ABMT. The problem in this setting is that the likelihood of completely eradicating leukemia in the body is very low, less than 25%.

I suggest we turn to the question of ALL, for which we have a similar dilemma. The question is, when can one do a transplant and when can one determine whether transplantation is a useful modality or not? I wonder if Dr. Kolb wants to comment on this.

Dr. Hans-Jochem Kolb: Well, in the works of Dr. Netzel and Dr. Haas, who have been doing ABMT in the Children's Hospital, all patients had common acute lymphoblastic leukemia antigen (CALLA) and purging of the marrow was done with a polyclonal rabbit anti-CALLA antibody specifically absorbed to not suppress colony-forming units in culture (CFU-C) and erythroid burst-forming units (BFU-E) in vitro. Marrow was collected between 9 and 15 months after the diagnosis. All patients had relapsed twice; two patients had relapsed with central nervous system (CNS) disease too. ABMT was carried out 24 to 63 months from diagnosis. There were two early deaths and one patient failed to show platelet recovery. Three patients had remissions of 3, 4.5, and 27 months. Two patients are alive, one with a recurrence, and the other with leukoencephalopathy after repeated treatment for CNS leukemia. These data show that intensive chemotherapy, TBI, and ABMT can induce prolonged remission. Results may be improved by earlier transplantation. Thank you.

Dr. Gale: We should focus on ALL. I think the issue is that if we look again at allogeneic transplants as a control, then the break point for relapse is after first and second remission. Patients in first and second remission have a 50% relapse rate. In later remissions relapse rates are higher. So again the question is not can one do an auto-transplant, but how do we know whether the autotransplant is in any way benefiting the patient. I think Dr. Bast's data illustrate the complexity. Some chemotherapists would argue that the results they

achieved with autotransplants in this highly selected group of patients with long first remissions and extramedullary relapses might have been achieved with chemotherapy alone. Furthermore, there is no evidence yet that the in vitro manipulation of the bone marrow had any effect on the outcome.

Dr. Ulrich Jehn: Looking at the results presented this morning, it seems that if you talk in terms of survival, there is no question that the ABMT has to be performed during remission and not in relapse. If you talk in terms of complete remission (CR), then it probably doesn't matter. On the other hand, if you treat patients with conventional chemotherapy, it is very difficult to beat the long-term survival rate of 40% or 50% in ALL with ABMT. So I think what really needs to be done is to randomize both modalities in a large cohort of patients with high-risk ALL in first remission as a consolidation treatment. That would probably answer some of these questions. In fact, we are planning to do so in the European Cooperative Leukemia Study Group.

Dr. Gale: I think that is an interesting proposition. One of the problems with this approach is if one accepts the results recently reported in adult ALL, 50% disease-free survival with intensive chemotherapy, one would have to achieve superior results with transplantation. If one calculates what the number of patients needed to show an improvement rate of 50% is, we are dealing with studies with at least 100 patients per arm. It is clear that no single institution could perform such a study; perhaps a group could.

Dr. Lawrence Frankel: In terms of childhood ALL the problems change a bit. First of all, we have at least a 60% long-term continuous CR rate in first remission. And it is very hard for the statisticians to tell us which patients would have a 80% or greater relapse risk at diagnosis. So we are unable to identify a patient whose risk would be so poor that we would be able to use this modality in the first remission. On the other hand, those patients who relapse have a promise of having a long-term second remission of less than 2%. So 98% of the patients who have a bone marrow relapse on therapy are likely to die of their disease. That is the pediatric population that we need to study. I think since we find very few patients with an allogeneic donor, ABMT is a very fine approach since chemotherapy is not effective by itself. It would seem to me that we ought to be pulling bone marrows for storage during first remissions on patients with the greatest risk of relapse. Then we could randomize between patients treated with nonpurged first remission marrows versus patients obtaining a purged-marrow second remission. My own feeling would be that first remission marrow taken 6 to 9 months after induction would probably be a highly successful marrow for transplant even if we do not purge it.

Dr. Gale: That is a good suggestion; probably one of the few that we would all agree on. Do you think that such a trial ought to be randomized because of the considerable probability of relapse in patients?

Dr. Frankel: I think the results of long-term remission after bone marrow relapse on therapy is so poor that even if the transplant center by itself is going to look at this and get extended remission rates in that population they could do very well.

Dr. Richard Champlin: I would feel strongly that randomization is required even in that admittedly extremely high risk group. The patients that were presented this morning illustrate that highly selected patients who had a prolonged first remission and were then engrafted are those that have the long posttransplantation remissions. These patient selection factors are really the major point of controversy in evaluating almost all BMT studies. I believe any type of definitive study really must be prospectively randomized, and it often requires large patient numbers, which is only possible in a cooperative group. Marrow purging techniques must also be shown to be efficacious by a randomized trial as well.

Certainly the AML data that were presented from numerous centers have shown that, at least in selected patients, long-term survival can be achieved without marrow purging. There is really no evidence from what we have seen so far that the marrow purging itself had anything to do with the success of treatment. A definitive study is definitely required to show that the marrow purging technique makes a difference.

Dr. Gale: The only point I am making is that one needs time to evaluate the results completely.

II. Clinical Autologous

Bone Marrow Transplantation Studies in Lymphoma

Which Patients with Lymphoma Can Be Salvaged with High-Dose Cytoreduction and Autologous Marrow Rescue?

J. O. Armitage, R. D. Gingrich, J. F. Foley, M. A. Kessinger, L. W. Klassen, P. O. Kumar, M. A. Tempero, and W. P. Vaughan

INTRODUCTION

Recent reports have demonstrated that treatment with high-dose chemotherapy or radiotherapy or both with rescue from otherwise lethal myelosuppression using autologous bone marrow infusion can be curative in some patients with end-stage, refractory lymphoma (1-4). Since this treatment modality can occasionally cure very poor-risk patients, it is expected that its use in lymphoma patients, who have been carefully selected and are in the early course of their disease, would yield better results.

There are several factors that need to be considered in choosing patients for high-dose cytoreduction and autologous bone marrow rescue (see Table 1). The patient's age, performance status, and the presence of serious illnesses other than lymphoma all have a direct bearing on the patient's ability to tolerate the rigors of high-dose cytoreductive therapy. Since lymphomas are an extremely heterogeneous group of illnesses, it would be expected that the exact histopathologic subtype would have an effect on the chances for successful therapy. Patients with bone marrow involvement would be good candidates only if effective ways to eliminate tumor cells from the bone marrow were available. The response of a patient to any preceding therapy has been a critical variable in other types of salvage therapy in lymphoma and is expected to be relevant here. In addition, the type of preceding therapy might affect the patient's ability to tolerate high-dose cytoreductive therapy. For example, a patient who had previous radiotherapy to the lungs would be expected to be at high risk for the use of total body irradiation (TBI). Since all forms of high-dose cytoreductive therapy are dangerous and associated with the risk of treatment-related mortality, it follows that if cure can be achieved with simpler, "conventional" therapy, high-dose cytoreductive therapy would be inappropriate. Finally, as the response rate to high-dose cytoreduction and autologous bone marrow rescue in the various types of lymphoma becomes clear, we will be better able to select patients for this treatment.

PATIENTS AND METHODS

Characteristics of Patients by Histopathologic Subtype

Diseases that fit into the broad category of "lymphoma" represent a wide spectrum. To produce clinically relevant recommendations, it is important to consider each histopathologic subtype individually. Table 2 lists several characteristics of three selected histopathologic subgroups that are pertinent to deciding which patients would be good candidates for high-dose cytoreductive therapy. The indolent non-Hodgkin's lymphomas are not entirely homogeneous, but are considered in one group. These diseases include diffuse well-differentiated lymphocytic non-Hodgkin's lymphoma, nodular poorly differentiated lymphocytic lymphoma, and nodular mixed lymphoma. These patients are relatively old--a median age of 66 years was noted in a large group of patients seen recently. Only 21% of the patients were younger than 50 years of age. We chose 50 years of age to be the approximate upper age limit for high-dose cytoreductive therapy. Certainly this is not an absolute limit and some patients older than 50 years of age would be good candidates, while some patients younger than 50

Table 1. Factors to Be Considered When Choosing Patients for High-Dose Cytoreduction and Autologous Bone Marrow Transplantation

Patient age
General health (performance status)
Other significant illnesses, major organ dysfunction
Histopathology of the lymphoma
Sites of disease
Marrow status
Response to previous therapy
Type of previous therapy
Prospects for further conventional therapy
Response rate to high-dose cytoreduction

Table 2. Characteristics of Patients with Various Types of Lymphomas

Category	Indolent Non-Hodgkin's Lymphomas	Hodgkin's Disease	Diffuse Histiocytic Lymphoma
Median age	66 years	29 years	64 years
<50 years	21%	73%	22%
Marrow involvement	40% (nodular mixed) to 90% (DWDL)	6%	12%
Cure with initial therapy	Only with localized disease, except nodular mixed (?)	Stages IA, IIA - 80% Stages IIIB, IVB - 40-50%	Stage I - >50% (XRT) Stages II-IV - 37% (CHOP)
Cure with conventional salvage therapy	Essentially none	Unusual after no response to systemic chemotherapy	Rare after no response to systemic chemotherapy

Note: *DWDL, diffuse well-differentiated lymphoma; XRT, radiotherapy; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone.*

years of age would be poor candidates. These tumors have a particularly high frequency of bone marrow involvement. Despite these negative aspects, some young patients do develop these disorders, and approximately half of the patients with nodular lymphomas will not have bone marrow involvement. Essentially none of these patients can be cured with currently available therapies, with the possible exception of patients with nodular mixed lymphoma. Young patients who have indolent non-Hodgkin's lymphomas without bone marrow involvement should be considered for high-dose cytoreductive therapy and autologous bone marrow rescue.

In contrast to the indolent non-Hodgkin's lymphomas, Hodgkin's disease is a tumor of young people. The median age in a series of patients in Nebraska was 29 years, with 73% of the patients younger than 50 years of age. Only 6% of these patients will have bone marrow involvement documented at diagnosis. While these factors would seem to make Hodgkin's disease the perfect illness to employ high-dose cytoreduction, the majority of patients with Hodgkin's disease will not need this treatment. Approximately 80% of patients with localized disease will be cured with radiotherapy. However, only approximately half of the patients with high-stage, symptomatic Hodgkin's disease will be cured with front-line chemotherapy. Results of salvage chemotherapy in these patients have been somewhat controversial. Although as many as 50-60% of patients can achieve remission with salvage chemotherapy regimens (5-9), a great majority of the patients, and in some series all the patients, eventually relapse and die of Hodgkin's disease. Patients who relapse after systemic chemotherapy for Hodgkin's disease should be considered for high-dose cytoreductive therapy and autologous bone marrow rescue.

The aggressive non-Hodgkin's lymphomas are extremely heterogeneous and should be considered separately. Because of constraints of space,

only diffuse histiocytic lymphoma (DHL) will be considered here. Patients with DHL tend to be old (e.g., the median age at our institution is 64 years). Only 22% of these patients are younger than 50 years of age, and only 12% of the patients had bone marrow involvement at diagnosis. A great majority of the patients with DHL will have disseminated disease, but for those few patients with stage I disease, radiotherapy is curative in the majority. Several chemotherapy regimens have been used to treat patients with disseminated DHL. In a series of 75 patients treated with the cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP) program (10), the long-term disease-free survival rate was 37% (11). This is comparable to that reported for other 4-5 drug chemotherapy regimens (12-16). A careful analysis of prognostic factors for CHOP-treated patients identified several clinical and laboratory features found at diagnosis that allowed for prediction of the eventual treatment outcome (17). Using this information, it was possible to predict a high cure rate in patients with favorable prognostic factors, but a cure rate of 16% or less was predicted in patients with a variety of poor prognostic factors. This information would make it possible to choose patients for high-dose cytoreduction very early in the course of their disease. However, newer, more effective chemotherapy regimens for DHL are complicating this picture. Several aggressive 6-8 drug chemotherapy regimens, including cyclophosphamide, vincristine, prednisone, bleomycin, doxorubicin (COP-BLAM) (18); cyclophosphamide, doxorubicin, etoposide, methotrexate, prednisone, mechlorethamine, vincristine, procarbazine, prednisone (ProMACE-MOPP) (19); methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, decadron (M-BACOD) (20); and alternating non-cross-resistant sequences of regimens (21) appear to eliminate several previously important prognostic factors. Until longer follow-up is available in patients treated with these new regimens, it will be very difficult to recommend high-dose cytoreduction and autologous bone marrow rescue to patients with newly diagnosed DHL.

The results of salvage therapy in DHL are dependent on the type of initial treatment. Patients who fail to respond to radiotherapy can frequently be cured with front-line, combination chemotherapy regimens. However, patients who fail to respond to treatment with systemic therapy have a poor outlook. Only an occasional report has described any long-term, disease-free survivors with salvage chemotherapy for DHL (22). Patients with DHL, who cannot be cured with their initial combination chemotherapy regimen, should be considered good candidates for high-dose cytoreductive therapy and autologous bone marrow rescue.

Identification of Clinical Characteristics of Patients

In addition to identifying histopathologic subtypes of lymphomas that are better treated with high-dose cytoreductive therapy, it is also important to identify the clinical characteristics that will allow a patient to survive the rigors of this therapy. We have recently treated 25 lymphoma patients with high-dose cytoreductive therapy with rescue from myelosuppression using cryopreserved autologous bone marrow. The characteristics of these patients are presented in Table 3. The majority of patients had DHL, but two patients each had Hodgkin's disease, diffuse undifferentiated lymphoma, and nodular poorly differentiated lymphocytic lymphoma.

For the purposes of this presentation, early death was considered death from any cause that occurred from the time of entering the study to 50 days after bone marrow infusion plus 50. Table 4 presents the frequency of death by several prognostic categories. The histopathologic diagnosis did not seem to be important. Early in our study we transplanted only patients with advanced, refractory lymphoma. These patients were much less likely to tolerate the treatment than a more recent group of patients with minimal, nonrefractory disease. Finally, the most important prognostic factor for tolerating the therapy was the patient's performance status. Patients who had a Karnofsky score of 80 or greater (i.e., they were able to carry on their usual activities) had a mortality rate of 18% in contrast to patients who were incapacitated and in which

Table 3. Characteristics of 25 Patients[a]

Category	No. of Patients
Histologic diagnosis	
Diffuse histiocytic lymphoma	19
Hodgkin's disease	2
Diffuse undifferentiated lymphoma	2
Nodular poorly differentiated lymphocytic lymphoma	2
Karnofsky score	
≥ 80	16
≤ 70	9

[a]*The median age of the patients was 31 years (range, 4-52 years).*

Table 4. Results with High-Dose Cytoreductive Therapy and Autologous Bone Marrow Rescue

Category	*N*	Early Death (%)	Complete Tumor Clearance (%)	Complete Remission (%)	Relapse
Histologic diagnosis					
Diffuse histiocytic	19	7(37)	10(53)	7/18(39)[a]	3
Other	6	2(33)	3(50)	2(33)	2
Responded to last treatment (minimal disease)					
No	19	8(42)	7(37)	5(26)	5
Yes	6	1(17)	6(100)	4/5(80)[a]	0
Karnofsky score					
≥ 80	16	3(18)	10(63)	8/15(53)[a]	4
≤ 70	9	6(67)	3(33)	1(11)	1

[a]*One patient with complete tumor clearance is still hospitalized and, thus, does not yet meet the criteria for complete remission, which are 1) complete tumor clearance and 2) being clinically well and discharged from the hospital.*

67% of the patients died. We no longer considered the latter group of patients appropriate candidates for this form of therapy.

Chances for Successful Therapy

The tumor responses to high-dose cytoreductive therapy are reported in Table 4. Twenty-three patients were treated with cytarabine (3 g/m^2, intravenously for 5-8 doses), cyclophosphamide (90 mg/kg for 1 dose), and TBI 9-Gy fraction, or 11-12.5 Gy in 5 daily fractions. Two patients were treated with the carmustine, cytarabine, cyclophosphamide, thioguanine (BACT) regimen (3). Complete tumor clearance (i.e., absence of tumor on restaging evaluation or at autopsy) was achieved in approximately 50% of patients regardless of histopathologic subtype. However, patients who responded to their last therapy and had minimal disease at the time of receiving high-dose cytoreduction had the tumor eliminated in contrast to only 37% of the patients with bulky, refractory disease. One of the patients with minimal, responsive disease died of Fournier's disease during the period of myelosuppression and one patient remains hospitalized. The other patients continue to respond well to the therapy (i.e., 4-64 weeks after the time of bone marrow infusion).

CONCLUSIONS

High-dose cytoreductive therapy and autologous bone marrow rescue are potential cures for patients with advanced lymphomas. The advanced age of many patients with non-Hodgkin's lymphomas and the presence of highly effective conventional therapy for many types of non-Hodgkins' lymphomas and Hodgkin's disease will limit the applicability of high-dose cytoreductive therapy to carefully selected subgroups of patients. These will include young patients with indolent non-Hodgkin's lymphoma, patients with Hodgkin's disease and diffuse histiocytic lymphoma who have failed to respond to their initial conventional chemotherapy regimen, and patients with DHL and a poor outlook, as identified by adverse prognostic factors. Currently, all young patients with advanced lymphoma who cannot be cured with conventional therapy should be considered for this approach. It is increasingly clear that patients who are ill and have a low performance status will frequently have treatment-related mortality and are not appropriate candidates for high-dose cytoreductive therapy. However, patients who are clinically well and who have minimal, responsive disease have a greater chance for complete remission.

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Natural History of Non-Hodgkin's Lymphomas: Implications for Marrow Transplantation Research

R. I. Fisher

INTRODUCTION

In 1966, Rappaport proposed a classification of the non-Hodgkin's lymphomas that, with relatively minor modifications, still defines the important histologic subtypes of these diseases (1). He divided the lymphomas into nodular and diffuse types based on the pattern of the malignant infiltrates in the lymph node and then subdivided each of these two categories based on the degree of differentiation of the malignant lymphocytes. In 1972, Jones et al were able to demonstrate that the Rappaport classification indeed defined different types of non-Hodgkin's lymphomas that had unique survival curves (2). It is probably accurate to consider these results as representative of the natural history of the non-Hodgkin's lymphomas, since the therapy given in the 1950's and 1960's had little long-term impact on the disease.

As a result of these and other similar studies, the non-Hodgkin's lymphomas have clinically been grouped into the following two categories: 1) the indolent or so-called favorable lymphomas that include nodular poorly differentiated lymphocytic, nodular mixed, diffuse well-differentiated lymphocytic, and diffuse intermediate lymphomas; and 2) the aggressive or unfavorable forms which include diffuse histiocytic or large cell, diffuse mixed, diffuse undifferentiated non-Burkitt's, and nodular histiocytic lymphomas. In recent years, little progress has been made in the therapy of the indolent lymphomas and because of this, their natural history remains unchanged. However, dramatic improvements have occurred in the treatment of the aggressive lymphomas such that their prognosis is now significantly improved.

NODULAR POORLY DIFFERENTIATED LYMPHOCYTIC LYMPHOMA

Nodular poorly differentiated lymphocytic lymphoma (NPDL) is the most common of the indolent lymphomas and has been considered the prototype of this category. NPDL patients almost always present with advanced disease (stages III or IV) and they are usually treated with radiation therapy, single-agent chemotherapy, combination chemotherapy, or combined modality therapy (3). Indeed, a policy of no initial therapy until the patient has significant symptoms or potentially life-threatening organ involvement has been advocated (4). The results of all the studies reported in the literature are remarkably similar. They show that approximately 60-80% of all newly treated patients will achieve a complete remission (CR), but that these remissions are not very durable. In fact, 50% of these complete responders will relapse within 4 or 5 years. In spite of this, 70-80% of all patients will be alive at 5 years, although only 30-40% will be alive at 10 years. The good survival figures at 5 years can be attributed to the fact that these patients can frequently be retreated after relapse. However, the percentage of complete responders and the duration of response decrease with each relapse. Finally, the patient will no longer achieve significant benefit from treatment and will die shortly thereafter.

NODULAR MIXED LYMPHOMA

The one possible exception to the behavior of the indolent lymphomas is the nodular mixed lymphoma (NML) category. Longo and colleagues at the National Cancer Institute (NCI) have reported that these patients have, after combination chemotherapy, durable CRs that result in longer survival

than that observed with NPDL (5). Of the complete responders, 52% remained disease free with a median follow-up of 7 years. Indeed, approximately 50% of all patients are still alive at 10 years. Although this date needs to be confirmed in other centers, the results suggest that patients with NML may have a different prognosis with current treatment than those with NPDL.

DIFFUSE HISTIOCYTIC LYMPHOMA

Diffuse histiocytic lymphoma (DHL) is considered the prototype of the diffuse aggressive lymphomas. Prior to the development of effective combination chemotherapy, this disease was rapidly fatal with few patients surviving more than 2 years (2). The first demonstration that long-term disease-free survival could be obtained in some patients with advanced stages of DHL after treatment with combination chemotherapy was published in 1975 (6). Subsequent studies from several other institutions have confirmed that a subset of patients with DHL could be cured (7-9). Thus, by 1977, it was well established that CRs, documented by reevaluation of all initially involved sites, could be achieved in approximately 40-50% of all patients with advanced stages of DHL and that 75-80% of these complete responders would have long-term disease-free survival. Several studies have shown that approximately 35% of all these patients were cured with follow-up in excess of 10-15 years. An analysis of prognostic factors in these patients enabled us to predict, with a high degree of accuracy, the ultimate survival of certain subsets of patients based on their initial clinical staging information (10,11). Several factors were associated with a poor prognosis. They included the following: male sex, constitutional or B symptoms, advanced stage, bone marrow involvement, huge abdominal mass greater than 10 cm with gastrointestinal involvement, hepatic involvement, and hemoglobin (12 g/dl) or serum LDH (250 units).

In 1977, we initiated the third generation of the NCI studies in the chemotherapy of the advanced stages of the diffuse aggressive lymphomas with a cyclophosphamide, doxorubicin, etoposide, methotrexate, prednisone, mechlorethamine, vincristine, procarbazine, prednisone (ProMACE-MOPP) treatment program. The results of this clinical trial, which have recently been published, suggest a significant improvement in the long-term survival of these patients (12). The CR rate for the entire group of patients was 74%. The durability of these CRs was excellent. Actuarial analysis predicts that 76% of the complete responders will remain disease free in excess of 3 years. Median survival has not been reached at this time. Median follow-up for this study now exceeds two and one-half years. Actuarial analysis predicts that the median survival will be in excess of 4 years, with 65% of all patients alive at that time. As expected, the prolonged survival observed in the entire population is a function of the survival of the complete responders. Eighty-two percent of the complete responders are predicted to be alive at 4 years, while only 10% of the partial responders and none of the nonresponders have survived 2 years.

Several other studies that were initiated at approximately the same time have also suggested a significant improvement in survival (13-15). Thus, in the 1980's it may now be possible to achieve long-term disease-free survival in approximately 60% of all patients with advanced stages of DHL. Studies are now being conducted to further improve the CR rate and long-term disease-free survival, while at the same time minimize the toxicity and cost to the patient.

IMPLICATIONS FOR TRANSPLANTATION RESEARCH

What are the implications of the previously described data for autologous bone marrow transplantation (ABMT)? The situation must be analyzed for each disease group separately. The majority of patients with indolent lymphomas, such as NPDL, are not cured by combination chemotherapy. However, survival is still excellent at 5 years. Thus, treatment of newly diagnosed patients with ABMT probably carries too high

a mortality rate, in light of the lack of proven therapeutic benefits. Treatment of refractory patients is certainly possible. However, this is complicated by the fact that approximately 50% of these patients have bone marrow involvement initially and the figure is higher at relapse. Thus, successful bone marrow purging of tumor cells would be essential. Treatment of DHL is so successful now that initial ABMT is not indicated. However, relapsed patients or patients who fail to achieve a CR almost never have long survivals, therefore, they are excellent candidates for ABMT. Furthermore, bone marrow involvement is less frequent in those with DHL since only 15% of the patients have bone marrow disease initially. Careful attention to the prognosis of patients with non-Hodgkin's lymphomas enables ABMT studies to select appropriate candidates for this new treatment modality.

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Autologous Bone Marrow Transplantation for Non-Hodgkin's Lymphoma: The Preliminary European Experience

A. H. Goldstone for the European Bone Marrow Transplant Working Party on Autologous Bone Marrow Transplantation

INTRODUCTION

The European Bone Marrow Transplant Group has opened a registry to collect European data on autologous bone marrow transplantation (ABMT) in lymphoma. In a preliminary survey, interested centers were asked to report all cases carried out before December 1983, indicating the histologic diagnosis, status of disease at autografting, ablative regimen, and current status of the patient as of January 1984. Eleven centers replied initially, reporting a total of 112 cases. The participating centers were Lyon, Marseilles, Besancon, Nantes, London (University College Hospitals (UCH), Middlesex & Guy's), Bologne, Parma, Bern, Paris (St. Antoine), Villejuif-Institut Gustave Roussy (IGR), and Genoa. The patients included both adults and children treated with a whole variety of different regimens.

MATERIALS AND METHODS

The patient characteristics are shown in Table 1. It is noteworthy that in the whole group of 112 patients, there were more children than adults and significantly more men than women, particularly in the pediatric group. For the purposes of this analysis, the cut-off age between adult and pediatric cases was at 15 years. The significant majority of the pediatric cases came from three main centers - Bern (Dr. Baumgartner), Villejuif (Dr. Hartmann), and Lyon (Dr. Philip).

The ablative regimens used were extremely variable, not only between but within centers and also with regard to number of grafts carried out in an individual patient. For instance, 23 of 112 patients received total body irradiation (TBI), in addition to chemotherapy (no patients received TBI alone), and these 23 included 15 of 15 from Bern. The chemotherapeutic ablative regimens were either single-agent ones (usually high-dose melphalan) (1), or, if combination chemotherapy, were largely based on the TACC (carmustine, cytarabine (ara-C), cyclophosphamide, thioguanine) (2) and BACT (6-thioguanine, aracytine (cytarabine), CCNU, cyclophosphamide) (3) regimens. One center (UCH) carried out a double transplant procedure on some patients (4). Techniques of bone marrow harvest varied in minor detail from center to center. Almost all reporting centers used cryopreservation before ABMT. Twenty-three of 112 (20.5%), i.e., 23 of 101 (23%) who survived grafting itself, including 7 of 11 (64%) grafted at diagnosis, received additional consolidation and maintenance therapy after ABMT.

An attempt is currently being made to redefine all patients histologically in terms of the International Classification, but this classification is not yet available. Table 5 shows the current classification being used. On the basis of received data, the patients were categorized by status at grafting into the following groups at the time of ABMT: 1) Grafted at diagnosis. This included patients in whom ABMT was the initial treatment modality for the lymphoma. 2) Grafted as consolidation. This included patients already in complete remission (CR) in whom ABMT was used to consolidate induction therapy. 3) Grafted as partial initial response. This included patients with a significant clinical response (50% at evaluable sites) who were not in CR after three or four initial induction courses. 4) Grafted in responding relapse. This included patients who had relapsed after first-line therapy but were still showing *significant* response to second-, third-, fourth-line therapy, etc. 5) Grafted in resistant relapse. This included patients whose

Table 1. Autologous Bone Marrow Transplantation in Non-Hodgkin's Lymphoma: 112 First European Patients

Group	Males	Females
Whole group	92	20
Adults[a]	40	15
Children[b]	52	8

[a]*Mean age: males, 35.7 years; females, 35.5 years.*

[b]*Mean age: males, 8.6 years; females, 8.5 years.*

Table 2. Patient Groups at Autologous Bone Marrow Transplantation

Stage of Treatment or Disease at Engraftment	Adults (52)	Children (60)
Diagnosis	11 (21%)	0 (0%)
Partial remission	8 (15%)	3 (5%)
Consolidation	9 (17%)	14 (23%)
Responding relapse	10 (19%)	23 (38%)
Resistant relapse	14 (27%)	19 (32%)
Total (excluding those in resistant relapse)	38 (72%)	41 (68%)

disease was totally refractory to conventional therapy that had included Adriamycin or those who had progressive disease with any form of conventional salvage therapy.

The disposition of individual patients in the above groups is shown in Table 2. Note that the disposition within the individual groups does not differ greatly between adult and pediatric cases. Disease-free survival was calculated in months from the date of marrow transplantation and was analyzed as of December 31, 1983, as far as the data were available.

Twelve of 112 patients (11%) had some form of attempted marrow purge, five patients with monoclonal B-cell antibody, and seven with Asta Z.

RESULTS

Table 3 shows those patients, both adults and children, who achieved CR, remained in CR, had a relapse, or had a procedure-related death.

Table 3. ABMT in Non-Hodgkin's Lymphoma

Category	No. Patients (%)				
	Grafted at Diagnosis	Partial Remission	Consolidation	Responding Relapse	Resistant Relapse
Achieving complete remission	9/11(82%)	10/11(83%)	21/23(91%)	29/33(88%)	20/33(61%)
Toxic death	1/11(9%)	0/12(0%)	2/23(9%)	4/33(12%)	4/33(12%)
Persisting complete remission	9/9(100%)	8/10(80%)	15/21(71%)	16/29(55%)	6/20(30%)
Relapse	0/9(0%)	2/10(20%)	6/21(29%)	10/29(34%)	14/20(70%)
Adults	11/11(100%)	9/12(75%)	9/23(39%)	10/33(30%)	14/33(42%)
Children	0/11(0%)	3/12(25%)	14/23(61%)	23/33(70%)	19/33(58%)

When relapse occurred after the achievement of CR, it almost always occurred in the first 6 months after ABMT. This is well shown in Table 4. Table 5 shows response by histology in the histologic categories given previously.

The further breakdown of the three groups with active disease at the time of ABMT is shown below:

Treated in PR - 12 Cases		Treated in Responding Relapse - 33 Cases	
9 Adults	*3 Children*	*10 Adults*	*23 Children*
7/9 CR	3/3 CR	9/10 CR	20/23 CR
2/7 relapsed	0/3 relapsed	2/9 relapsed	8/20 relapsed
5/7 persist in CR	3/3 persist in CR	5/9 persist in CR	11/20 persist in CR

Treated in Resistant Relapse - 33 Cases

14 Adults	*19 Children*
8/14 CR	12/19 CR
7/8 relapsed	7/12 relapsed
1/8 persist in CR	5/12 persist in CR

In an attempt to see whether the large number of children in the whole group biased the results, a disease-free survival curve was plotted for children versus adults. This is shown in Fig 1 and indicates that in this group of patients, there seems to be little difference between ABMT in children and adults.

Table 4. Time of Relapse in Patients Achieving Complete Remission Post-ABMT

Group	No. of Patients Relapsing	No. of Patients Relapsing (within 6-12 mo post-ABMT)
Partial remission	2/10	2/2
Consolidation	6/21	5/6
Responding relapse	10/29	10/10
Resistant relapse	14/20	11/14
Total	32/80 (40%)	28/32 (87.5%)

Table 5. Patient Response by Histology

Category	No. of Patients	Achieving CR[a]	Relapse	Alive in CR
Histiocytic	3	3	1	2
Immunoblastic	8	7	4	2
NHL[b] type not given	9	8	1	7
Burkitt's	28	24	8	14
NHL (T-cell)	22	16	9	8
NHL (B-cell)	42	31	12	21
Total	112	89 (79%)	35 (39%)	54 (61%)

[a]*CR, complete remission.*

[b]*NHL, non-Hodgkin's lymphoma.*

The 12 lymphomas purged with either monoclonal antibody or Asta Z were too few in number and too heterogeneous to enable any meaningful analysis to be made at this stage.

In 23 patients who relapsed, sufficient data were available to enable an analysis of patterns of relapse. At least 14 of 23 (61%) patients, and probably more, showed clear-cut relapse at sites of previous disease. In patients with previously localized disease, there was no tendency to widespread sites of relapse, which might indicate a contaminated autograft. Information on effect of numbers of cells infused on patient recovery figures and toxic death incidence was obtained for 60 of 112 patients with non-Hodgkin's lymphoma and was compared with that from 21 Hodgkin's disease patients not otherwise described in this chapter. The details are shown in Tables 6 and 7. Figures 2 and 3 show disease-free survival curves for those patients in the different groups who achieved CR

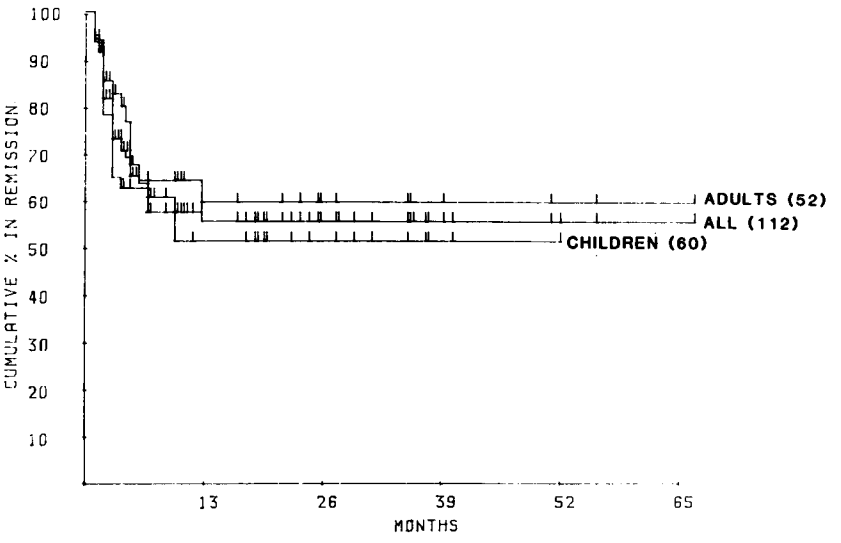

Figure 1. Disease-free survival of children and adults, both with non-Hodgkin's lymphoma who underwent autologous bone marrow transplantation in European Bone Marrow Transplantation group trials (data compiled January 1984).

Table 6. Number of Nucleated Cells Infused/kg vs. Number of Toxic Deaths

	Patients Receiving Infusions (%)				
Disease	0-1.0 x10^8/kg	1.1-2.0 x10^8/kg	2.1-3.0 x10^8/kg	3.0+ x10^8/kg	Total (%)
NHL[a]	16 (26.7)	24 (40)	16 (26.7)	4 (6.7)	60
Toxic deaths	1	3	1	0	5/60 (8.3)
Hodgkin's disease	7 (33.3)	9 (42.9)	4 (19)	1 (4.8)	21
Toxic deaths	5	1	0	1	7/21 (33.3)

[a]*NHL, non-Hodgkin's lymphoma.*

at ABMT. In Fig 3, it is shown that there was a significant difference between the group with refractory, fully resistant disease and all the other groups combined.

DISCUSSION

This retrospective study of 112 cases of ABMT for non-Hodgkin's lymphoma is very difficult to analyze because of the heterogeneity of case content and therapeutic approach. Nonetheless, some significant patterns emerge from it and are consistent with what has been reported previously in the literature (5). It is quite clear from these data that patients with relapsed disease and many of those with disease totally refractory to conventional therapy can be brought into a further remission of the

Table 7. Mean Time to White Blood Cell Recovery to 1000 Leukocytes Post-ABMT for Lymphoma

Disease	Mean Time (days) to WBC Recovery 0-1.0 x10⁸/kg	1.1-2.0 x10⁸/kg	2.1-3.0 x10⁸/kg	3.0+ x10⁸/kg	Overall Mean (days)
NHL[a] (*N* = 60)	15.3	15.0	19.0	13.5	16.1
Toxic deaths	1	3	1	0	
Hodgkin's disease (*N* = 21)	30.6	20.0	14.5	27	22.8
Toxic deaths	5	1	0	1	

[a]*WBC, white blood cell; NHL, non-Hodgkin's lymphoma.*

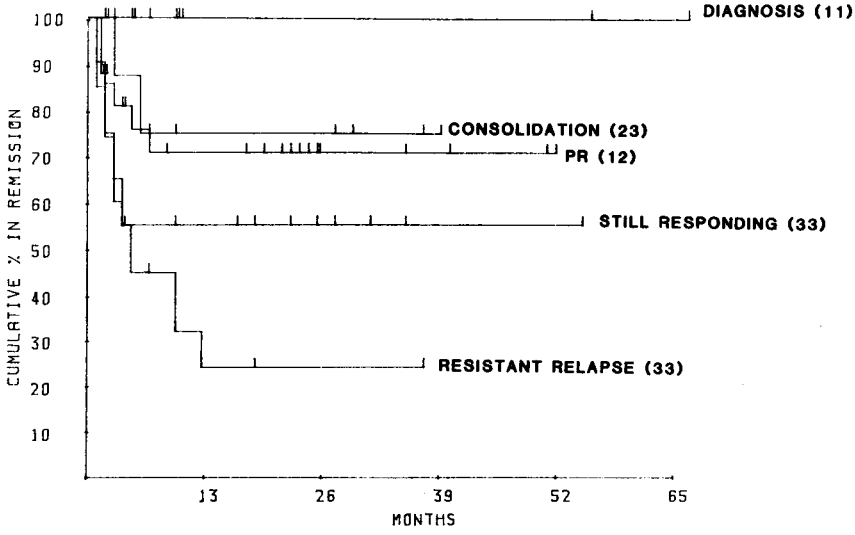

Figure 2. Disease-free survival of non-Hodgkin's lymphoma patients in various categories of disease who underwent autologous bone marrow transplantations in the European Bone Marrow Transplantation group trials (data compiled January 1984).

disease by significant escalation of ablative therapy followed by ABMT. This is difficult to evaluate in the context of modern conventional salvage chemotherapy; however, ABMT in the relapsed but still responding patient may be a better option for treating some lymphomas than is salvage chemotherapy (6).

This study shows that the majority of patients with truly refractory disease who achieve remission with massive therapy followed by ABMT experience another relapse within 6 months. The pattern of this relapse, which occurs predominantly in sites of previous disease, suggests that in the patient with refractory disease, the failure of ABMT is a failure to eradicate host disease; this is consistent with findings for any form of grafting in relapsed resistant leukemia or lymphoma (7). Relapse patterns

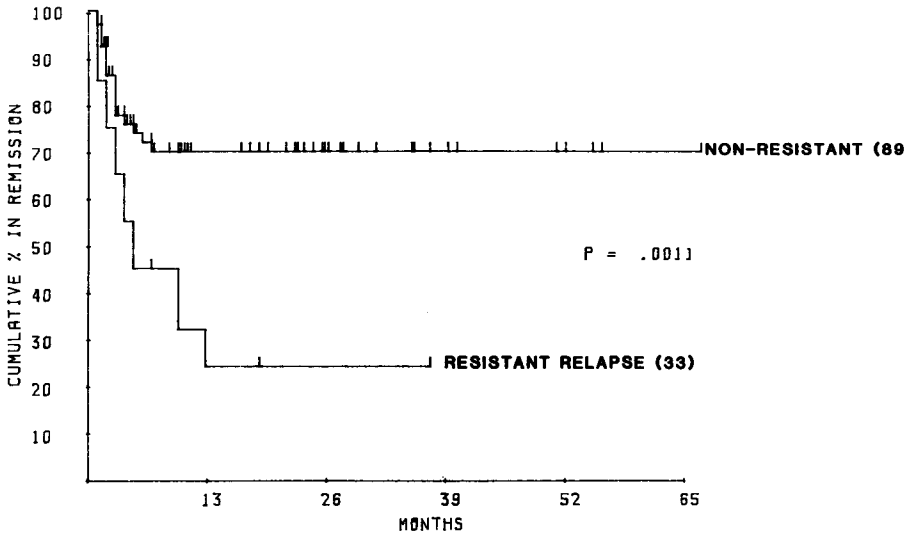

Figure 3. Comparison of non-Hodgkin's lymphoma patients with refractory, fully resistant disease and all other non-Hodgkin's lymphoma patients treated in the European Bone Marrow Transplantation group trials (data compiled January 1984). All underwent autologous bone marrow transplantation.

in this group of patients give no suggestion of reinfusion or contaminated marrow, although the criteria for normal marrow have been clearly defined only under the light microscope. In the vast majority of patients in this study (89%), no attempt was made to purge the marrow. For the small group (11%) whose marrow was purged, content was so heterogeneous that no conclusion can be drawn about the value of purging. However, the facts that in advanced disease eradication of host disease appears to be the major problem and that nonpurged lymphoma marrow seems satisfactory in many cases (even on occasion in patients with known previous marrow involvement) indicate that the exact setting for studies of purging of lymphoma marrow must be determined before extensive investments of time and technology are initiated.

It is extremely difficult to evaluate the usefulness of ABMT carried out at diagnosis or during consolidation in patients with lymphoma. This is because many such patients will be cured by conventional therapy and because their individual disease characteristics, such as staging, histology, etc., are insufficiently defined in this study to indicate the patient's true prognosis without ABMT. In addition, many of these patients, particularly those given ABMT at diagnosis, received further consolidation and maintenance therapy after ABMT, making it difficult to interpret the role of ABMT in any long-term survival. It is interesting to note that if Fig 3 (disease-free survival for resistant relapse versus all nonresistant cases) is redrawn to exclude patients grafted at diagnosis or as consolidation therapy, the significant difference between the two groups becomes very marginal indeed.

Although the patient content of this accrued group seems unbalanced because of the large number of children (53.6%) and patients with Burkitt's lymphoma (25%), this appears not to have seriously skewed the results and might suggest that when stage and status of the disease at ABMT are taken into account, children with lymphoma are not terribly different from adults with lymphoma.

It is important to review the numbers of toxic or treatment-related deaths in these patients, and here the numbers seem acceptable. Eleven (9.8%) of the entire 112 patients and 8 of 66 (12.1%) patients in relapse had a toxic death, comparable to syngeneic marrow transplantation for relapsed leukemia (7) and salvage chemotherapy for non-Hodgkin's lymphoma (8) and important in evaluating the risks for an individual patient from

salvage chemotherapy, ABMT, and allogeneic bone marrow transplantation. In ABMT for lymphoma, the role of TBI in contributing to toxic death may be more relevant for Hodgkin's disease, in which there has been a tendency to perform ABMT at a much later stage in more unfit patients (9). Certainly in this group, in which at least 15 of 23 patients (65%) were given TBI when in remission, there is no suggestion of an adverse effect from irradiation. The number of nucleated cells/kg infused in these non-Hodgkin's lymphoma patients also does not seem to influence either toxic deaths or days to recovery, although in Hodgkin's disease both toxic death and prolonged recovery in some patients may be related to the small number of cells reinfused in the autograft (Tables 6 and 7) (AH Goldstone, manuscript in preparation). This might reflect the difficulty in obtaining adequate marrow for ABMT in heavily pretreated patients with Hodgkin's disease.

In summary, this heterogeneous group of 112 patients with non-Hodgkin's lymphoma confirms the possibility of producing durable responses in some patients responding poorly to conventional therapy but also indicates that patients with resistant relapse are unlikely to benefit. In lymphomatous disease, it has always proved difficult to identify poor-prognosis patients early but it may be that this is becoming possible. The British National Lymphoma Investigation studies (BNLI) are indicating that patients with advanced high-grade non-Hodgkin's lymphoma who are not in remission after 3 courses of chemotherapy or who are in any stage of relapse have a very poor prognosis (A Jelliffe and G Vaughan-Hudson, personal communication). These findings are now spurring prospective ABMT lymphoma protocols in which patients younger than 60 years of age with normal marrow (by light microscopy) who are not in CR after 3 courses of chemotherapy or who are in early relapse after adequate chemotherapy are considered relatively early-disease patients for high-dose chemotherapy and ABMT.

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Autologous Stem Cell Transplant for Poor Prognosis Diffuse Histiocytic Lymphoma

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Significant improvements have been made in the treatment of adult and pediatric non-Hodgkin's lymphoma (NHL). Newer combination chemotherapy using cyclophosphamide, doxorubicin, etoposide, methotrexate, and prednisone plus mechlorethamine, vincristine, and procarbazine (Pro-MACE-MOPP) (1), methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, and decadron (M-BACOD) (2), or the revised intermediate methotrexate dose m-BACOD (3) results in a high complete remission (CR) rate and significant improvement in disease-free survival. The LSA_2-L_2 protocol appears particularly promising for treatment of pediatric NHL (4). Long-term follow-up and confirmation of the initial success with these protocols will be very important.

Recent diagnostic improvements in classification of NHL using histopathological criteria, cell surface markers, and biochemical enzyme levels have made it possible to identify patients who have a poor prognosis with increasing reliability. Several institutions have now shown that patients with large cell immunoblastic lymphoma (diffuse histiocytic lymphoma by Rappaport classification or DHL), lymphoblastic lymphoma, and small noncleaved cell (undifferentiated or Burkitt's lymphoma) malignant lymphomas are destined to do poorly, especially if the patient presents with bone marrow involvement or with a large mass (mediastinal or abdominal) (5-8). Patients with DHL who have high serum lactate dehydrogenase (LDH) levels (>500 U/l) have a poor long-term response rate on conventional therapy (8).

Since the major dose-limiting side effect of chemotherapeutic agents used to treat patients with lymphoma is hematopoietic toxicity, bone marrow transplantation can help circumvent this toxicity, and then escalated doses of such agents can be used therapeutically. Allogeneic bone marrow transplantation (BMT) has shown significant promise in the therapy of leukemias and lymphomas, but these benefits have been limited to young patients (age below 35 years) with human leukocyte antigen-mixed leukocyte culture (HLA-MLC) matched siblings, and graft-versus-host disease has been a significant problem (9-13). Autologous stem cell transplantation (ASCT) has wider applicability, and the cryopreserved bone marrow can be reinfused after intensive treatment to rescue the patient from hematopoietic toxicity. Several institutions have performed ASCT for patients with lymphoma (see recent review, ref. 14). Most previous reports involved patients engrafted at the time of relapse, and the best results were in patients with Burkitt's lymphoma (14-18). Because of generally poor results in heavily pretreated patients, 3 years ago we decided to assess the role of ASCT early in the course of disease in patients with DHL who were identified as having a poor prognosis based on presenting features. In this report we present our initial results which suggest improved therapeutic results in such patients using total body irradiation (TBI), followed by high-dose cyclophosphamide therapy and hematopoietic rescue by cryopreserved bone marrow.

PATIENT SELECTION AND METHODS

From July 1981 to July 1984 previously untreated patients less than 40 years old who had DHL and presented with bulky mediastinal or abdominal disease (> 8 x 8 cm) or a serum LDH level of greater than 500 U/l with no bone marrow involvement or only minimal marrow infiltration at presentation were considered for this study. The highest pretreatment serum LDH value was taken for this analysis. In the review of the results of

recent trials with combination chemotherapy protocols at our institution, patients with DHL presenting with either one of these features had less than 20% probability of long-term survival (6,8). All the protocols used in this study were approved by the Institutional Review Board of Memorial Hospital. The data analysis is as of August 1984.

The initial design of the protocol called for randomization between two arms as described below, but we found most patients preferred to choose one arm over the other and refused randomization. Therefore, patients were allowed to choose which arm they preferred, continuing chemotherapy or induction immediately followed by ASCT.

Arm I

The L-17M protocol is a combination chemotherapy protocol used principally for patients with acute lymphoblastic leukemia (ALL) and lymphoblastic lymphoma (LL) (19). Induction therapy (usually requiring 35-40 days) consists of cyclophosphamide, weekly vincristine, intrathecal methotrexate x 4, Adriamycin on days 15-17, and cyclophosphamide and Adriamycin. After completion of induction, the bone marrow is evaluated for colony-forming unit in culture (CFU-C) and erythroid burst-forming unit (BFU-E) growth; and if this is satisfactory, approximately 1 liter of marrow is taken and cryopreserved for possible use later if the patient subsequently relapses. Patients are then continued on the consolidation and maintenance phases of the protocol.

Arm II

After induction therapy on the L-17M protocol, the bone marrow is cryopreserved. The patients then receive one course of DAT (daunomycin 60 mg/m^2 intravenously on days 1-3, cytarabine (ara-C) 20 mg/m^2 bolus, 150 mg/m^2 intravenously as 24-h infusion on days 1-5, and 6-thioguanine 100 mg/m^2 by mouth every 12 h for 5 days), following which they are allowed 2-3 weeks to recover. During this time, computed tomography simulation for radiation therapy, extent of disease workup, and dental evaluations are performed in preparation for ASCT. All but two patients received additional irradiation to the site of the bulky disease (usually 3 Gy/day x 4 days). Bactrim (double strength by mouth) twice a day on day -8 to -1 is given as prophylaxis against *Pneumocystis carinii* infection. TBI with partial shielding of the lungs is given at 1.2 Gy/treatment 4 times a day for 11 treatments on day -7 to -4 (20). On day -3 and -2 under adequate hydration and alkalinization of urine, cyclophosphamide at 60 mg/kg per day x 2 is given. Two days later the cryopreserved bone marrow is infused. Patients are managed in single rooms during TBI, high-dose chemotherapy, and subsequent nadir sepsis.

Patients who failed to achieve CRs on the L-17M protocol (Arm I) or who relapsed during the consolidation or maintenance phases were also considered for ASCT and were subgrouped accordingly. They received the same preparative treatment as patients on Arm II. Five additional patients who have been heavily pretreated on various conventional protocols also received transplants.

Bone marrow aspiration and biopsy on all patients was evaluated at initial presentation and then 10-14 days prior to bone marrow cryopreservation. The bone marrows were assessed for lymphoma involvement by examination of the smears and surface marker analysis, including light chain immunoglobulin clonal excess by flow cytometry (21). Flow cytometric analysis for RNA-DNA content and chromosomal analysis were also performed when indicated. If there was no evidence of lymphoma involvement of the marrow at presentation, the marrow was not purged, but if there was minimal lymphoma involvement at presentation and even though it was no longer detectable after induction therapy, the marrow was treated with 4-hydroperoxycyclophosphamide (4-HC) prior to freezing on the assumption that there might still be some residual lymphoma cells present (21). Nine patients (nos. 4,5,6,7,10,18,20,21,23) received 4-HC-purged marrow; the marrow of patient 20 was purged at 60 μM 4-HC and all others

were purged at a concentration of 100 μM for 30 min at 37°C. In all cases where purged bone marrow was used, 66% of the marrow dose infused was cryopreserved untreated to be used in the event that the 4-HC-treated marrow did not engraft.

All patients receiving untreated and 4-HC purged marrow have so far shown good hematopoietic engraftment although one of the heavily pretreated patients (no. 21) had delayed recovery of her platelet count.

RESULTS

Only patients with DHL (B-cell) are considered in this report; Table 1 shows the results of all such patients entered into the study. The dimensions of the tumor mass at presentation, stage of disease, and highest serum LDH level prior to treatment and at time of ASCT are also shown. The cell dose infused in patients who underwent ASCT and the representative CFU-C and BFU-E doses are shown in Table 2. Table 2 also shows the number of days required for counts to recover from the nadir. It was not necessary to use the untreated reserve marrow in any of the patients who received 4-HC-purged marrow.

Ten patients elected to receive Arm I of the protocol, of whom seven achieved CR and three achieved partial remission (PR). In all PRs on Arm I and all except patient 11 on Arm II, the serum LDH level fell below 250 U/l on the L-17M, although it later often increased at the time of relapse. The usual reason for designation of PR rather than CR was incomplete shrinkage of the mediastinal mass on chest x-ray. Seven of these patients (4 CR and 3 PR) relapsed 4-17 months after the start of the L-17M therapy. One (case 4) of the seven patients who relapsed on the L-17M therapy did not receive ASCT, as he refused to have his bone marrow cryopreserved; this patient subsequently relapsed and died. The other six patients received ASCT after they relapsed.

Accurate staging in the posttransplant period is difficult since a residual mass often persists and surgical confirmation of residual lymphoma is often not possible for months because of the increased risk of infection (as the immunological recovery is slow). Two of these six patients were alive and disease-free at 10+ and 6+ months, two patients relapsed and one of them has died at 9 months (case 7), and the other two patients died of peritransplant complications, one of disseminated herpes-zoster infection (before the availability of intravenous acyclovir) and the other of pulmonary complications.

Nine patients elected to have ASCT after completion of the L-17M induction (Arm II), of whom two achieved CR, six had PR, and one had progressive disease (case 19). The eight patients in PR or CR who were engrafted shortly after the L-17M induction therapy were alive with no known evidence of disease at follow-up of 9+ to 33+ months from initial diagnosis or 5+ to 31+ months from the time of transplantation. In one patient (no. 11), the residual mass after completion of ASCT was surgically removed and was found to be necrotic and fibrous tissue. This patient is the longest survivor after ASCT. The one patient whose disease progressed after L-17M induction therapy and in whom ASCT was inadvertently delayed for 1 month died of pulmonary complications associated with paratracheal lymphoma 1 month after ASCT.

Five additional patients who relapsed on various conventional lymphoma protocols were also engrafted. One patient died of peritransplant complications (case 22) and another died of recurrent lymphoma. The other three patients are alive with no evidence of disease at 24+, 13+, and 3+ months after ASCT.

All patients who received ASCT showed good hematopoietic engraftment, but one patient had a long delay (6 months) before her platelet count increased to over 50,000/mm^3 (case 21).

DISCUSSION

From these initial results, it appears that the L-17M protocol, which is effective in treating patients with ALL and LL (19,22), is also

TABLE 1: CLINICAL COURSE OF PATIENTS WITH POOR PROGNOSIS LYMPHOMA

Case Number	Age	Sex	Primary Site in cm	Bone Marrow* Initial	Bone Marrow* ASCT	Stage	LDH Initial	LDH ASCT	Repsonse L-17M (Relapse)**	Survival in months from Initial DX	Survival in months from ASCT (Relapse)**
ARM 1											
Still on L-17M											
1	20	M	Med 14 X 13	+	-	IV	705		CR	40+	
2	38	M	Med 12 X 11	-	-	III	254		CR	25+	
3	24	F	Med 12 X 11	±	-	II	281		CR	22+	
4	37	M	Med 8 X 8	±	-	IV	251		CR (6)	7	
ASCT at Relapse on L-17M											
5	29	M	Med 8 X 8	-	-	IV	150	380	CR (17)	24+	6+
6	34	M	Med 12 X 11	±	-	IV	508	352	PR (4)	22+	18+(6)
7	21	F	Med 10 X 10	±	-	IV	566	343	CR (5)	16	9 (8)
8	30	F	Med 10 X 10	±	-	IV	564	304	PR (5)	15+	10+
9	24	M	Rib 12#	-	-	IV	160	383	CR (5)	7	1
10	25	F	Med 8 X 9	±	-	IV	254	322	PR (4)	5	1
ARM 2											
ASCT After Induction											
11	17	M	Med 11 X 13	-	-	IV	777	424	PR	33+	31+
12	24	F	Med 12 X 13	-	-	IV	623	235	CR	30+	25+
13	36	M	Med 10 X 8	-	-	II	426	225	PR	26+	21+
14	32	F	Med 12 X 14	-	-	III	834	171	CR	23+	20+
15	34	M	Med 13 X 14	-	-	IV	182	217	PR	20+	16+
16	28	M	Med 13 X 14	-	-	III	528	307	PR	19+	16+
17	24	M	Med 18 X 14	-	-	IV	501	233	PR	11+	8+
18	17	M	Bone 3 x 3	±	-	IV	669	192	PR	9+	5+
19	30	M	Med 16 X 14	±	-	IV	379	240	Prog.	6	1
ASCT in Heavily Pretreated											
20	21	M	Med 10 X 14	±	-	IV	230	401	PR (2)	34+	24+
21	29	F	Med 14 X 12	±	-	IV	388	144	PR (4)	27+	13+
22	24	F	Med 11 X 13	±	-	IV	155	927	CR (5)	7	1
23	35	F	Breast 10 X 12	±	±	II	364	236	PR (15)	21	7
24	41	M	Abd 8 x 8	±	±	IV	558	181	PR (1)	14+	3+

* + = Suspicious by morphology and/or clonal excess, + = definite by morphology and/or surface markers.
** (Relapse) = Number of months for relapse to occur after start of L-17M or after ASCT.
# Soft tissue involvement and with multiple lymph nodes. Effusion at relapse.

Table 2 LABORATORY DATA FOR PATIENTS UNDERGOING ASCT

Patient	(Cell dose/kg) x 10^{-8}	AT FREEZE Untreated CFU-c*	BFU-e*	Treated CFU-c*	BFU-e*	At Infusion CFU-c*	BFU-e*	Number of Days Post BMT WBC 1,000	ANC 500	Platelets 50K
ASCT at Relapse on L-17M										
5	2.89 (Tx)			57.8	20.2	54.9	4.8	11	12	24
6	4.42 (Tx)			77.4	35.4	28.7	2.9	13	13	17
7	4.86 (Tx)			213.8	197.6	37.7	1.6	36	40	82
8	4.25 (Tx)	97.8	56.3	—	—	39.3	62.3	15	16	18
9	3.42 (UnTx)	48.7	NR	—	—	43.6		13	13	+
10	2.4 (Tx)			9.6	24.0	3.6	40.0	16	14	16
ARM 2 ASCT After Induction										
11	3.8 (UnTx)	168.2	0.0	—	—	62.7	0.0	10	12	18
12	3.4 (UnTx)	75.9	NR	—	—	71.4	NR	12	8	13
13	3.95 (UnTx)	105.7	0.0	—	—	121.5	NR	12	11	18
14	5.52 (UnTx)	305.0	230.0	—	—	121.4	0.0	16	15	18
15	2.89 (UnTx)	8.7	1.0	—	—	41.9	0.0	12		24
16	3.07 (UnTx)	59.9	76.8	—	—	66.0	45.0	13	15	26
17	2.08 (Tx)			128.4	30.5	72.8	43.7	22	32	33
18	2.2 (Tx)			16.5	0	12.8	0.0	12	13	31
19	3.27 (UnTx)	40.2	32.7	—	—		0.0			
ASCT in Heavily Pretreated										
20	1.51 (Tx)			23.8	NR	14.7	NR	18	17	30
21	2.60 (Tx)			9.8	34.7	60.5	6.1	+	+	+
22	3.9 (UnTx)	218.4	24.4	—	—	150.2	0.0	+	9	+
23	3.45 (Tx)			42.3	87.4	95.7	3.5	15	17	24
24	2.4 (UnTx)	117.6	23.3	—	—	35.5	122.4	16	18	+

* Colonies per 100,000 cells plated x 10^{-3}/kg.

\+ Signs of engraftment by bone marrow aspirate analysis.

NR Not reliable due to laboratory error or contamination of the plates, BFU-e's are particularly sensitive and prone to laboratory error.

effective for the initial therapy of DHL. However, from our experience so far with Arm I of the current protocol, and as was also true with the earlier similar cyclophosphamide L-2 protocol (8), it appears that patients with poor-prognosis DHL tend to relapse within the first 6 months after the start of therapy, indicating that the consolidation and maintenance phases of the L-17M protocol are often inadequate to eradicate the lymphoma when it is extensive (Table 1). The need for more intensive therapy of such patients is supported by the data on patients who received ASCT on Arm II (cases 11-19). Of the nine patients who received transplants after completion of induction therapy, one patient with extensive paratracheal disease who had progression of disease on the L-17M protocol died of pulmonary decompensation, but the other eight patients remain free of disease with a median follow-up of 20+ months from initial diagnosis and 16+ months from the time of ASCT (Table 1).

Fractionated TBI (20) (plus additional radiotherapy to bulky tumor masses) and high-dose cyclophosphamide were used to treat the patients in this study. This combination has shown promising results in allogeneic bone marrow transplantation for acute nonlymphocytic leukemia (9) and ALL (12), and it is of interest that the same combined therapy also appears to be effective in treating patients with poor-prognosis DHL. The results of ASCT when it is performed after the patients have relapsed on the L-17M (Arm I) or in heavily pretreated patients are not as impressive as when ASCT is performed immediately after (L-17M) induction therapy. Perhaps for these two subgroups of patients, more-aggressive combination chemotherapy will be necessary, if it can be tolerated.

Minimal involvement of the bone marrow by lymphoma in patients with DHL can often be missed by routine morphological examination. It can be calculated that 2-5% lymphoma cells in the marrow can represent 2-5 x 10^5 tumor cells/ml. In the doses used for purging in this study, 4-HC is probably effective in producing at least a 3-4 $\log_{10}$ cell kill of human lymphoma cells (23,24), but is probably inadequate to eradicate the tumor cells from marrow densely infiltrated with lymphoma. The clinical trial of 4-HC-purged bone marrow has therefore been restricted to patients who had minimal bone marrow involvement at presentation and whose bone marrow had no detectable lymphoma at the time of bone marrow cryopreservation. The clinical effectiveness of the purging procedure cannot be proved by our studies (because of the inability to detect slight marrow involvement by lymphoma), and the induction treatment may have been sufficient to clear the marrow of lymphoma. Nine patients have so far received 4-HC purged ASCT. ALL patients showed good hematopoietic engraftment with no significant delay except in one case compared to patients receiving untreated marrow. The number of days needed for recovery of white blood cell, granulocyte, and platelet counts is depicted in Table 2.

From the preliminary data presented in this study, it appears that TBI, when given with high-dose cyclophosphamide and followed by rescue with cryopreserved marrow, is effective therapy for patients with poor prognosis lymphoma, especially when transplantation is performed early.

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Role of Autologous Transplantation in Hodgkin's Disease

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INTRODUCTION

Combination chemotherapy has dramatically improved the prognosis of patients with advanced Hodgkin's disease. However, long-term follow-up results of mechlorethamine, vincristine, procarbazine, prednisone (MOPP) chemotherapy, as reported by DeVita et al (1), show that there is still a 20% primary induction failure rate and also that one-third of the patients who achieve a complete remission (CR) will relapse. Thus, there is still a subgroup of patients with Hodgkin's disease who will need effective salvage therapy.

Several combination chemotherapies have been shown to induce CRs in patients failing to respond to MOPP chemotherapy. A higher incidence of CRs is seen in patients who have failed to respond to MOPP chemotherapy but are not truly MOPP resistant. Despite this high CR rate, the long-term disease-free survival rate is approximately 22% or less in this population (2,3).

High-dose chemotherapy with or without total body irradiation (TBI) and autologous bone marrow transplantation (ABMT) is known to produce prolonged remissions in patients with hematological malignancies (4-6). However, most of the patients who relapse with Hodgkin's disease have had radiation to the chest, abdomens, or both, thereby increasing the risk of severe morbidity for TBI-based programs.

We report here the successful use of combination chemotherapy with autologous bone marrow rescue for patients failing to respond to prior multiple salvage therapies.

PATIENTS AND METHODS

Sixteen adult patients (10 men and 6 women) with progressive or relapsed Hodgkin's disease, who were examined at the bone marrow transplantation clinic of The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, have been entered in this study. Informed consent was obtained. The median age was 30 years (range, 20-44 years). The median duration of follow-up of surviving patients was 9 months (range, 3-38 months). Twelve patients (75%) had progressive disease on their last salvage chemotherapy at the time of transplantation. Four patients (25%) relapsed after chemotherapy and underwent transplantation. Half the patients had extranodal disease, the more common extranodal sites being lung (six patients), bone (two patients), and pleura (two patients). The other sites involved were bone marrow, kidney, liver, and thyroid. Sixty percent of the patients also had constitutional "B" symptoms.

All but one patient had good performance status (Karnofsky scale, 60 or above) and no associated disease that was significantly compromising to cardiac, renal, pulmonary, or liver functions. The exception was a patient who had lung, liver, and bone marrow involvement and was accepted into this study while she was febrile, neutropenic, and had an active herpes-zoster infection.

All but three patients had three or more prior salvage chemotherapies (Table 1). They were usually given MOPP as the initial chemotherapy; one patient was allergic to procarbazine and was given Adriamycin, bleomycin, vinblastine, dacarbazine (ABVD) as the initial chemotherapy. There were five primary MOPP-resistant patients in this group. At relapse they received regimens containing Adriamycin, some of which included ABVD (nine patients); Adriamycin (doxorubicin), bleomycin, dacarbazine (ABDIC) (three patients); and bleomycin, cyclophosphamide, Adriamycin (doxorubicin), Velban (vinblastine) (B-CAVe) (two patients). On further relapse a

Table 1. Prior Therapy and Complete Remissions

Prior Therapy	No. of Patients	Complete Remission
MOPP[a]	15	10
Adriamycin combination	13	4
VP-16-213 combination	11	4
Radiotherapy	13	

[a]*MOPP, mechlorethamine, vincristine, procarbazine, prednisone.*

regimen containing etoposide (VP-16-213)--like AMSA, ifosfamide, VP-16-213 (AIVP-16-213); ifosfamide, methotrexate, VP-16-213 (IMVP-16-213); or mitoguazone, ifosfamide, methotrexate, etoposide (MIME)--was given. Of the 11 patients who were given etoposide, eight had MIME chemotherapy. Several patients received other combination chemotherapies, etoposide, and single-agent chemotherapies prior to entering this study. Thirteen patients had prior radiotherapy including 11 who had mediastinal irradiation. Response to prior chemotherapy is shown in Table 2. Three patients had continuous disease activity and never achieved a CR. Eight patients who had a prior CR had a maximum disease-free interval of less than 12 months.

The median time from bone marrow collection to transplantation was 1 month (range, 1-15 months), whereas the median time interval from diagnosis to transplantation was 39 months (range, 15-111 months). The patient must have adequate bone marrow function at the time of marrow collection. This was defined as absolute granulocyte count of greater than 2000/mm^3, platelet count of greater than 100,000/mm^3, and a marrow cellularity of greater than 25%. The process of aspiration and storage was previously described (7).

The preparative regimen consisted of cyclophosphamide (1.5 g/m^2/day intravenously on days 1-4), 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU) (300 mg/m^2 intravenously on day 1), and etoposide (100 mg/m^2 every 12 h intravenously for six doses on days 1-3) (CBV). Patients were well hydrated immediately before and during the chemotherapy administration. Most of the patients were nursed in a protective environment and prophylactic oral antibiotics were administered.

Table 2. Prior Chemotherapy and Disease-free Intervals

		Maximum Disease-free Interval	
Disease Activity[a]	No. of Patients	<12 mo	>12 mo
Continuous	3	3	-
1 CR	6	4	2
2 CR	6	4	2
3 CR	1	-	1
Total	16	11	5

[a]*CR, complete remission.*

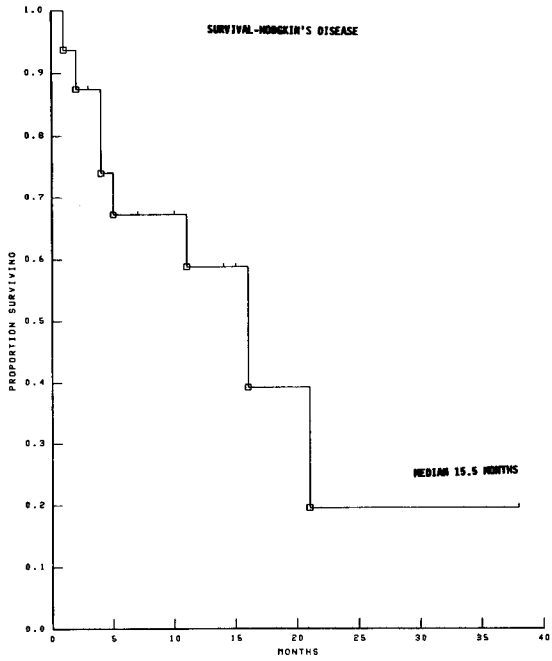

Figure 1. Overall survival curve from the time of bone marrow transplantation.

RESULTS

Of the 16 patients treated, 6 achieved a CR (3+, 4+, 5, 6, 6+, 38+ months), 6 achieved a partial response (2+, 3, 4, 4, 4+, 14+ months), and 4 failed to respond. Median follow-up is 9 months. The overall survival curve is shown in Fig 1. Median survival time is 15 months.

Among the complete responders, two patients died: one patient died of *Pneumocystis carinii* and cytomegalovirus (CMV) pneumonia 4 months posttransplantation and had no evidence of Hodgkin's disease at autopsy; the other patient relapsed and died of his disease. Another patient relapsed but is still alive and undergoing further therapy. Thus, there were only three patients alive and free of disease at the time of this writing (i.e., 38+, 8+, and 10+ months posttransplantation).

One partial responder, who received additional radiotherapy to residual nodal disease, is alive and free of disease after 14 months. Three patients showed progression of disease and one of them died. All patients with no response died of their disease within 4 months of transplantation.

Five patients failed to respond to initial MOPP chemotherapy. Their site of disease at the time of transplantation is given in Table 3. Three of these patients achieved a CR and all three are alive and free of disease at this time. Patients 1, 2, and 5 had never achieved a CR in prior salvage therapy attempts.

For the 16 patients undergoing CBV chemotherapy and ABMT, the prior disease-free interval had been less than 12 months for 11 and greater than 12 months for 5. Among the 11 patients with fewer than 12 months free of disease, 6 achieved a CR, 1 a partial response, and 4 had progressive disease. The 5 patients who had achieved longer disease-free intervals in the past achieved only a partial response with this therapy.

Patients with extranodal disease had a poorer prognosis and all nonresponders were in this group. All four patients who relapsed when they were no longer receiving chemotherapy responded and two achieved a CR.

Table 3. Response of MOPP-Resistant Patients to CBV

Patient	Disease Site	Response[a]	Survival (mo)
1[b]	Lung	CR	38+
2[b]	Lung + Mediastinum	CR	8+
3	Abdomen	CR	10+
4	Abdomen	PR	14+
5[b]	Mediastinum + Kidney	PD	2

[a]*CR, complete remission; PR, partial remission; PD, progressive disease.*

[b]*Patients who never achieved complete remission in prior salvage therapy.*

High-dose CBV produces significant neutropenia and thrombocytopenia in these heavily pretreated Hodgkin's disease patients. All patients had a leukocyte count of less than 100/mm^3 for a median of 13 days (range, 5-28 days) and platelet counts of less than 20,000/mm^3 for a median of 17 days (range, 2-28 days). All patients required platelet and packed RBC transfusions. There was one failure to engraft. This patient's bone marrow was harvested 19 months before transplantation, but transplantation was not done until the patient had progressive disease in the lungs, liver, and bone marrow. Her Hodgkin's disease persisted in her marrow until the time of her death. Marrow recovered in all other patients. It took a median of 22 days (range, 16-50+ days) for the total WBC count to reach greater than 1000/mm^3; and a median of 35 days (range, 16-50+ days) for the platelets to reach greater than 100,000/mm^3.

Nonhematologic toxicities included fever, infections, and inflammations. All patients had febrile episodes during neutropenia, which warranted intravenous antibiotic therapy. Fifty percent of the patients had documented infections like pneumonia or bacteremia. Five patients had severe mucositis, four had severe hematuria secondary to hemorrhagic cystitis, and three had moderate-to-severe diarrhea. Two patients had diffuse gallium uptake by the lungs, suggestive of BCNU pulmonary toxicity, that resolved over a few months. None had clinical evidence of pulmonary fibrosis.

DISCUSSION

Our results show that high-dose CBV induces a high response rate in relapsed, previously treated Hodgkin's disease patients and that it may induce some long-term unmaintained remissions. There is no need to purge the bone marrow. These results continue to update our preliminary observations, which have been reported elsewhere (9,10).

We have extensively studied the combination of high-dose cyclophosphamide, BCNU, and VP-16-213 for its activity and toxicity in leukemia, lymphoma, and other solid tumors (11,12). Use of the combination for Hodgkin's disease has advantages. Individually, all three drugs have shown activity in Hodgkin's disease. Many relapsed patients with Hodgkin's disease never received cyclophosphamide. On the contrary, relapsed patients have quite often received prior radiotherapy to the mediastinum or abdomen, which has compromised the application of the TBI program. The cyclophosphamide dose is at least six to eight times the conventional dose used in combination chemotherapy (e.g., CHOP); while the doses of BCNU and VP-16-213 are double that used in conventional combination chemotherapy. This would give high-dose CBV the biological equivalence of at least three standard chemotherapy courses by dosage

alone. Administration of conventional-dose chemotherapy may permit lower cell kill and thereby emergence of tumor regrowth between therapies, as well as the opportunity for the development of resistant clones. Since cell repair at lower doses may be overcome by higher doses, the administration of high-dose chemotherapy in one course, like TBI, may have a biological equivalence to that of several courses of conventional chemotherapy. This may account for the consistent results of long-term unmaintained remissions seen in Hodgkin's disease following such high-dose chemotherapy programs.

Fisher et al had reported that the likelihood of achieving second CR could be predicted by the duration of the first remission (13). With second and subsequent relapses, the number of relapses and the maximum disease-free interval immediately before salvage therapy would also influence the response (14). Analysis of our data, in light of the above, shows that all four patients who relapsed after receiving chemotherapy responded, and all failures were in the group whose maximum disease-free interval at any time was less than 12 months. However, patients with a prolonged disease-free interval achieved only partial remissions. Extranodal disease also had a poor prognosis in our study.

Patients who failed to respond to primary induction with MOPP had a poor prognosis; however, high-dose chemotherapy was successful in salvaging 60% of these patients.

Hodgkin's disease patients who failed to respond to MOPP and combination chemotherapy containing Adriamycin, like ABVD or ABDIC, still responded to alternative conventional-dose salvage chemotherapies like MIME (15) and CEP (16). However, many of the patients in this study have even failed salvage therapy programs containing etoposide. Results of MIME salvage therapy show a continued relapse pattern with no plateau.

Dumont et al reported the results of treating 18 patients with high-dose chemotherapy and ABMT (17). All patients received therapy with either 6-thioguanine, aracytine (cytarabine), CCNU, cyclophosphamide (TACC) or carmustine, cytarabine (ara-C) BACT protocols, and four patients even received TBI. There were two long-term unmaintained remissions (17). TBI-based programs also have a high response rate, but there have been more treatment-related deaths and no long-term survivors (18,19).

In summary, the combination of high-dose cyclophosphamide, BCNU, and VP-16-213 is an effective salvage regimen and may play a more important role in the salvage of patients with advanced Hodgkin's disease who fail sequential non-cross-resistant induction chemotherapies.

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Massive Chemotherapy with Autologous Bone Marrow Transplantation in 50 Cases of Non-Hodgkin's Lymphoma with Poor Prognosis

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INTRODUCTION

Recent reports on intensive cytoreductive regimens followed by autologous bone marrow transplantation (ABMT) in non-Hodgkin's malignant lymphoma (NHML) with poor prognosis show that durable complete responses can be achieved occasionally (1-17). The place of this approach in the management of NHML is not yet determined and the overall experience is still minimal (16). In a recently established collaborative group, the participating institutions (i.e., Centre Leon-Berard, Lyon; Institut Paoli-Calmettes, Marseilles; Centre Gauducheau and Centre Hospitalier, Nantes; Hopital Saint-Jacques, Besancon; Hospices Civils de Lyon et Saint-Etienne; and University College Hospital and Middlesex Hospitals, London) have been evaluating this method of treatment since 1980. This report represents our combined experience in treating 50 patients with NHML. Patients who had no chance of being cured with conventional therapy were treated. The patients, who had diffused NHML (49 of 50) in treatment-resistant and treatment-responsive relapses, included those with progressive disease (PD) during conventional therapy and those who were not in complete remission (CR) after four courses of initial induction chemotherapy (i.e., delayed responders). The results are reported and prospective studies are discussed in this chapter.

PATIENTS AND METHODS

Patients

The group of patients (37 males and 13 females) consisted of 31 adults (ages 15-60 years, median 41 years) and 19 children (ages 3-15 years, median 7 years). Of the 50 patients, 16 had treatment-resistant relapse (i.e., resistance to second- or third-line rescue protocols with PD); 19 patients were in relapse but still responded to rescue protocols (i.e., responders after relapse); 7 patients were in partial remission (PR) after at least 2 months of conventional therapy (i.e., partial responders to initial conventional therapy); and 8 received high-dose therapy as intensification because of a long delay to CR (2-8 months; median, 4 months).

NHML was diagnosed in all cases. Of the 50 patients, 32 had high-grade and 18 had intermediate-grade non-Hodgkin's lymphomas (NHLs), according to the international working group (18). Among the high-grade group, 18 patients had B small noncleaved lymphomas, including 16 Burkitt's, 8 lymphoblastic, and 6 immunoblastic lymphomas. Among the intermediate group, seven patients had diffuse mixed lymphomas, five had large cell (histiocytic) lymphomas, and six other patients had three large noncleaved, one follicular large cell, one small cleaved cell, and one polymorphic T cell.

Details of the patients' clinical course and treatment before ABMT are given in Tables 1-4. All patients with treatment-resistant relapse (Table 1) had resistant and PD during treatment and ABMT. In this group of patients, only two were children. The ages of the adult patients ranged from 24 to 60 years (median, 44 years). All had undergone prior

Table 1. Analysis of 16 Patients with Treatment-Resistant Relapses

Patient/ Age/Sex	Pathologic Diagnosis	Interval Diagnosis/ Massive Therapy (mo)	Previous Therapy	Status Prior to Massive Therapy
1/24/F	T-cell mixed intermediate-grade diffuse	23	VCR, CPM, ADR, CYT TG, ASP, MTX (IT), MTX (HD), BLEO Bone marrow harvesting after relapse	Relapse *Subcutaneous diffuse tumors*
2/40/M	Mixed intermediate-grade diffuse	11	CPM, VCR, ADR, BLEO, CYT, TG, MTX (HD), MTX (IT), ASP Bone marrow harvesting after relapse	Relapse *Mediastinal local pleuritis*
3/60/F	T-cell multiform intermediate-grade diffuse (Pinkus)	15	ADR, VCR, CPM, 5FU, CDDP, PRED, Radiotherapy Bone marrow harvesting at relapse	Relapse *Subcutaneous diffuse tumors*
4/59/F	Immunoblastic high-grade diffuse	9	ADR, VCR, CPM, PRED, Radiotherapy Bone marrow harvesting at relapse	Relapse *Abdominal* mesenteric lymph nodes Axillary lymph nodes
5/44/M	Immunoblastic high-grade diffuse	12	ADR, VCR, CPM, BLEO, PRED MTX (HD), MTX (IT), VDS Bone marrow harvesting before relapse (PR)	Relapse Cervical *lymph nodes*
6/41/M	Large cells intermediate-grade follicular	108	ADR, VCR, CPM, PRED, BLEO, VDS, Radiotherapy Bone marrow harvesting at relapse	Relapse *Subcutaneous tumor* (15-cm diam) (head)
7/44/M	Immunoblastic high-grade diffuse	16	ADR, VCR, CPM, VDS, PRED, MTX (HD), MTX (IT), BLEO, ACT, Radiotherapy Bone marrow harvesting before relapse	Relapse *Inguinal lymph nodes* Mesenteric lymph nodes
8/44/F	Immunoblastic high-grade diffuse	6	ADR, VCR, CPM, MTX (HD), MTX (IT), BLEO Bone marrow harvesting before relapse (in CR)	Relapse *Nasopharynx tumor*
9/7/F	T-cell lymphoblastic high-grade diffuse	12	VCR, CPM, ADR, MTX (IT), CYT, TG, ASP, MTX (HD) Bone marrow harvesting after relapse	Relapse Cervical lymph nodes Local *mediastinal* pleuritis
10/38/M	Small cleaved intermediate-grade diffuse	28	CPM, ADR, VCR, PRED, Radiotherapy Bone marrow harvesting after relapse	Relapse Large *abdominal masses*
11/29/M	Small cleaved intermediate-grade diffuse	7	CPM, VCR, PRED, ADR, VP-16-213, VDS Bone marrow harvesting before relapse	Relapse *Abdominal masses*
12/39/M	Small cleaved intermediate-grade diffuse	12	VCR, CPM, ADR, PRED, BCNU, MTX (HD), CCNU Bone marrow harvesting at relapse	Relapse Abdominal masses Cervical *lymph nodes*
13/49/M	Lymphoblastic high-grade diffuse	12	VCR, CPM, ADR, PRED, MTX (HD), Radiotherapy Bone marrow harvesting at relapse	Relapse Head and neck *lymph nodes*
14/28/F	Small cleaved intermediate-grade diffuse	9	VCR, CPM, ADR, MTX (HD) Bone marrow harvesting at relapse	Relapse *Subcutaneous tumor* Lymph nodes Chest involvement
15/11/F	Burkitt's lymphoma high-grade diffuse	1	VCR, CPM, ADR, MTX (HD), PRED Bone marrow harvested at relapse, cleansing Y29/55	*CNS relapse*
16/53/M	Immunoblastic high-grade diffuse	6	VCR, CPM, ADR, PRED, MTX (HD), CCNU, BLEO, Radiotherapy Bone marrow harvesting at relapse	Relapse Biologic signs of inflammation *CNS relapse*

Note: *VCR, vincristine; CPM, cyclophosphamide; ADR, Adriamycin; CYT, cytosine; TG, thioguanine; ASP, asparaginase; MTX, methotrexate (HD, high dose; IT, intrathecal); BLEO, bleomycin; BCNU, 1,3-bis-(2 chloroethyl)-1-nitrosourea; CNS, central nervous system; CSF, cerebrospinal fluid.*

chemotherapy regimens that included Adriamycin--with an average of seven agents administered before ABMT. The major sites of relapse before ABMT were subcutaneous tumor (four patients), lymph nodes (four patients), abdominal tumor (three patients), mediastinal tumor (two patients), central nervous system (CNS) (two patients), and nasopharyngeal tumor (one patient). Histology was intermediate grade (eight patients) and high grade (eight patients) (Table 1). The median time from diagnosis to ABMT was 12 months. In eight patients, bone marrow was harvested at the time of relapse; patients with marrow involvement were excluded. In four patients, bone marrow was harvested in PR after initial induction. Using conventional staining methods, results from aspiration and biopsy of the marrow were normal in all patients.

Data on patients who were responders after relapse are presented in Table 2. Of 19 patients, 16 relapsed during therapy and 3 relapsed posttherapy (patients 29, 33, and 34). All patients received a regimen containing Adriamycin prior to relapse. The number of drugs or modalities of therapy prior to ABMT ranged from 4 to 10 (mean, 7). Four patients had intermediate-grade lymphomas and 15 had high-grade lymphomas, i.e., 7 Burkitt's lymphomas, 7 lymphoblastic lymphomas (including 4 T-cell lymphoblastic), and 1 B-cell immunoblastic lymphoma. The interval between diagnosis and ABMT was 3-46 months (median, 11 months). The group consisted of 8 adults and 11 children, 16-50 years of age for adults and 3-14 years of age for children. The clinical status of the patients at the time of ABMT is shown in Table 2.

The three patients who relapsed posttherapy were partial responders to cyclophosphamide, Adriamycin, vincristine, prednisone (CHOP) at the time of ABMT.

The seven Burkitt's lymphoma patients, who relapsed during therapy, received high-dose methotrexate (MTX [HD]) (3 g/m^2), vincristine (1.5 mg/m^2), and asparaginase (1000 U/kg x 5) every week for 3 weeks (with CNS irradiation for cerebrospinal fluid [CSF] relapses) as the initial treatment of the relapse. In the seven patients with lymphoblastic lymphoma, high-dose cytosine was intravenously administered initially. The four patients with intermediate-grade lymphoma were initially treated with a variety of drug regimens. In all patients, high-dose therapy and ABMT were begun as soon as possible after response to conventional rescue chemotherapy regimens--usually within 3 weeks. Of the 19 patients, 17 had bone marrow harvested either after relapse or at relapse, and only 2 patients had the marrow harvested before relapse.

Data on the group of partial responders to initial conventional therapy are listed in Table 3. These patients (two children and five adults) ranged in age from 4 years to 57 years. Two of the patients had intermediate-grade lymphoma and five had high-grade lymphoma (4 Burkitt's, 1 lymphoblastic). The interval between diagnosis and ABMT was 2-5 months (median, 4 months). All seven patients received Adriamycin prior to ABMT. Patients with Burkitt's lymphoma were treated according to the French Society of Pediatric Oncology protocols (19) (CHOP plus intrathecal methotrexate [MTX] [IT]) before 1981 and CHOP plus MTX (HD) plus asparaginase plus cytosine plus thioguanine and 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU) after 1981. All other patients were treated with CHOP combinations plus MTX (HD). Bone marrow was always harvested after at least 2 months of chemotherapy, except for patient 37, who presented with 5% Burkitt's lymphoma cells in the bone marrow prior to high-dose therapy. This particular patient received carmustine, cytarabine (ara-C), cyclophosphamide, thioguanine (BACT) without ABMT (1). At the time of high-dose treatment, three patients had CNS involvement, two had residual abdominal disease, one had residual mediastinal disease, and one had persistent monoclonal gammapathy with minor bone marrow involvement (patient 37).

Data on the group of eight patients who received massive therapy as consolidation are summarized in Table 4. Of the eight patients, three had intermediate-grade lymphoma and five had high-grade lymphoma (4 Burkitt's, 1 immunoblastic). In six patients, high-dose therapy was given because of a long delay to CR on conventional therapy (3-5 months). Patient 50 was treated with high-dose therapy as consolidation of CR for a stage-IV Burkitt's lymphoma with initial CNS and bone marrow involvement. Patient

Table 2. Analysis of 19 Responders after Relapses

Patient/ Age/Sex	Pathologic Diagnosis	Interval Diagnosis/ Massive Therapy (mo)	Previous Therapy	Status Prior to Massive Therapy
17/10/M	T-cell lymphoblastic high-grade diffuse	28	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, CYT, TG Bone marrow harvesting after relapse	Relapse {Bone marrow, Facial palsy} Responder to first-line salvage therapy Bone marrow Normal Persistence of facial palsy
18/10/F	Burkitt's high-grade diffuse	4	VCR, CPM, ADR, MTX (IT), MTX (HD), PRED Bone marrow harvesting after relapse	Relapse {CSF, Facial palsy} Responder to first-line salvage therapy Persistence of facial palsy
19/4/M	Burkitt's high-grade diffuse	4	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED Bone marrow harvesting after relapse	Relapse CSF and facial palsy Responder to first-line therapy Persistence of facial palsy
20/3/M	T-cell lymphoblastic high-grade diffuse	6	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, BLEO, CYT, TG Bone marrow harvesting after relapse	Relapse Responder to first-line therapy Cervical lymph nodes persistence
21/3/M	Burkitt's high-grade diffuse	5	VCR, CPM, ADR, MTX (IT), ASP, PRED Bone marrow harvesting after relapse	Second CR CSF relapse
22/11/M	Burkitt's high-grade diffuse	7	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, Radiotherapy Bone marrow harvesting after relapse	Second CR CNS relapse
23/7/M	Burkitt's high-grade diffuse	3 12	VCR, CPM, ADR, MTX (IT), PRED, MTX (HD), ASP 24 Gy CNS - Bone marrow harvesting after relapse 24 Gy CNS	Second CR Abdominal local relapse PR to second relapse Intracranial BL with normal CSF
24/27/M	B-cell immunoblastic high-grade diffuse	12	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, CYT, TG, BCNU Bone marrow harvesting after relapse	Second CR After abdominal relapse (Diffuse all abdomen with ascites)
25/9/M	T-cell lymphoblastic high-grade diffuse	39	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, CYT, TG Bone marrow harvesting after relapse	Second CR Local mediastinal relapse + CSF
26/14/M	Burkitt's high-grade diffuse	11	VCR, CPM, ADR, MTX (IT), PRED, CNS, Radiotherapy Bone marrow harvesting after relapse	Second CR (CSF and facial palsy)
27/16/F	T-cell high-grade diffuse	11	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, BLEO, CYT, TG, Radiotherapy Bone marrow harvesting after relapse	Second CR Local mediastinal relapse
28/4/M	Lymphoblastic high-grade diffuse	7	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, CYT, TG Bone marrow harvesting prior to relapse	Second CR (Bone marrow relapse)
29/28/M	Mixed intermediate-grade diffuse	39	VCR, CPM, ADR, PRED, Bone marrow harvesting after relapse	Relapse Crural lymph nodes 8x8 cm (PR to CHOP)
30/31/M	Large cell intermediate-grade diffuse	12	VCR, CPM, ADR, PRED, VM-26, VDS, Radiotherapy Bone marrow harvesting at second CR	Second CR Mediastinal local relapse
31/14/M	Burkitt's high-grade diffuse	3	VCR, CPM, ADR, MTX (HD), CYT, ASP Bone marrow harvesting prior to relapse	Relapse Local relapse (PR) Cervical lymph nodes Nasopharynx tumor

Table 2 (Continued)

Patient/ Age/Sex	Pathologic Diagnosis	Interval Diagnosis/ Massive Therapy (mo)	Previous Therapy	Status Prior to Massive Therapy
32/47/M	Lymphoblastic high-grade diffuse	13	VCR, CPM, ADR, Ara-C, TG, MTX (HD), Radiotherapy Bone marrow harvesting after relapse	Relapse (Cervical lymph nodes) Second CR
33/47/M	B-cell small cleaved intermediate-grade diffuse	46	VCR, CPM, ADR, Radiotherapy, PRED Bone marrow harvesting at relapse	Relapse Residual disease Post-CHOP x 6 Initially radiotherapy (40 months ago) for stage I
34/50/M	Small cleaved intermediate-grade diffuse	8	VCR, CPM, ADR, PRED Bone marrow harvesting at relapse	Relapse (testicular) of stage I PR to CHOP x 6
35/26/M	Lymphoblastic high-grade diffuse	4	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, CYT, TG, VM-26, CDDP, Radiotherapy Bone marrow harvesting at relapse	Relapse Mediastinal initial persistence of a phrenic palsy

Note: VCR, vincristine; CPM, cyclophosphamide; ADR, Adriamycin; MTX, methotrexate (HQ, high dose; IT, intratheca1); ASP, asparaginase; PRED, prednisone; CYT, cytosine; TG, thioguanine; BLEO, bleomycin; CSF, cerebrospinal fluid; CR, complete remission; CNS, central nervous system; PR, partial remission; BL, Burkitt's lymphoma; BCNU, 1,3-bis-(2 chloroethyl)-1-nitrosourea; CHOP, cyclophosphamide, Adriamycin, vincristine, prednisone; CDDP, cisplatin.

Table 3. Partial Responders to Initial Conventional Therapy[a]

Patient Age/Sex	Pathologic Diagnosis	Interval Diagnosis/ Massive Therapy (mo)	Previous Therapy	Status Prior to Massive Therapy
36/30/M	Large noncleaved intermediate-grade diffuse	2	VCR, CPM, ADR, PRED, BLEO	PR Clinical remission Persistence of abdominal mediastinal lymphoma
37/4/M	Burkitt's high-grade diffuse	3 (No ABMT)	VCR, CPM, ADR, MTX (IT), PRED	PR Persistence of Bence-Jones gammapathy and 5% Burkitt's cells at cytology of BM
38/4/M	Burkitt's high-grade diffuse	5	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, CYT	PR Persistence of a facial palsy
39/57/M	B-cell mixed intermediate-grade diffuse	3	VM-26, MTX (IT), CPM, ADR, CYT, TG, Radiotherapy	PR CSF disease persistent
40/17/M	Lymphoblastic high-grade diffuse	4	VCR, CPM, ADR, MTX (HD), CYT, TG, ASP	PR Persistence of abdominal lymph nodes (positive laparotomy)
41/25/M	Burkitt's high-grade diffuse	5	VCR, CPM, ASP, ADR, TG, MTX (HD), MTX (IT), CYT, Radiotherapy	PR (Minor persistent palsy)
42/24/M	Burkitt's high-grade diffuse	4	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, CYT, TG	PR (Persistent abdominal masses CT scan)

[a] *All bone marrows were harvested while patients were in PR. Bone marrow was cleansed by Asta Z in patient 42.*

Note: *VCR, vincristine; CPM, cyclophosphamide; ADR, Adriamycin; PRED, prednisone; BLEO, bleomycin; PR, partial remission; BM, bone marrow; MTX, methotrexate (IT, intrathecal; HD, high dose); ASP, asparaginase; CYT, cytosine; TG, 6-thioguanine; CSF, cerebrospinal fluid; CT, computed tomography.*

Table 4. Massive Therapy as Consolidation[a]

Patient/ Age/Sex	Pathologic Diagnosis	Interval Diagnosis/ Massive Therapy (mo)	Previous Therapy	Status Prior to Massive Therapy
43/32/F	Mixed intermediate-grade diffuse	5	VCR, CPM, ADR, BLEO, MTX (HD), MTX (IT), Radiotherapy	First CR obtained at 4 mo
44/32/F	Large noncleaved intermediate-grade diffuse	7	VCR, CPM, ADR, MTX (IT), PRED, BLEO, CYT, TG	First CR obtained at 3 mo
45/36/M	Large noncleaved intermediate-grade diffuse	8	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, BLEO, PRED	First CR obtained at 5 mo
46/4/M	Burkitt's high-grade diffuse	8	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, CYT, TG	First CR obtained at 5 mo
47/23/M	Immunoblastic high-grade diffuse	11	Radiotherapy CPM, VM-26, ADR	First CR obtained at 3 mo
48/10/M	Burkitt's high-grade diffuse	7	VCR, CPM, ADR, MTX (HD), MTX (IT)	First CR obtained at 1 mo, but no treatment between 1 and 7 mo (toxicity)
49/4/F	Burkitt's high-grade diffuse	5	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, CYT	First CR obtained at 4 mo
50/14/M	Burkitt's high-grade diffuse	3	VCR, CPM, ADR, MTX (IT), MTX (HD), ASP, PRED, CYT, TG	First CR obtained at 2 mo, with initial CNS and bone marrow involvement

[a] *Bone marrow from patients 46, 49, and 50 was cleansed by Y29/55 antibody.*

Note: *VCR, vincristine; CPM, cyclophosphamide; ADR, Adriamycin; BLEO, bleomycin; MTX, methotrexate (HD, high dose; IT, intrathecal); CR, complete remission; PRED, prednisone; CYT, cytosine; TG, 6-thioguanine; ASP, asparaginase; CNS, central nervous system.*

48 received 200 mg/kg of cyclophosphamide 7 months after diagnosis because of a long delay without therapy (6 months), which followed a major hepatic and neurologic toxicity at induction. There were four children and four adults and the interval between diagnosis and massive therapy was short (3-11 months; median, 7 months).

Drug Regimens

The high-dose chemotherapy regimens were not identical among the centers, but there were strong similarities in both the indications for treatment and the methods employed.

Group A

According to protocols of which the details are shown in Table 5 (10); of 31 patients treated in Lyon, 15 received BACT IGR (Table 5) (9); 13 received either BACT (10) or TACC (2) (Table 5) (1); and 3 received cyclophosphamide alone (2 received 60 mg/kg and 1 received 50 mg/kg every 4 h). Dosages of BCNU were administered intravenously, infused slowly over 10 min; ara-C was administered in continuous infusion over 12 h or as a 30-min infusion over 12 h, cyclophosphamide was infused in 250 ml of 5% dextrose intravenously for 30 min, and thioguanine was given orally every 12 h. Eight patients received 10 Gy of nonfractionated total body irradiation (TBI) after chemotherapy (Table 5). In 30 of 32 courses (22 patients received two courses of massive therapy) and ABMT was performed to combat chemotherapy-induced aplasia. In two patients (patients 37 and 22) marrow was not administered because of suspicion of bone marrow contamination by malignant cells. Freezing procedures and detection of bone marrow contamination were previously described (10; I Philip, T Philip, GM Lenoir et al., unpublished observations). All patients were treated in a sterile care unit and given parenteral nutrition. After oral decontamination, sterile nutrition was allowed. Folinic acid and vitamin

Table 5. High-Dose Chemotherapy Regimens Used in Lyon

Regimens/Drugs		Days -8	-7	-6	-5	-4	-3	-2	-1	0	N
Appelbaum BACT[a]											
BCNU	200 mg/m²			x							
Ara-C	200 mg/m²				x	x	x	x			10
CPM	50 mg/kg				x	x	x	x			
6-thioguanine	200 mg/m²				x	x	x	x			
ABMT										x	
Modified IGR BACT											
BCNU	200 mg/m²			x	x	x					
Ara-C	200 mg/m²				x	x	x	x			15
CPM	50 mg/kg				x	x	x	x			
6-thioguanine	200 mg/m²				x	x	x	x			
ABMT										x	

Note: *BACT, carmustine, cytarabine, cyclophosphamide, 6-thioguanine; BCNU, 1,3-bis-(2 chloroethyl)-1-nitrosourea; ara-C, cytarabine; CPM, cyclophosphamide; ABMT, autologous bone marrow transplantation; IGR, Institut Gustave Roussy; TACC, 6-thioguanine, aracytine (cytarabine), CCNU (1-[2-chloroethyl]-3-cyclohexyl-1-nitrosourea), cyclophosphamide; TBI, total body irradiation.*

[a]*TACC is identical to BACT, except that CCNU replaces BCNU. When the TBI regimen begins 1 day before and TBI is realized on day 1, it has been realized in eight patients.*

B12 were systematically administered during aplasia. Day 0 was defined as the day of ABMT or, in the absence of ABMT, as the proposed day of marrow reinfusion. No further chemotherapy or radiotherapy was administered after ABMT until disease progression.

Group B

Twelve patients were treated according to the Marseilles protocols (Table 6). Among this group, five patients received high-dose melphalan alone, and seven were treated with the BAVM protocol (Table 6). The patients received the following dosages: BCNU (300 mg/m²) was administered intravenously and infused (slowly) over 10 min; vindesine (1.3 mg/m²), infused continuously every 24 h for 5 days; and ara-C (100 mg/m²) infused continuously every 12 h for 5 days. Nonfrozen marrow was used in five patients and frozen marrow was used in seven patients. Freezing procedures were similar to those in group A. Sterile case units were used for patient care.

Group C

Seven patients were treated in accordance with the University College Hospital protocol (Table 7). High-dose therapy consisted of two different courses followed by ABMT in each case (Table 7). All patients received the first course, but only three patients received the second course followed by a second ABMT. The bone marrow of the patients in London was harvested after relapse, except in one patient, who had enough for storage of two reinfusions. Freezing procedures were previously reported (20). Patients were kept in an isolated normal room. The therapeutic approach both during and after ABMT did not differ from that of other teams in the group.

Table 6. Protocols Used in Marseilles

Regimens/Drugs		Days -8	-7	-6	-5	-4	-3	-2	-1	0	N
BAVM											
BCNU	300 mg/m²	x									
Ara-C	200 mg/m²/day		x	x	x	x	x				7
Vindesine	1.3 mg/m²/day		x	x	x	x	x				
Melphalan	140 mg/m²							x			
ABMT										x	
Melphalan											
Melphalan	140 mg/m²								x		
ABMT										x	5

Note: *BAVM, BCNU, ara-C, vindesine, melphalan; BCNU, 1,3-bis-(2 chloroethyl)-1-nitrosourea; ABMT, autologous bone marrow transplantation.*

Table 7. High-Dose Chemotherapy Regimens Used in University College Hospital (London)

Regimens/Drugs		Days -8	-7	-6	-5	-4	-3	-2	-1	0	N
London 1											
BCNU	300 mg/m²			x							
Ara-C	200 mg/m²			x	x	x	x	x			7
Cyclophosphamide	1.5 g/m²			x	x	x					
ABMT										x	
London 2											
BCNU	300 mg/m²			x							
MTX	1 g/m²			x							
Leucovorin	rescue			x	x	x	x				(3/7)
Ara-C	200 mg/m²			x	x	x	x	x			
ABMT										x	

Note: *BCNU, 1,3-bis-(2 chloroethyl)-1-nitrosourea; ara-C, cytarabine; ABMT, autologous bone marrow transplantation; MTX, methotrexate.*

RESULTS

In accordance with groups A, B, and C, the disease-free survival of all patients is shown in Fig 1. In the patients with treatment-resistant relapse (Table 8), 9 of 16 were in CR 30 days after ABMT, 2 of 16 were in PR, 2 were nonresponders, 1 had progressive disease, and 2 were not evaluable because of early death but clearly responded (patient 4 died of small bowel rupture caused by rapid tumor lysis and patient 13 died on day 9 of sepsis in clinical clear response). Among the 14 evaluable patients, 11 (78.5%) had disease that responded to massive therapy (9 CR, 2 PR), 2 had no response (NR), and 1 had progressive disease. The duration of CR ranged from 44 to 394 days (median, 100 days; average, 141 days). One patient is still alive in CR 394 days after ABMT. ABMT was performed for relapse of an intermediate-grade histiocytic lymphoma. It may be of

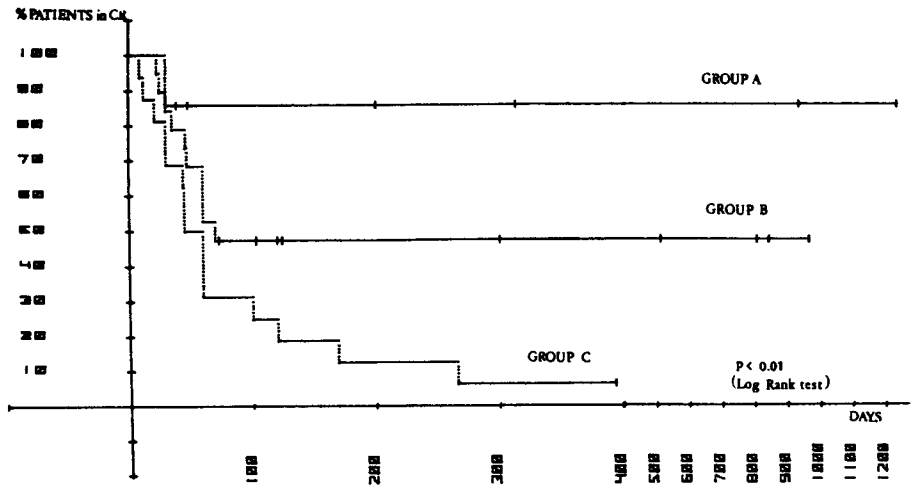

Figure 1. Disease-free survival for groups A, B, and C.

interest that this patient received "boost" radiotherapy after ABMT to the site of major intra-abdominal disease. Overall survival in this group is 6% (Fig 1, group A).

The ABMT procedure produced significant morbidity in 10 of 16 patients. As mentioned earlier, one patient died of septic shock after small bowel rupture and one died of sepsis at day 9. In the other eight patients, complications included toxicity (i.e., hemomediastinum, pulmonary infection [3], interstitial pneumonitis, active chronic hepatitis, cardiac failure, and subclavian thrombosis).

Details of the responses in patients who were responders after relapse are shown in Table 9. The disease-free survival is shown in Fig 1, group B. Nine of the 19 patients are alive with no evidence of disease 73-962 days (median, 300 days) after ABMT. The relapses occurred before day 70, except for patient 22 who is alive in third CR. Nine patients, who were in second CR at the time of massive therapy, were not evaluable for response--except one patient whose disease progressed after ABMT. Among the 12 evaluable patients, 7 were in CR at day 30, 2 were in PR, and 3 had progressive disease at the time of massive therapy. In the 9 of 12 evaluable patients the response rate was 75%.

No lethal major toxicity occurred in seven patients. One cyclophosphamide-induced cardiomyopathy and cystitis, one tubulopathy, one Moschowitz syndrome, one viral interstitial pneumonitis, one leukoencephalitis, one major diarrhea, and one veno-occlusive disease were observed in this group. Three patients (3 of 19; 16%) died of toxicity--one with *Aspergillus* endocarditis, one with veno-occlusive disease, and one with *Escherichia coli* sepsis and pneumonitis after recovery from veno-occlusive disease.

The group of partial responders to first-line therapy (Table 10, Fig 1, group C) consisted of seven patients only. These patients were not very different from the nonresistant relapse group. Of seven patients, six had CR and one failed with progressive disease at day 55. There were no treatment-related deaths, but 4 of 7 patients had severe complications: one tuberculosis, one *Candida* pneumonitis, one subarachnoid hemorrhage, and one presumed viral infection. Six of 7 patients are alive 39-1230 days (median, 200 days) after ABMT.

Eight patients with long delays to CR received high-dose therapy as consolidation (Table 11). Of eight patients, three died with lymphoma despite intensification, which indicated that the definition of poor prognosis CR was correct. Of these eight patients, two died of treatment-related causes (interstitial pneumonitis and Moschowitz syndrome), one of whom had lymphoma at autopsy, two relapsed at days 21 and 46. Four

Table 8. Treatment-Resistant Relapses: Details of Treatment and Evolution Post-ABMT

Patient	Type of Therapy	TBI	Stay in Sterile Care	No. of Days <1000 WBC	<500 PMN	<200 PMN	<50,000 Platelets	Status after Massive Therapy	Additional Therapy	Complications E: Early L: Late	Status on 1/9/83
1	CPM	12 Gy	56	53	53	42	>100	CR	No	Hemomediastinum (E) *Pseudomonas aeruginosa* sepsis (E)	Relapsed day 100 Died day 150
2	IGR BACT	No	27	15	18	15	30	PR (clinical)	Radiotherapy (Boost)	Bacterial pulmonary infection (E)	Alive day 45 Progressive disease
3	HDM	No	38	24	15	6	22	CR	No	Active chronic hepatitis (L)	Relapsed day 266 Died day 421
4	HDM	No	12	NE	NE	NE	NE	NE (dramatic response)	No	Septic shock (E) (small bowel rupture)	Died day 12
5	HDM	No	28	9	12	5	19	CR	No	Small bowel volvulus (E)	Relapsed day 60 Died day 300
6	HDM	No	29	44	24	12	52	PR	No	No	Relapsed day 45 Died day 270
7	BAVM	No	42	15	13	10	40	CR	No	Aspergillosis (E)	Relapsed day 60 Died day 240
8	BAVM	No	45	28	12	10	45	CR	No	Interstitial pneumonitis (E) (resuscitation)	Local relapse day 120 Alive with disease day 150
9	Appelbaum BACT	No	30	27	27	24	32	CR	No	*Streptococcus pneumonitis* (resuscitation) (E)	Relapsed day 44 Died day 95
10	London (Course 1)	No	NE	13	13	NE	14	CR (clinical)	No	Subclavian thrombosis (E)	Relapsed day 169 Died day 260
	London (Course 2)	No	NE	15	16	NE	20	CR (clinical)		No	

11	London (Course 1)			32	34	NE	43	NR	No	No	Died day 44
12	London (Course 1)			11	20	NE	21	CR (clinical)	Radiotherapy (boost) (abdomen)	No	Alive NED 394+ days
	London (Course 2)	No	NE	17	20	NE	25			No	
13	London (Course 1)	No	NE	NE	NE	NE	NE	Massive response (NE)	No	Sepsis	Dead day 9
14	London (Course 1)	No	NE	13	15	NE	16	NR	Yes	No	Died while in relapse day 68
15	IGR BACT	No	10	6	5	3	13	PD	Yes Radiotherapy (very good PR)	No	Alive in PD day 21
16	BAVM	No	23	21	10	8	NE	CR	No	No	Relapse day 60 Alive with disease day 230

Note: *CPM, cyclophosphamide; CR, complete remission; IGR, Institut Gustave Roussy; BACT, carmustine, cytarabine, cyclophosphamide, 6-thioguanine; PR, partial response; HDM, high-dose melphalan; NE, not evaluable; BAVM, BCNU, Ara-C, vindesine, melphalan; NR, no response; NED, no evidence of disease; PD, progressive disease.*

Table 9. Details of the 19 Patients Still Responding after Relapse and before Massive Therapy

Patient	Type of Therapy	TBI	Stay in Sterile Care	No. of days <1000 WBC	<500 PMN	<200 PMN	<50,000 Platelets	Status after Massive Therapy	Additional Therapy	Complications E: Early L: Late	Status on 1/9/83
17	IGR BACT	No	35	14	24	16	32	CR	No	No	Relapse day 70 Died day 90
18	IGR BACT	No	21	14	19	12	NE	CR	No	Cyclophosphamide myocardiopathy Cyclophosphamide cystitis (E)	Relapse day 35 Died day 45
19	IGR BACT	No	30	28	32	27	35	CR	No	No	Relapse day 60 Died day 83
20	Appelbaum BACT	No	25	15	25	15	21	PD	No	Hypokalemia with tubulopathy (E)	Died day 40
21	Appelbaum BACT	No	25	13	16	14	NE	PD	No	Reinjected bone marrow contaminated? (E)	Died day 25
22	IGR BACT	No	25	20	22	16	25	CR	No	Leukoencephalitis (L)	CR 840+ days
23	Appelbaum BACT	No	24	9	11	3	3	CR	CHOP x 3	No	Relapse day 210 Alive NED; 3rd CR Day 804+
	IGR BACT	No	21	11	11	9	13	CR	Interferon		
24	Appelbaum BACT	10 Gy	31	11	20	14	NE	CR	No	Aspergillus endocarditis (E)	Died day 23 (No lymphoma at autopsy)
25	Appelbaum BACT	10 Gy	47	5	8	2	NE	CR	No	Veno-occlusive disease (E)	Died day 47 CR
26	IGR BACT	-	30	24	43	29	70	CR	No	No	Alive NED day 962+

27	Appelbaum BACT	10 Gy	24	18	20	16	NE	CR	No	Veno-occlusive disease (E) with regression Escherichia coli pneumopathy	Died day 46 (No lymphoma at autopsy)
28	CPM	10 Gy	30	23	29	26	22	CR	No	Diarrhea (E)	Alive CR NED 300+ days
29	TACC	No	29	12	21	16	22	CR	No	Moschowitz syndrome (L)	Alive NED day 510+
30	HDM	No	17	14	8	7	13	CR	No	No	Relapse day 60 Died day 104
31	IGR BACT	No	25	12	21	11	23	CR	No	Interstitial viral pneumonitis (E)	Alive NED day 103+
32	BAVM	No	28	14	10	7	21	CR	No	No	Alive NED day 120+
33	London (Course 1)	No	NE	18	20	NE	22	PR	No	No	Alive day 73+ NED (Doubtful CT scan)
34	London (Course 1)	No	NE	16	19	19	15	PR	MTX (HD)	No	Alive day 124+ CR
35	BAVM	No	24	17	13	7	21	CR	No	No	Relapse day 60 Alive day 120

Note: *TBI, total body irradiation; IGR, Institut Gustave, Roussy; BACT, carmustine, cytarabine, cyclophosphamide, 6-thioguanine; CR, complete remission; NE, not evaluable; PD, progressive disease; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; NED, no evidence of disease; CPM, cyclophosphamide; TACC, 6-thioguanine, aracytine (cytarabine), CCNU, cyclophosphamide; HDM, high-dose melphalan; BAVM, BCNU, Ara-C, vindesine, melphalan; CT, computed tomography; MTX (HD), methotrexate (high dose).*

Table 10. Analysis of Responders after Initial Induction Therapy Prior to Autologous Bone Marrow Transplantation

Patient	Type of Therapy	TBI	Stay in Sterile Care	No. of Days				Status after Massive Therapy	Additional Therapy	Complications	Status on 1/9/83
				<1000 WBC	<500 PMN	<200 PMN	<50,000 Platelets			E: Early L: Late	
36	Appelbaum BACT	10 Gy	23	14	15	11	32	CR	No	Tuberculosis (L)	Alive NED 1230+ days
37	Appelbaum BACT	No	45	9	16	11	11	CR	No	Toxic agranulocytosis (Duration, 17 days) (E) Candida pneumopathy (E)	Alive NED 930+ days
38	IGR BACT	No	28	20	22	17	25	CR (Disappearance of facial palsy day 10)	No	CSF bleeding (E)	Alive NED 200+ days
39	IGR BACT	No	28	11	14	10	12	CR	No	No	Alive NED 313+ days
40	TACC	No	40	33	34	29	>48	CR (clinical)	No	Viral infection (E)	Alive NED 48+ days
41	BAVM	No	25	16	14	5	35	SD	No	No	Progression CSF bone marrow day 55 died day 60
42	IGR BACT	No	26	17	17	16	24	CR	No	No	NED 39+ days

Note: *TBI, total body irradiation; BACT, carmustine, cytarabine, cyclophosphamide, thioguanine; CR, complete remission; IGR, Institut Gustave, Roussy; NED, no evidence of disease; CSF, cerebrospinal fluid; TACC, 6-thioguanine, aracytine (cytarabine), CCNU, cyclophosphamide; BAVM, BCNU, ara-C, vindesine, melphalan; SD, stable disease.*

Table 11. Analysis of Patients Receiving Massive Therapy as a Consolidation of First and Complete Remission

Patient	Type of Therapy	TBI	Stay in Sterile Care	No. of days <1000 WBC	<500 PMN	<200 PMN	<50,000 Platelets	Status after Massive Therapy	Additional Therapy	Complications E: Early L: Late	Status on 1/9/83
43	BAVM	-	30	NE	12	9	NE	CR	No	VOD - Interstitial pneumonitis (Pneumococcus) (day 7) (E)	Died day 30 clinical CR (no autopsy)
44	Appelbaum BACT	10 Gy	20	16	16	12	15	CR	No	Moschowitz syndrome (L)	Died day 147 (lymphoma at autopsy)
45	Appelbaum BACT	10 Gy	27	14	16	16	17	CR	No	No	NED 731+ days
46	IGR BACT	No	25	18	17	13	30	CR	No	Burkitt's interstitial pneumonitis (E)	Relapse day 46 died day 47
47	TACC	No	22	19	17	14	22	CR	No	Infectious hepatitis (E) Thoracic herpes (L)	NED 763+ days
48	CPM	No	25	6	11	6	0	CR	No	No	NED 124+ days
49	IGR BACT	No	24	17	20	17	16	CR	No	No	Alive NED 126+ days
50	IGR BACT	No	27	17	11	10	27	PD Day 30 (CSF +)	No	Strepto D sepsis (E) Cyclophosphamide vesical toxicity	Alive in relapse 21+ days

Note: *TBI, total body irradiation; BAVM, BCNU, ara-C, vindesine, melphalan; NE, not evaluable; CR, complete remission; VOD, veno-occlusive disease; IGR, Institut Gustave, Roussy; BACT, carmustine, cytarabine, cyclophosphamide, 6-thioguanine; TACC, 6-thioguanine, aracytine (cytarabine), CCNU, cyclophosphamide; NED, no evidence of disease; PD, progressive disease; CSF, cerebrospinal fluid.*

patients are still alive with no evidence of disease (124+, 126+, 731+, 763+ days). Two of the four patients have experienced severe morbidity.

In summary, in these groups of patients with poor prognoses who were still responsive to chemotherapy, 19 of 34 (55; 8%) are still alive and have no evidence of disease. The response rate, when evaluable, was high (15 of 19; 78%). These results were obtained at the cost of nonfatal toxicity in 16 of 34 (47%) and 5 of 34 deaths directly related to therapy (14; 7%).

Among the survivors, 10 are adults and 9 are children. In the patients with Burkitt's lymphoma, 9 of 16 (56%) are still alive with no evidence of disease, whereas 8 of 20 (40%) of the patients with other histologies are still alive with no evidence of disease. The trend for better results in Burkitt's lymphoma is not significant ($P > .05$).

No correlation could be established between the number of granulocyte-macrophage colony-forming cells (CFC-GM) per kilogram and recovery or between the number of injected nucleated cells and recovery, as previously reported (12). The BACT regimen did not cause irreversible aplasia since patients 22 and 37, who received BACT treatment but no ABMT, recovered quickly (12).

Of the 33 patients who died or relapsed, 9 had bone marrow involvement at the time of relapse. It was not possible to make a distinction between failure of the high-dose therapy or reinjection of contaminated bone marrrow in these patients. Of these nine patients, seven had their marrow harvested after relapse, one at the time of CSF involvement and only one while in CR (patients 1, 9, and 20 - lymphoblastic lymphoma; and patients 15, 18, 19, 21, 40, and 46 - Burkitt's lymphoma). Bone marrow contamination, therefore, appears to be a potential problem for late-harvested marrow. Bone marrow purging was done in four patients and details have previously been reported (21).

Hematologic Toxicity

Details of hematologic toxicity are given in Tables 8-11. There was a tendency toward a longer duration of aplasia in patients whose marrow was harvested after relapse, but this was not statistically significant.

DISCUSSION

Results of this retrospective study of 50 cases of advanced non-Hodgkin's lymphoma (NHL) treated with high-dose chemotherapy may help us to formulate protocols for prospective studies.

Although the cases are heterogeneous with respect to diagnosis, prior treatment, and details of the high-dose regimens employed, there are sufficient similarities to allow several conclusions to be drawn.

In patients with relapsed disease resistant to conventional chemotherapy, high-dose treatment will still produce responses, many of which are complete. These remissions are not durable (i.e., only 1 of 16 are in CR at 1 year). Nevertheless, this number of CRs represents a remarkable figure in such a group of resistant patients and has hitherto not been obtainable by any conventional chemotherapy regimen (22,23). This conclusion is supported by the work of others (4,5,9-12,14,17,21,24). It seems reasonable, therefore, to draw the conclusion that although reasonably safe, high-dose chemotherapy has little to offer patients with drug-resistant relapse NHL.

Of the 19 patients who were responding to conventional chemotherapy after relapse, high-dose therapy produced responses in 9 of 12 evaluable patients. Toxicity was significant in this group with three deaths, five major complications, and three minor complications. Nevertheless, 9 of 19 are in continuous unmaintained remission with a median follow-up of 300 days. These results in relapsed but still responding patients appear superior to conventional rescue protocols published at this time (22,23). In this group, and possibly also in the fully resistant group, there may be a potential role for local boost radiotherapy (12) to major involved anatomic sites of disease. The only adult long-term survivor

with resistant disease was given boost abdominal radiotherapy immediately post-ABMT.

In the present study, the harvested marrow was free of tumor, as judged by conventional diagnostic means. In our patients, there was some evidence that marrow involvement at death was more frequent in those whose marrow was harvested at relapse and it seems possible that marrow harvest during the first remission would be preferable. At the time of harvest in first remission in vivo, purging may already have occurred by the induction chemotherapy, and the role of additional chemotherapeutic or immunologic purging would not be clear. Our clinical results may well indicate that the problem is of residual host disease (i.e., the early relapse of most patients in the resistant group achieving CR) since our nonresistant patients achieve an early plateau with minimal evidence of late relapse (see Fig 1).

The practical problem for the future is one of logistics. The marrow of patients harvested in a well-defined CR will very likely be free of disease and never need purging. But such patients have a very high chance of cure by conventional chemotherapy and will never need ABMT. However, marrow harvested from the patient with an incomplete initial response or in relapse may be more likely to require purging. Even for the latter group and because of the early plateau of response, the results did not show clear evidence that marrow contamination could be a major problem. Similar results are seen in the seven patients who had had a PR to first-line chemotherapy. There was considerable treatment-related morbidity (4 of 7 patients) during the period of aplasia, but 6 patients have been in CR with a median follow-up time of 200 days. The long-term survival of patients achieving only PR by conventional induction therapy was very poor; even in the best series, it was approximately 15-20% (25). In the present series, although numbers are extremely small, 6 of 7 patients are still in continuous complete remission (CCR) and the median observation time is 7 months. ABMT might represent a significant advance for these patients. Of the eight patients who required prolonged first-line chemotherapy to achieve a complete response, three died of lymphoma in spite of high-dose treatment and two deaths were treatment related. Four patients remain in remission. In this group of eight patients, three still died of lymphoma after achieving a transient CR with massive therapy and ABMT. Of the other five patients in this group, four are still in CCR. It cannot yet be excluded that the length of CCR in those four patients is identical.

Follow-up is too short to conclude whether the length of CR in those patients is longer than the remission duration achieved by conventional dose chemotherapy. Therefore, at the moment, the role of high-dose cytoreductive therapy with ABMT as intensification on first CR cannot yet be assessed.

A potential difficulty with the present series of patients is the mixture of adult and pediatric patients, but an analysis of a larger series of 112 NHML patients, in which 50 of these patients are included, shows no difference between adult and pediatric results in NHML, although there is a tendency for fewer children to be grafted in resistant relapse (26). The larger series also shows that there is no difference in the results between Burkitt's and other types of NHML.

In conclusion, high-dose chemotherapy and ABMT are capable of producing durable responses in patients who are responding poorly to conventional chemotherapy. Those with drug-resistant relapses seem unlikely to benefit. The use of the procedure will depend on the early selection of poor-risk patients who are not responding to treatment with modern intensive chemotherapy regimens. Although results with conventional therapy are improving, patients not in CR after 3-4 months of treatment are probably not curable with conventional dose drugs much of the time (25). A parallel can be drawn with the introduction of allografting as part of intensive treatment in acute leukemia--its use earlier in the disease has led to improved long-term survival (16). A multicenter study in which the initial chemotherapy is uniform and high-dose therapy and ABMT are introduced after early failure of treatment is now in progress. This multicenter study may establish whether similar conclusions, as the above, can be drawn for individual lymphomas, such as

Burkitt's, immunoblastic, lymphoblastic, and intermediate-grade lymphomas, which are clearly separate entities.

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Burkitt's Lymphoma and Autologous Bone Marrow Transplantation: An Overview

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INTRODUCTION

The overall survival of children with Burkitt's lymphoma (BL) increased from 42% to 78% with aggressive conventional chemotherapeutic regimens in a 3-year period in Lyon, France (1-3). During the same period, we investigated the effect of massive therapy with autologous bone marrow transplantation (ABMT) rescue in patients with poor prognoses, treatment initially proposed for Burkitt's lymphoma by Appelbaum (4), as did several other teams in Europe (5-7).

Massive therapy and ABMT induced a cure rate of 40-50% in relapsed BL patients. The majority of patients who relapsed but were cured had central nervous system (CNS) relapses, which accounted for 20% of the relapses in 1980 (2). However, in 1984, only 5% of the relapses were in the CNS (8), and because of variations in definition of poor prognosis BL, confusion still persists concerning the indication for ABMT.

We investigated high-dose cytoreductive therapy with ABMT in 22 BL patients who entered the study from February 1980 to May 1984. Patients with no chance of being cured by conventional therapy were selected. The criteria for this selection were chosen based on experience in our previous studies (1,2)--all had BL after relapse and were patients with progressive disease under conventional therapy, patients not in complete remission (CR) after 2 months of initial induction therapy, or patients with CNS involvement at diagnosis.

PATIENTS AND METHODS

A summary of the clinical data of the 22 patients is given in Table 1. Four patients initially had stage III disease (patients 8, 10, 12, and 16). Massive therapy was indicated because of partial remission (PR) after initial induction therapy (patient 16) or long delay to CR (patients 8, 10, and 12). Six patients had stage IV disease (patients 2, 11, 17, 18, 20, and 21), and massive therapy was indicated because of PR after initial induction in patients 17, 18, and 21. All others, except patients 14 and 15, were in early relapse of BL and were still sensitive to conventional rescue protocols.

The high-dose chemotherapeutic regimens that were used are listed in Table 2. Appelbaum's BACT (BCNU, cytarabine, cyclophosphamide, and 6-thioguanine) was used in four cases, the IGR BACT in 15, BEAM (BCNU, cytarabine, melphalan, and VP-16-213) in two, and cyclophosphamide alone in one (patient 10). BCNU was given intravenously, infused slowly over 10 min; ara-C was given in a 30-min infusion in 5% dextrose every 12 h; cyclophosphamide was dissolved in 250 ml 5% dextrose intravenously for 30 min; and thioguanine was given by mouth every 12 h. Patient 4 received two courses--one Appelbaum's BACT and one IGR BACT.

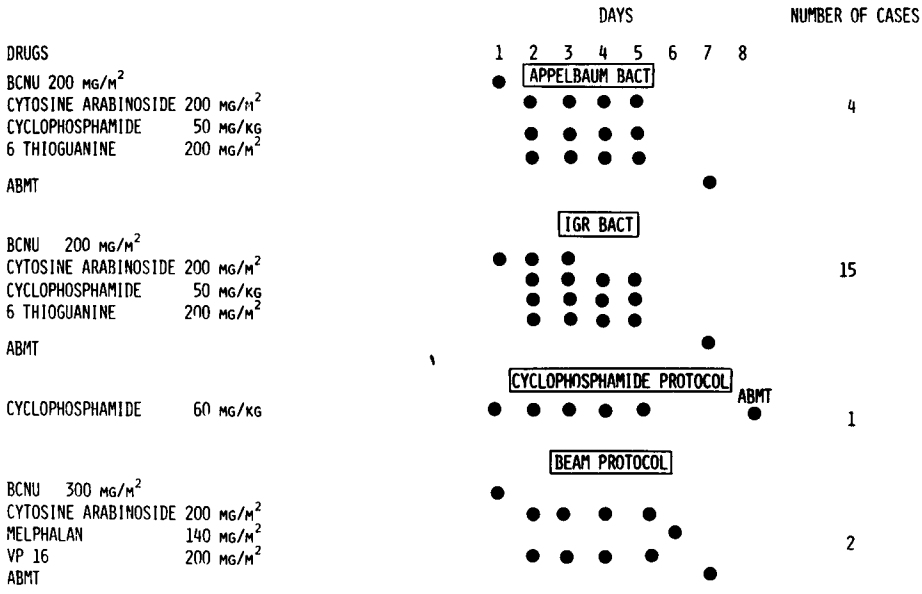
In 20 of 22 courses, ABMT was performed in order to ameliorate chemotherapeutically induced aplasia. However, in two cases (patients 2 and 4, first course) bone-marrow cells that were likely to be contaminated by malignant cells were not reinjected (see the chapter "Detection of Burkitt's Cells in Remission Marrow by a Cell Culture Monitoring System"). This occurred in 1980 before purging procedures had been developed. Bone marrow was harvested prior to relapse in 11 cases and in 11 cases after extramedullary relapse (Table 1). In all cases, four bone marrow smears and four bone marrow biopsies were analyzed at the time of

TABLE I

PATIENT	AGE	SEX	DIAGNOSIS AND STAGING (Murphy)	PREVIOUS THERAPY	INTERVAL DIAGNOSIS MASSIVE THERAPY	TIME OF BM COLLECTION	STATUS PRIOR MASSIVE THERAPY
CLB 3683 1	3	M	BL STAGE III	VCR-CPM-ADR MTX-IT-ASP PRED-CNS-X RAY	5 MONTHS	AFTER RELAPSE	2nd CR AFTER CSF RELAPSE
CLB 3176 2	4	F	BL STAGE IV	VCR-CPM-ADR MTX-IT-ASP PRED-CNS-X RAY	3 MONTHS	NO ABMT	PR AFTER INITIAL INDUCTION THERAPY (MONOCLONAL GAMMA-PATHY AND PARTIAL BM INVOLVEMENT)
CLB 3433 3	11	M	BL STAGE III	VCR-CPM-ADR MTX IT-MTX HD ASP-PRD	7 MONTHS	AFTER RELAPSE	2nd AFTER CSF AND CNS RELAPSE
CLB 2054 4	7	M	BL STAGE III	VCR-CPM-ADR-MTX IT&HD-ASP-PRED CNS-X RAY	3 MONTHS	NO ABMT	2nd CR AFTER ABDOMINAL RELAPSE
				30 GRAYS CNS	12 MONTHS	AFTER RELAPSE	PR AFTER CNS RELAPSE
BES 1 5	10	F	BL STAGE IV	VCR-CPM-ADR MTX IT-PRED CNS-X RAY	4 MONTHS	AFTER RELAPSE	PR AFTER CNS AND CSF RELAPSE
BES 2 6	14	M	BL STAGE III	VCR-CPM-ADR MTX IT-PRED CNS-X RAY	11 MONTHS	AFTER RELAPSE	2nd CR AFTER CSF RELAPSE
HCL DEB.1 7	4	M	BL STAGE II	VCR-CPM-ADR MTX IT-MTX HD ASP-PRED	4 MONTHS	AFTER RELAPSE	PR AFTER CSF AND CNS RELAPSE
CLB 4560 8	4	M	BL STAGE III	VCR-CPM-ADR MTX IT-MTX HD ASP-PRED-CYT-TG	8 MONTHS	IN CR	1st OBTAINED AFTER 5 MONTHS OF CONVENTIONAL THERAPY
MARS 2 9	4	M	BL STAGE II	VCR-CPM-ADR MTX IT-MTX HD CYT-ASP-TG	11 MONTHS	IN CR	2nd CR AFTER CSF RELAPSE
SE 3 10	10	M	BL STAGE III	VCR-CPM-ADR HDM TX-IT MTX	7 MONTHS	IN CR	1st CR
CLB 3571 11	4	M	BL STAGE IV	VCR-CPM-ADR MTX IT-MTX HD ASP-PRED-CYT	5 MONTHS	INITIAL PR	PERSISTENT FACIAL PALSY AFTER 5 MONTHS OF THERAPY
CLB 278 12	4	F	BL STAGE III	VCR-CPM-ADR MTX IT-MTX HD ASP-PRED-CYT	5 MONTHS	CR DECONTAMINATION Y/29/55	1st CR OBTAINED AFTER 4 MONTHS OF CONVENTIONAL THERAPY
DEB 2 13	14	M	BL STAGE II	VCR-CPM-ADR HDMTX	3 MONTHS	PRIOR TO RELAPSE	PR AFTER EARLY LOCAL RELAPSE (HEAD and NECK)
MARS 1 14	9	M	BL STAGE III	VCR-CPM-ADR PRED-CYT-ASP HD MTX-TG	10 MONTHS	AFTER RELAPSE	PROGRESSIVE DISEASE (abdominal relapse)
DEB 3 15	11	F	BL STAGE III	VCR-CPM-ADR HDMTX-PRED	1 MONTH	AFTER RELAPSE DECONTAMINATED Y/29/55	PROGRESSIVE DISEASE CNS
BES 6 16	24	M	BL STAGE III	VCR-CPM-ADR HDMTX-ASP PRED-CYT-TG	4 MONTHS	1st PR DECONTAMINATED BY ASTA 2	PARTIAL RESPONSE TO INITIAL TREATMENT(RESISTANT ABDOMINAL MASSES)
SE 4 17	14	M	BL STAGE IV	VCR-CPM-ADR HDMTX-ITMTX PRED-CYT	3 MONTHS	1st CR DECONTAMINATED Y/29/55	CR(INITIAL ABDOMEN +CNS + BONE MARROW)
DEB 6 18	10	M	BL STAGE IV	VCR-CPM-ADR HDMTX-ITMTX ASP-PRED-CYT	3 MONTHS	1st CR DECONTAMINATED Y/29/55	CR (INITIAL ABDOMINAL + CNS + BONE MARROW)
SE 5 19	24	F	BL STAGE I	CPM-ADR-VCR PRED-BLEO	7 MONTHS	AFTER RELAPSE	2nd CR OF ABDOMINAL AND BM RELAPSE
CLB NANC 20	4	M	BL STAGE IV CNS	CPM-ADR-VCR-PRED HD MTX-AS-CYT HD CYT-CNS-X RAY	4 MONTHS	PR	PR OF INITIAL CNS : PERSISTENCE OF FACIAL PALSY
DEB 7 21	9	F	BL STAGE IV CNS BM	CPM-ADR-VCR-PRED HD MTX-CYT-CNS-X RAY	5 MONTHS	CR	1st CR
CLB 83 2766 22	14	M	BL STAGE I	CPM-ADR-VCR-PRED HD MTX-CNS-X RAY	7 MONTHS	AFTER RELAPSE	2nd CR OF CNS RELAPSE

Note: *BL, Burkitt's lymphoma; BM, bone marrow; VCR, vincristine; CPM, cyclophosphamide; ADR, Adriamycin; MTX, methotrexate; IT, intrathecally; ASP, asparaginase; PRED, prednisone; CNS, central nervous system; CR, complete remission; CSF, cerebrospinal fluid; ABMT, autologous bone marrow transplantation; PR, partial remission; HD, high dose; CYT, Cytoxan; TG, 6-thioguanine; HDM, high-dose melphalan; HDMTX, high-dose methotrexate; ITMTX, intrathecally administered methotrexate; BLEO, bleomycin.*

Table 2. Massive Chemotherapy Regimens



aspiration. No abnormal cells could be detected. In 17 cases, bone marrow was also studied by means of a liquid culture monitoring system (see the chapter "Detection of Burkitt's Cells in Remission Marrow by a Cell Culture Monitoring System"), and again, no tumor cells could be detected. Since 1983, bone marrow was found to be free of Burkitt's cells cytologically and in culture in eight patients, but the risk of bone marrow contamination remained. Bone marrow of these patients was harvested and purged by monoclonal antibodies (9) or Asta Z (ifosfamide) (4) as part of a phase I, II study (patients 12, 15, 16, 18, and 19-21).

RESULTS

Bone marrow recovery after ABMT and the clinical status of each patient as of January 1984 have been summarized in Table 3 and Figs 1 and 2. The results follow. Five of 12 patients who underwent transplantation while in relapse, three of four patients who underwent transplantation in PR, and three of six patients who underwent transplantation in CR are alive with no evidence of disease at a median observation time of 530 days after ABMT (Fig 1). As shown in Fig 2, 16 patients were engrafted either while in relapse or in PR. Eight of 16 are alive with no evidence of disease. The results in the patients who suffered relapse but responded to chemotherapy are clearly better than those who relapsed and did not respond, as previously shown in a larger group of lymphoma patients (9).

The patients treated with purged marrow are obviously a group of selected patients with poor prognoses compared with the whole group. Survival of this group (37.5%) is worse than the group who received nonpurged bone marrow (57%). However, three findings are especially noteworthy. First, the recovery after ABMT is not statistically different for patients treated with purged or nonpurged marrow (Table 3). Second, in the group treated with nonpurged marrow, five of six relapses were seen in the bone marrow (two patients had additional CNS relapse). Bone marrow involvement as a first sign of relapse in BL is exceptional (3%, according to Patte et al [7]), and it may well be that bone marrow relapse is due to infused bone marrow contaminated with tumor cells. However, this seems contradicted by the third finding: all four patients who received

TABLE 3

PATIENT	TYPE OF THERAPY	STAY IN STERILE UNIT(DAYS)	NUMBER OF DAYS <1000 WBC	<500PN	<200PN	≤50.000 Platelets	STATUS AFTER MASSIVE THERAPY	COMPLICATIONS E : EARLY L : LATE	STATUS ON 1/5 /84
CLB 3683 1	BACT APPELBAUM	25	13	16	14	NE	PROGRESSIVE DISEASE	--	DIED DAY 25 EXPLOSIVE RELAPSE (CSF CNS BM ABDOMEN CHEST)
CLB 3176 2	BACT APPELBAUM	45	9	16	11	11	CR	TOXIC AGRANULOCYTOSIS (DURATION 17 D) (E)INTERSTITIAL PNEUMONITIS (L)	CR 990 +
CLB 3433 3	BACT IGR	25	20	22	16	25	CR	LEUKOENCEPHALITIS(L)	CR 1170 +
CLB 2054 4	BACT APPELBAUM	24	9	11	3	3	CR	--	RELAPSE DAY 210 CNS
	BACT IGR	21	11	11	9	13	CR	--	CR 894 +
BES 1 5	BACT IGR	21	14	19	12	NE	CR	CYCLOPHOSPHAMIDE MYOCARDIOPATHY AND CYSTITIS (E)	RELAPSE DAY 35 DIED DAY 45
BES 2 6	BACT IGR	30	24	43	29	70	CR	--	CR 410 +
HCL DEB.1 7	BACT IGR	30	28	32	27	NE	CR	--	RELAPSE DAY 60 DIED DAY 83
CLB 4560 8	BACT IGR	25	18	17	13	30	CR	--	RELAPSE DAY 46 DIED DAY 67
MARS 2 9	BACT IGR	34	19	21	9	23	CR	INTERSTITIAL PNEUMONITIS (E) LEUKOENCEPHALITIS(E)	CSF RELAPSE DAY 60→CR→ ALIVE IN RELAPSE (3th) DAY 113
SE 3 10	CE60mg/kg 95	25	6	11	6	0	CR	--	CR 186 +
CLB 3571 11	BACT IGR	28	20	22	17	25	CR	CSF BLEEDING (E)	CR 260 +
CLB 278 12	BACT IGR	24	17	20	17	16	CR	--	CR 186 +
DEB. 2 13	BACT IGR	25	12	21	11	23	CR	INTERSTITIAL VIRAL PNEUMONITIS (E)	CR 163 +
MARS 1 14	BACT IGR	22	17	20	11	15	PROGRESSIVE DISEASE	--	DIED DAY 39
DEB. 3 15	BACT IGR	10	6	5	3	13	PROGRESSIVE DISEASE	--	DIED DAY 54
BES 6 16	BACT IGR	26	17	17	16	24	CR	--	RELAPSE AM CSF DAY 70 ALIVE DAY 100 PD
SE 4 17	BACT IGR	27	17	11	10	27	PROGRESSIVE DISEASE	SEPSIS (E) CYCLOPHOSPHAMIDE CYSTITIS (E)	RELAPSE D 30 ALIVE D 90 PD
DEB. 6 18	BACT IGR	22	14	19	14	23	CR	--	CR 30 +
SE 5 19	BACT IGR	15	10	13	10	NE	CR	CANDIDA SEPSIS (E)	DIED DAY 15
CLB NANC 20	BACT IGR	40	27	20	20	44	CR	--	ALIVE NED 190 +
DEB. 7 21	BEAM	30	24	21	7	39	CR	--	ALIVE NED DAY 29
CLB 2766 22	BEAM	25	20	20	14	33	CR	--	ALIVE NED DAY 40

Note: *WBC, white blood cell; PN, polynuclear cells; NE, not evaluable; CR, complete remission; CSF, cerebrospinal fluid; CNS, central nervous system; BM, bone marrow; D, day; PD, progressive disease.*

monoclonal antibody-purged bone marrow and who relapsed after ABMT did not show recurrence of disease in the bone marrow.

DISCUSSION

In our study, five of 12 patients in relapse are alive with no evidence of disease; four have had no disease for more than 8 months (a

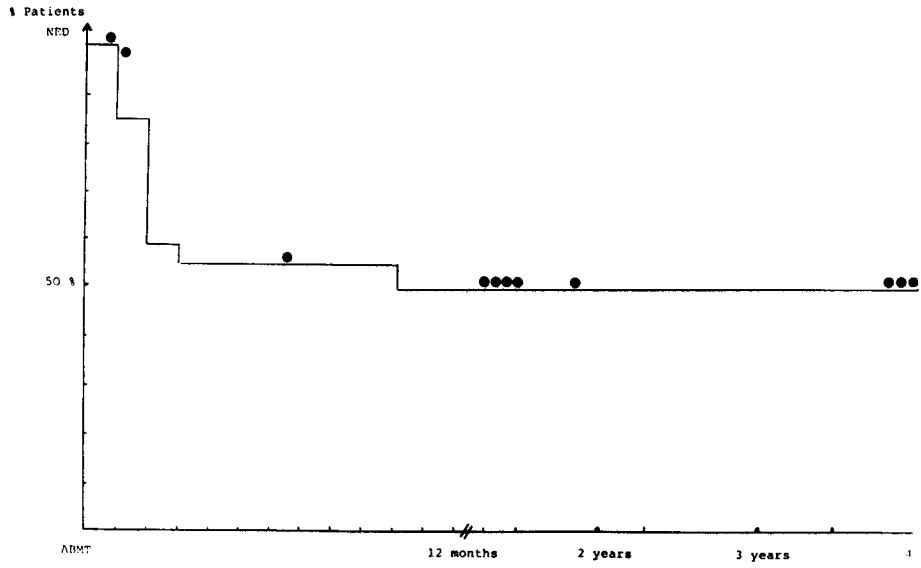


Figure 1. Autologous bone marrow transplantation (ABMT) in 22 cases of Burkitt's lymphoma (BL) with very bad prognosis. Of 6 in complete remission, 3 had no evidence of disease (NED); of 4 in partial remission, 3 had NED; of 12 in relapse, 5 had NED.

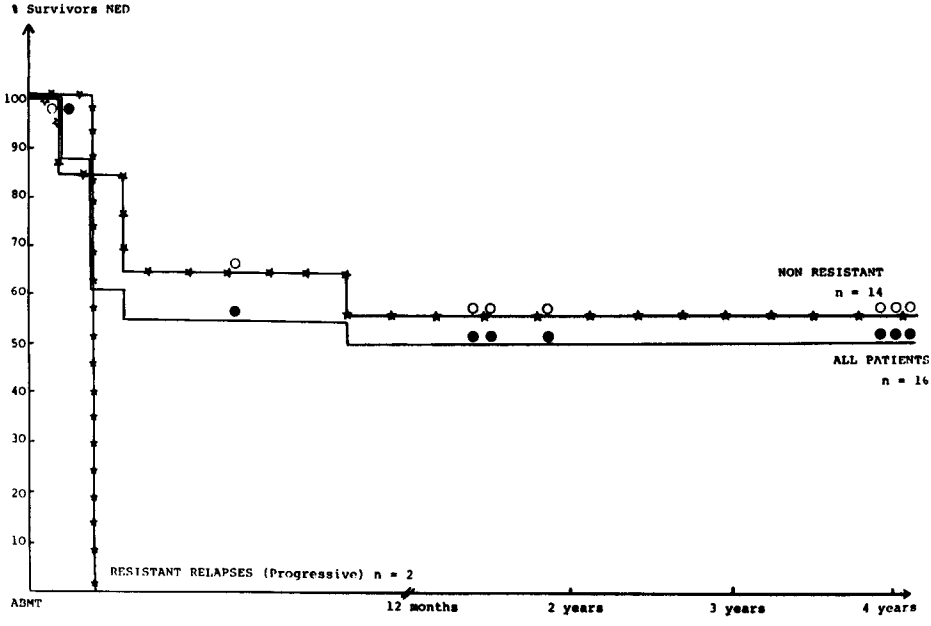


Figure 2. Autologous bone marrow transplantation (ABMT) as a rescue protocol for Burkitt's lymphoma (BL). (NED, no evidence of disease.)

probable cure for BL) (2). This result cannot be achieved with any conventional chemotherapeutic rescue regimen. These results support the hypothesis that the BACT regimens can cure BL patients after relapse. Failure to cure progressive disease in two patients confirmed the previous conclusions of our group (9).

Three of four patients who were rescued by massive therapy and ABMT when in PR after initial induction therapy are alive with no evidence of disease and are considered to be long-term survivors. These results are favorable compared with the fatal outcome of 100% of the patients in that category treated with conventional therapy. Massive therapy is indicated for this small group of patients.

Six patients received massive therapy and ABMT while in CR of initial CNS disease. This group still has a poor prognosis with conventional chemotherapeutic regimens (30% survive) (8). Despite marrow purging, three of six did experience early relapse in the CNS, indicating that chemotherapy is responsible for treatment failure in these cases.

Our three conclusions, based on this experience of 22 patients with BL and very poor prognoses treated by massive therapy and ABMT and our experience of 50 cases of non-Hodgkin's lymphoma treated during the same period by the French Conventional Cooperative protocol (3,8) follow. First, massive therapy and ABMT are clearly effective for relapses of stages I, II, and III BL and for patients not in CR after 2 months of conventional therapy. This procedure should not be used when the patient is in first CR because of the high cure rate by conventional therapy. Bone marrow could be harvested in all CR patients, but in 80% of the cases, harvesting the marrow would be useless. We prefer to harvest the bone marrow only when ABMT is clearly indicated after relapse. In this situation, bone marrow decontamination is necessary in case of CNS relapse and probably useful in all other cases (see the chapter "Detection of Burkitt's Cells in Remission Marrow by a Cell Culture Monitoring System"). Second, massive therapy and ABMT are not necessary in stage IV BL with initial bone marrow involvement prior to relapse in remission because long-term disease-free survival is achieved with conventional therapy (8). However, for stage IV disease with initial CNS or cerebrospinal fluid disease (13% of BL patients), conventional therapy is not yet satisfactory. Massive therapy and ABMT, purging procedure in vitro (see the chapter "Monoclonal Antibodies and Complement as Purging Procedure in Burkitt's Lymphoma") and new therapeutic regimens for CNS disease in vivo need to be investigated because the leads are promising. Finally, the role of purging is not clear and needs to be assessed.

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The Role of Total Body Irradiation in the Treatment of Lymphoma

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BACKGROUND AND RATIONALE

Clinical trials using autologous bone marrow transplantation (ABMT) have been most successful in malignant lymphoma (1); the sensitivity of malignant lymphoid cells to cytotoxic therapy and the variable pattern of bone marrow involvement are undoubtedly contributing factors. Although occult tumor contamination of the autologous bone marrow may ultimately lead to relapse, the high relapse rate noted with normal bone marrow transplantation (BMT) in patients with advanced hematologic malignancies indicates that the main problem is inadequate cytoreduction (2). Ablative regimens requiring BMT for hematologic malignancy frequently use marrow-lethal doses of total body irradiation (TBI), mainly because of the pioneering efforts of the Seattle group with syngeneic and allogeneic BMT in acute leukemia (3). Not only is TBI a major component of most preparative regimens, but few long-term survivors have been seen with relapsed leukemia when an ablative regimen without TBI was used (4). The rationale for the use of TBI in leukemia and lymphoma is identical (3): (*a*) experimental models indicate a steep dose-response effect of ionizing radiation; (*b*) clinical experience has proved radiation to be effective; (*c*) pharmacologic sanctuary sites are treated; (*d*) radiation therapy is non-cross-resistant with most chemotherapeutic drugs; and (*e*) TBI is a potent immunosuppressive agent.

However, major problems exist with TBI. Chiefly, the dose-limiting nonmyeloid toxicity (usually pulmonary) that often occurs is presumably cumulative and may be additive (or worse) with certain chemotherapeutic agents (5). Also, the potent immunosuppression seen with TBI is neither required nor desirable in syngeneic transplantation or ABMT.

Given these considerations, it is important to examine the role of TBI in the treatment of lymphoma with ABMT. Unfortunately, this is not simple because the term "TBI" does not denote a uniform therapy, but rather a wide variety of total doses, dosage rates, sources, and schedules (6). These are radiobiologic points (7) that are beyond the scope of this discussion. Suffice it to say that there is not unanimous agreement among radiobiologists in these areas. TBI has been used infrequently as a single agent with BMT, and the contribution of the components in various TBI regimens is uncertain. Few lymphoma patients have been treated with TBI regimens and syngeneic BMT (8), a model that excludes an effect of reinoculated occult lymphoma cells. Most trials have reported small numbers of patients that may obscure the known heterogeneity of the lymphomas, both inherent and treatment induced. While these factors preclude a clear evaluation of the role of TBI alone as a treatment of lymphoma, clinical trials may be reviewed to answer the following questions:

1. Are TBI-containing regimens and ABMT potentially curative for relapsed lymphoma? If so, is TBI a necessary component of these regimens, and which TBI regimen is the best?
2. What is the incidence of (and what factors affect) the chief nonmyeloid toxicity of TBI, interstitial pneumonitis (IP), following the use of TBI in this circumstance?
3. What recommendations can be made concerning the use of TBI in future trials?

This chapter will concentrate on the Southeastern Cancer Study Group (SECSG) experience in treating 52 relapsed lymphoma patients between 1977 and 1984 (9,10); and selected groups of these patients will be used to illustrate certain points.

CURABILITY OF RELAPSED LYMPHOMA

We initially chose to evaluate ablative therapy and ABMT in lymphoma patients who had failed primary (and often subsequent) therapy because their clinical course with conventional salvage therapy was well known. Two regimens were used, cyclophosphamide (120 mg/kg over 2 days) and either single-fraction TBI (11) or fractionated TBI (6). Study I was a phase I-II evaluation. After 13 patients received single-fraction TBI from a (single) cobalt source at 0.05-0.09 Gy/min to an (uncorrected) nominal dose of 10 Gy, 19 other patients received fractionated TBI in 2-Gy, twice-daily fractions from a linear accelerator at 0.15-0.45 Gy/min to (uncorrected) nominal doses of 12, 14, or 16 Gy. The fractionated TBI regimen of 12 Gy was relatively safe and effective and was used in study II after local radiotherapy (15-20 Gy) for patients with bulk lymphoma. (Results from radiotherapy further complicate an evaluation of TBI alone.) We have treated 32 relapsed patients in study I. As noted in Table 1, complete remission (CR) was achieved in 56%, including 47% with diffuse large cell lymphoma, 75% with diffuse undifferentiated lymphoma, 2 of 3 with miscellaneous non-Hodgkin's lymphomas, and 2 of 4 with Hodgkin's disease. Five CR patients are free of lymphoma at 3.5, 28.5, 48.5, 54, and 65.5 months. Two long-term survival patients were not in continuous CR. One was a Burkitt's lymphoma patient who died from complications of asthma after 6 years, and the other was a large cell lymphoma patient who was successfully treated for a local relapse at 12 months and who continues in a second post-BMT CR at 30 months.

The median survival for all patients was 4 months (range, <1-73 months); the median disease-free survival was 10 months (range, 2-73 months). Relapse occurred in 10 of the 18 patients in CR, an actuarial relapse rate of 67% (excluding three patients who died of toxicity while in CR). Our definition of CR included a disease-free state of at least 1 month. Relapses in our patients began immediately thereafter, peaked early, and continued to 19 months. There was only one relapse beyond 1 year. Indirect data suggest that relapses were chiefly owed to inadequate ablation of residual lymphoma (i.e., regimen failures). First, our (actuarial) relapse rate was similar to the figure reported by the Seattle group whose lymphoma patients were given cyclophosphamide plus TBI and allogeneic BMT (12). Second, nearly all relapses occurred at previous sites of lymphoma or in a leukemic pattern, as might be expected in the case of contaminated bone marrow relapses.

Table 1. Antitumor Results (Southeastern Cancer Study Group Study I)

Diagnosis	*N*	No. in Complete Remission (%)	Number Alive[a] (Continuous Remission in mo)
Non-Hodgkin's lymphoma			
Diffuse large cell lymphoma	17	8 (47)	2 (48.5, 28.5)
Diffuse undifferentiated lymphoma	8	6 (75)	1 (54)
Miscellaneous	3	2 (67)	1 (65.5)
Hodgkin's disease	4	2 (50)	1 (3.5)
Total	32	18 (56)	5 (3.5-65.5)

[a] *As of August 1, 1984.*

Table 2. Curability of Relapsed Lymphoma

Regimen	Burkitt's N	Burkitt's CR	Burkitt's CCR (yr)	Other N	Other CR	Other CCR (yr)
Chemotherapy alone						
National Cancer Institute (USA)	18	10	4 (>5)	4	-	-
France/United Kingdom	8	6	4 (3>2)[a]	22	12	3 (<1.5)
France (Paris)	-	-	-	2	1	-
The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston (USA) (4)	-	-	-	4	2	-
Total	26	16	8	32	15	3
Chemotherapy and TBI						
Southeastern Cancer Study Group (USA)	8	6	1 (4.5)	24	12	4 (3>2)[a]
Memorial Sloan-Kettering Cancer Center (USA)	-	-	-	10	10	5 (<2.5)
France/United Kingdom	-	-	-	5	5	2 (1)
France (Paris)	-	-	-	1	-	-
The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston (USA)	2	2	1 (<0.5)	-	-	-
University of California at Los Angeles (USA)	1	1	-	2	1	1 (>2)
Total	11	9	2	42	28	12

Note: *CR, complete remission; CCR, continuous complete remission.*

[a]*Three patients in CCR for more than 2 years.*

These data indicate that cyclophosphamide plus TBI and ABMT is capable of producing long-term disease-free survival (and perhaps cure) in some relapsed lymphoma patients. The contribution of TBI alone to these data is difficult to determine; however, all of our non-Hodgkin's lymphoma patients had received a cyclophosphamide regimen (at a standard dose) as primary therapy, and most were clinically resistant to it. The CR rate in these resistant patients after receiving cyclophosphamide plus TBI was similar to patients not resistant to cyclophosphamide before BMT. In a more general sense, few long-term survivors with lymphomas (other than Burkitt's lymphoma) have been reported in relapsed patients using non-TBI regimens (13; see the chapter "Massive Chemotherapy with Autologous Bone Marrow Transplantation in 50 Cases of Non-Hodgkin's Lymphoma with Poor Prognosis") (Table 2). Burkitt's lymphoma and other lymphomas were analyzed separately, because some cases of even relapsed Burkitt's lymphoma remain very sensitive to high-dose cyclophosphamide alone (14). Although it appears that TBI-containing regimens may be better, small

numbers and possible selection bias prevent concluding that TBI is necessary in this situation. Rather, the data emphasize that TBI regimens are the standard against which other regimens must be measured, at least in lymphomas other than Burkitt's.

Approaches to the problem of resistant lymphoma have been twofold. First, an augmentation of the drug component of drug plus TBI regimens has been used by several investigators (1,15), and some of these regimens are discussed elsewhere in this book. Second, the other major tactic has been the fractionation of TBI, a procedure expected to be advantageous in the treatment of lymphohematopoietic cells, which, unlike cells of normal tissues, have a limited capacity for repair (6). Larger total amounts of radiation, therefore, can be administered without an increase in normal organ toxicity. Fractionation is also practical; dosimetry and both patient and physician convenience are facilitated. After initial experience with single-fraction TBI, most centers now use fractionated regimens. Nevertheless, only the trial of Thomas et al (16) directly compared single-fraction and fractionated TBI in an allogeneic BMT series for patients with acute myelogenous leukemia in first remission. Although improved survival was noted in the fractionated TBI group, differences in relapse rate (or any other single parameter) were not significantly different between the groups. Our data are limited and not randomized; eight of our thirteen patients given cyclophosphamide plus TBI (10 Gy in single-fraction regimen) achieved CR, and one is alive. The 12-14-Gy fractionated regimens produced 10 CRs in 19 patients, four of whom are alive in continuous CR. Therefore, while fractionated TBI is associated with both theoretical and practical benefits, it has not been clearly demonstrated as a superior antitumor tactic when compared to single-fraction therapy.

INTERSTITIAL PNEUMONITIS

In allogeneic BMT series, IP is a chief cause of death (3). It is a complex lesion, presumably related to the composition of the preparative regimen, source of the graft, graft-versus-host disease, post-BMT immunosuppression, and viral infections (17). In syngeneic transplantation (and presumably ABMT), IP is less frequent and presumably more directly related to the preparative regimen (5). Two questions are posed. First, Does TBI produce a higher incidence of IP than "ablative" combination chemotherapeutic regimens without TBI? Second, Is fractionated TBI safer than single-fraction regimens? Answers will be tentative, since possible selection bias and the heterogeneity of both prior therapy and preparative regimens complicate this analysis.

An approximation of the incidence of IP with TBI and non-TBI regimens may be gauged by comparing trials that used cyclophosphamide plus TBI with those that used BACT (BCNU [carmustine], Aracytine [cytarabine], cyclosphosphamide, and 6-thioguanine) or variants (Table 3). In the experience with cyclophosphamide plus TBI, 20% of these patients developed IP compared with 7% in the chemotherapeutic trials, which suggests that TBI is associated with an increased incidence of IP. Our experience confirms this finding. A 19% incidence of symptomatic IP was noted in our trials using TBI administered by both a single fraction and the fractionated regimens, as noted below.

It is unclear whether fractionation is a safer method of administering TBI. The randomized study by Thomas et al (16) who used allogeneic BMT did not show a clear decrease in IP with fractionation. As noted in Table 4, our (nonrandomized) experience in treating lymphoma using ABMT also does not suggest a decreased incidence of IP with fractionation; 2 of 13 (15%) with a single dose and 5 of 30 (17%) with fractions were noted.

It is unlikely that another randomized trial comparing single-fraction TBI and fractionated TBI will be conducted to answer these questions. Both theoretical and practical reasons exist for using fractionated TBI, and available antitumor and toxicity data using fractionated TBI appear at least no worse than single-fraction TBI. There is, therefore, little reason to return to the older method. The maximum

Table 3. Incidence of Interstitial Pneumonitis

Series	Regimen	No.	IP (%)
Chemotherapy alone			
France (Paris)	TACC	12	0
France/United Kingdom	BACT (or variant)	42	3 (7)
France	BACT	16	3 (19)
Italy (Trieste)	BAEC	5	0
The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston	CBV	3	1 (33)
National Cancer Institute	BACT	22	0
Total		100	7 (7)
Chemotherapy and TBI			
France/United Kingdom	BACT (or CY)/TBI	8	0
Iowa/Nebraska	High-dose ara-C + CY/TBI	14	2 (14)
The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston	CY/TBI	2	0
Memorial Sloan-Kettering Cancer Center	TBI/CY	14	1 (7)
Seattle	CY/TBI	11	6 (55)
Southeastern Cancer Study Group	CY/TBI	45	7 (16)
Total		94	16 (17)

Note: *IP, interstitial pneumonitis; TACC, 6-thioguanine, aracytine (cytarabine), CCNU, and cyclophosphamide; BACT, BCNU (carmustine), aracytine (cytarabine), cyclophosphamide, and 6-thioguanine; CBV, cyclophosphamide, BCNU, and VP-16-213 (etoposide).*

amount of fractionated TBI that can be given is unknown, since dose exploration has not been systematically carried out in most studies. With our 12- and 14-Gy twice-daily fractionated TBI regimens, serious nonmyeloid toxicity was only occasional. These data contrast with data from our experience in treating four patients (two with lymphoma) given 16 Gy in 8 fractions; all died of IP at or before 60 days passed. Data from Seattle indicate that the TBI regimen of 2.25 Gy daily in 7 fractions (15.75 Gy) at a low dose rate can be given safely (2), but results with higher doses have not been favorable. Therefore, though an absolute

Table 4. Interstitial Pneumonitis (Southeastern Cancer Study Group Studies I and II)

Radiation Dose (Gy)	Fractions	No.	No. Evaluable[a]	Interstitial Pneumonitis	
				Symptomatic	Fatal
10	1	13	8	2	1
12	6	22	20	3	2
14	7	6	6	0	0
16	8	2	2	2	2
Total		43	36	7 (19%)	5 (14%)

[a]*Survival to 90+ months.*

answer cannot be given, fractionated doses of 16 Gy may be excessively toxic using either regimen.

The possibility should be noted that certain treatment-related factors may modify the incidence of IP (e.g., the addition of certain chemotherapeutic agents [5] or prior chest radiotherapy). Although it seems likely that prior chest radiotherapy would increase the risk of IP after TBI, this cannot be concluded with certainty, perhaps because few patients with histories of mediastinal radiotherapy have received TBI. It is clear that some patients who had prior chest radiotherapy successfully underwent transplantation following TBI without developing IP (8,12). However, data from our study II of local radiotherapy (15-20 Gy) immediately before cyclophosphamide plus TBI (12 Gy) to treat bulk disease reveal that three of six patients who received the additional local radiotherapy to the mediastinum developed IP compared with two of thirteen who did not. This finding suggests that patients who have received mediastinal radiotherapy (at least immediately before treatment with cyclophosphamide plus TBI) may be at increased risk to develop IP.

CONCLUSIONS AND RECOMMENDATIONS

We may summarize these trials in four points. First, the lack of single-agent data and the variability of TBI use preclude a precise evaluation of the role of TBI in the treatment of lymphoma, especially in ABMT. Second, TBI-containing regimens produce long disease-free survival even in relapsed lymphoma patients and remain the "gold standard" by which subsequent therapies will be judged; however, no single TBI regimen (e.g., fractionated) is clearly superior to others. Third, TBI-containing regimens are probably not optimal for all circumstances, possibly including cases of Burkitt's lymphoma. Fourth, IP occurs in 10-20% of patients and is not clearly predictable. Patient selection and TBI fractionation may be useful.

Finally, attempts should be made to standardize the use of TBI. Uniform reporting of data and phase I-II trials, uniform patient characteristics, and a single method of administering TBI should be used for a single trial. Ultimately, randomized trials will be useful. In the end, these variables of the radiobiology of TBI must be clearly elucidated before optimal use of this modality can be realized.

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Panel Discussion: Session II

T. Philip and R. I. Fisher, Moderators

Dr. Thierry Philip: We will try to separate the discussion into two parts. In the first part we will address the questions raised by Dr. Fisher: What are the results of the conventional rescue regimen in non-Hodgkin's lymphoma? Then we will go to the results of autologous bone marrow transplantation (ABMT). We have decided that as a specialist in ABMT I will lead the discussion of the results of conventional therapy, and Dr. Fisher will do the same for ABMT. First, I wanted the specialists in conventional rescue protocols to define resistant relapse. My definition of resistant relapse is progressive disease under the best conventional rescue protocol I know. Secondly, I will ask the specialists in conventional rescue protocol to define nonresistant relapse, which for me is response to the rescue protocol for patients who did not progress before ABMT. Third, when you present your data tell us if the patient relapsed on treatment and how many patients relapsed after completing therapy. Fourth, is it possible to give the response rate, the duration of response, and the survival of patients that were included in the rescue protocols after they have received Adriamycin-containing regimens? For example, the resistant relapse in our group of 50 patients who all relapsed on therapy and received Adriamycin before ABMT had a response rate of 78.5% and a median duration of response of 100 days, and they all died. We could conclude from these data that a dose-effect relationship does exist in this group. What are the results of conventional therapy for this group by other teams? I think Dr. Cabanillas wants to open the discussion.

Dr. Fernando Cabanillas: I do not really know if I can answer all those questions, but I will go over the figures and see if I can give you an idea of what kind of results we get with a normal-dose salvage regimen for patients with recurrent lymphoma. I am not going to talk about Hodgkin's disease. Table 1 will just show the regimen that Dr. Jagannath already alluded to. It is called the MIME (methyl-GAG, ifosfamide, methotrexate, and etoposide) regimen. Of course, this regimen is used on patients who have not been previously exposed to VP-16-213 (etoposide). We wouldn't use this kind of regimen for patients who would relapse on cyclophosphamide, doxorubicin, etoposide, methotrexate, and prednisone, plus mechlorethyamine, vincristine, procarbazine, and prednisone (ProMACE-MOPP); I think that we have to make that clear from the beginning. The patients who go on this treatment have not been exposed to any of these drugs except maybe low-dose methotrexate in some instances. As can be seen in Table 2, we divided the patients into two major categories, those with aggressive and those with indolent lymphomas. The aggressive category corresponds to the intermediate- and high-grade lymphomas in the working classification. I have included the transformed lymphomas within the indolent category. That is an arbitrary decision since there is no slot for those patients in the working classification. The same table shows the results of the regimen for 208 patients that we have treated. If the patients are divided into the aggressive and the indolent groups, something interesting can be noticed, which is that the aggressive lymphomas have a higher complete remission (CR) rate than the indolent lymphomas. This is a phenomenon that we have observed before with ifosfamide-VP-16-213 combinations. The overall response rate is also a bit higher so that the aggressive lymphomas in our hands respond better to salvage treatment than the indolent lymphomas.

With regard to the question concerning resistant relapse, I don't really use that term. I think that a resistant relapse the way Dr. Philip defines it is a patient who responds to a salvage regimen but then relapses while on that salvage regimen. That does not necessarily mean that it is a resistant relapse because it is not known how the patient would respond to a third-line regimen, and so in that sense we cannot say

Table 1. MIME Salvage Regimen

Drug	Dose	Days[a]
Methyl-GAG	500 mg/m²	1, 14
Ifosfamide	1 g/m²	1-5
Methotrexate	30 mg/m²	3
Etoposide (VP-16-213)	100 mg/m²	1-3

[a]*Repeat every 21 days.*

it is resistant. I prefer to use the following classification: in the first place, we talk about patients who fail on frontline treatment; those are patients who never achieved any significant response, not even a partial remission (PR) on frontline treatment. Those patients respond poorly to MIME, with only 10% of them achieving a CR and only one-third of them achieving any kind of significant response, as can be seen in Table 3. Patients whose best response to frontline treatment is a PR also tend to do poorly on second-line salvage; only 12% achieve a CR. However, the overall response rate is not as bad; it is about the average for the whole group. Then there is another group of patients, those who relapsed while on treatment, and I think this is an important group. These are patients who responded to frontline therapy with either a CR or a PR, but then developed progressive disease while they were still receiving frontline treatment. These, I think, are the ones referred to by Dr. Philip as resistant relapses. The response rate in those patients is also somewhat low, the CR rate being 19%, although it is a little better than for the others that we have discussed so far.

Table 2. MIME Salvage Regimen:
Response Rate According to Histological Type

Cell Type	*N*	% CR	% CR+PR
Aggressive	123	32	64
DLCL (DHL)	94	33	62
Others	29	28	71
Indolent	85	12	74
Transformed	31	10	61
FCC (NPDL)	27	11	63
Others	27	15	37
Total	208	24	60

Note: *CR, complete remission; PR, partial remission; DLCL, diffuse large cell lymphoma; DHL, diffuse histiocytic lymphoma; FCC, follicular center cell; NPDL, nodular poorly differentiated lymphoma.*

Table 3. MIME Salvage Regimen
Cross-Resistance with Frontline Therapy

Type of Response to Frontline Rx	*N*	% CR	% CR+PR
Failure	39	10	33
PR	42	12	64
Relapsed on treatment	59	19	53
Relapsed >6 mo off Rx	42	40	76

Note: *CR, complete remission; PR, partial remission.*

Of course the best group is the patient population that responds to frontline treatment, usually with a CR, but then relapse after having been off frontline therapy for more than 6 months. In those patients we can achieve a 40% CR with MIME and 76% overall response rate. Figure 1 shows the disease-free survival of the complete responders to the MIME regimen compared to previous controls. The controls here consist of patients who have been treated with ifosfamide and VP-16-213 combinations. As you can see, these results are very similar to our previous experience. I do not know if this trend is going to continue to be less favorable for the MIME patients, because there are a lot of patients who are still very early in the evaluation phase. The important message here is that 50% of the complete responders have relapsed by approximately 18 months. However, there seems to be a tail, a plateau to this curve and I do not know exactly where we are going to hit that plateau with the MIME regimen. In previous regimens it was around 40% of the patients who achieved CR. However, since only approximately one-third of the patients with aggressive lymphoma achieve CR, of which approximately 60% will relapse, we are left with approximately a 15% overall cure rate at the very best. So that is still very unfavorable.

I think that we need to improve these results. What we might be able to do is induce a remission with this kind of regimen (MIME), and while the patient is in remission try to consolidate it with an ABMT using high-dose chemotherapy.

Dr. Philip: I was always disturbed, Dr. Cabanillas, by your mixture of indolent and aggressive lymphomas in the same data. I think I understand for the first time that the transformed lymphomas are in the indolent group. In your data the patients who relapse on therapy with an Adriamycin-containing regimen are comparable to our patient population treated by massive therapy and ABMT. Your CR rate is 19%; our CR rate is 56%. This is for me a clear demonstration of what we call the dose-effect relationship, which is the basis of all our trials. In the group of nonresistant relapses, if my understanding of your data is correct, your response rate with MIME is 76% whereas we reach 80% with massive therapy. The conclusion is that the response rate is comparable. However our survival is 47%, compared to less than 20% in your patient groups. It is obvious that some patients included in your data will be refused for ABMT, but it is also correct to point out that our results are based on patients with aggressive high-grade lymphomas who were heavily pretreated, a group that I think is more difficult to salvage. I still think that the results of ABMT, which are comparable in the United States, England, and France, are the best rescue results in this field at this time. Do you think your results could be reproducible in patients treated with an aggressive protocol as first-line therapy?

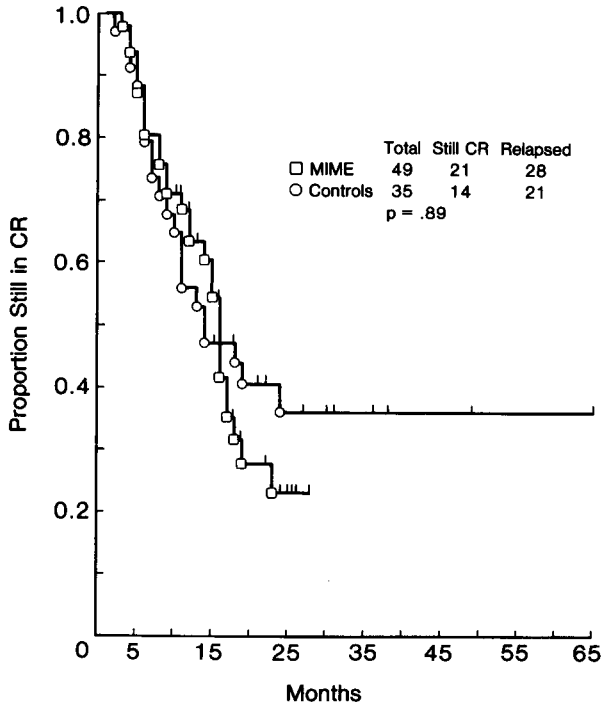

Figure 1. Relapse-free survival of complete responders to MIME salvage regimen compared to controls.

Dr. Cabanillas: I think that the answer to that last question is very clear: No. I think that patients who relapse on ProMACE-MOPP or in similar regimens have a much more unfavorable outlook because we do not have any salvage regimens that are effective for those patients. In regard to the results of ABMT being superior to the results that I presented here, I think that if you just look at numbers, yes, there is no question that the results are better; however, I don't think the question is precisely that. I think it is whether these patients are comparable to the patients that you treated with ABMT, and you already pointed out one difference, which is the fact that some of these patients have relapsed while off treatment. I don't know exactly how many of those patients are included in ABMT protocols. The other point I think is also very important has to do with the prognostic factors in these patients. In the first place, we do not exclude patients based on age, based on performance status, based on organ failure. I think those patients in the vast majority of instances are excluded from ABMT, particularly patients who are elderly, patients who present with renal failure or pulmonary failure, patients who have very poor performance status or who have bone marrow involvement. So I do not think that we are talking about similar patients.

Dr. Philip: Dr. Fisher will chair the second part of the discussion: Dr. Fisher did you have the experience of patients treated in another institution with regimens like cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) and referred to the NCI at time of relapse for treatment with ProMACE-MOPP as rescue protocol?

Dr. Richard Fisher: At the NCI we have not accepted previously treated patients, so I have no personal experience in that area. I have spent a lot of time on the phone with physicians around the country who are in that situation frequently, but I don't always hear about the final results so the data are probably not worth a lot. However I think it is

clear that after previous CHOP therapy or even in the situation where people have not received what we would call modern, optimal chemotherapy, the secondary responses to the new regimens are not nearly as good. There are few complete remissions, and there are few long-term survivors.

During the last part of the discussion, I thought that we would have enough data to call on the other participants and have them comment on whether they thought there were any routine indications for the use of ABMT. I would like to give you my viewpoint of what I have learned this morning and then ask a few questions. First I think we have a lot less data than we have in leukemia. I do believe we have some very tantalizing pieces of information. We have more data in certain areas than we do in others; for example in Burkitt's lymphoma we have a little more data. Remember that the message I wanted to give you in my initial talk is that we are not talking about one disease in non-Hodgkin's lymphoma. The histology and previous therapy were not clearly delineated in some papers. I think if there is a message from this meeting perhaps it should be that the BMT people should work closer with the chemotherapists and clearly define what groups of patients should receive ABMT. I think that all of the transplanters in this room would tell me that all transplantation is not alike. Dr. Dicke already alluded to the fact that in various centers many things are done differently. That is certainly true in chemotherapy. Also the fact that you tell me a patient is refractory in fact may or may not meet my definition. I rarely find patients with indolent lymphomas who are totally refractory to cyclophosphamide (Cytoxan). Their bone marrow may be more responsive than their tumor, but you are usually able to get transient responses. Biochemical resistance is rare, although that is not necessarily true in the diffuse aggressive lymphomas. So I think we have to work very carefully to define a homogeneous patient group. I believe that where we introduce ABMT depends on its mortality. And I would like to ask some of the speakers what is the initial mortality of ABMT in this patient population? Dr. Armitage?

Dr. James Armitage: It seems that whenever we have a discussion about lymphomas it always revolves around definitions. Before answering the question I would like to make one brief comment about another way to look at the problem. It seems with the four- and five-drug regimens we are talking about we get a 35-40% cure rate. We just saw that you can salvage with the MIME regimen approximately 10% of the failures. So it may add up to be a 50% cure rate. With the new regimens like ProMACE-MOPP we have about three-fourths CRs as opposed to one-half CRs with the four- and five-drug regimens, and about two-thirds of those CRs are durable. We end up with about the same, 50% long-term survival rate. And so the question is, can we identify the other half destined to do badly at a time early enough to treat them successfully with high-dose therapy? Our data showed that about 40% of the patients with advanced disease don't survive 2 months after an autologous transplant. About 10-20% are toxic deaths. This is similar to data for children with Burkitt's that Dr. Philip showed. When you treat end-stage patients, they die of lymphoma, or they die a toxic death, or they die of something else. It is not a very productive undertaking. If we want to improve the cure rate and cure that other half of patients that we can't cure with combination chemotherapy, we have to be able to identify them early so we can treat them when they might still survive the treatment.

Dr. Fisher: Jim, did you see the data that convinced you that any of the current high-dose cytoreductive regimens for the non-Hodgkin's lymphomas of a diffuse aggressive type are good enough that you think we are in a position to move those regimens earlier in therapy?

Dr. Armitage: There are a number of regimens that can cause complete tumor clearance in one-half or more of the patients and would be reasonable regimens to use. My prejudice is to use regimens that include radiation, but there is considerable data using chemotherapy alone to show that approach can also be effective. If we knew which patients to give them to, we would be in business.

Dr. Fisher: Do you think there are any data in the studies today that show that any of those patients are curable?

Dr. Armitage: Yes, I think that Gordon Phillip's data have sufficiently long follow-up to show that, even in end-stage disease, a small proportion of patients is cured. In diffuse histiocytic lymphoma (DHL) a remission that lasts for more than 2 years after therapy has a greater than 90% chance to translate into a cure.

Dr. George Santos: Up to now, in the 18 patients treated by us death is related to tumor recurrence. These were patients who failed at least one or possibly two or three regimens that at the time were thought to be the most effective regimens. They had DHL of the aggressive type; two had far-advanced Burkitt's lymphoma. The immediate mortality, I would have to say, was very low. The conditioning regimen consisted of 4 days of cyclophosphamide (50 mg/kg x 4 days), hyperfractionated irradiation (1.8 Gy, twice a day x 4 days; lung shielded for the third dose), and some boost treatments to sites of residual disease (1.2 Gy a day). Some patients were in PR; a number were in their second or third or fourth remission.

Dr. Anthony Goldstone: In non-Hodgkin's lymphoma in Europe the mortality across the board of ABMT procedure-related death is about 10% including a small number of cases in the group done at diagnosis. This just serves to make the point that of patients who would otherwise have responded to conventional therapy, one or two have died because of ABMT done at diagnosis. Otherwise the toxic deaths are very much spread across the groups that I mentioned. We have also gathered together 39 cases of Hodgkin's disease autografted both from European centers and from two centers here in the United States. The procedure-related mortality is very different but may have many explanations. First of all, in the Hodgkin's disease patients, about 75% of them had end-stage, refractory, resistant disease--call it what you will. And of that group, although there was a complete response rate of around 50% there was also a 50% procedure-related death. Procedure-related mortality is much higher in Hodgkin's disease patients, probably because they were done at the end-stage of the disease. And possibly many more of them had total body irradiation (TBI) in the ablative regimen (10.0 Gy). Most were of course heavily pretreated patients. When you pull out the Hodgkin's disease patients who had TBI (I have 11 in the initial analysis), it seems that 10 of them died a procedure-related death. This may be very different within the last 12 months as other cases come in. But that was certainly the case at the end of last year.

Dr. William Velasquez: People are constantly asking what group of patients benefit from ABMT? We have raised that question in our group of patients. The people who can be identified as such, with the prognosis of less than 10% that they will be alive at 3 or 5 years, are people who have high tumor burdens measured by two areas of bulky nodal disease; mediastinal and extensive abdominal involvement are two areas of extranodal disease.

Dr. Robert Gale: Dr. Gulati, how were patients evaluated for CR or PR after L-17M induction therapy and is the difference in arm I and arm II statistically significant?

Dr. Subhash Gulati: In 1981 the treatment for patients with bulky DHL was poor when CHOP or bleomycin plus CHOP combinations were used. Since then, newer combinations like ProMACE-MOPP and methotrexate plus bleomycin, doxorubicin, cyclophosphamide, vincristine, and decadron (M-BACOD) have produced significant improvement in survival. If we had to design a protocol now, we would consider these as potential models for patients with DHL. We began a pilot study in 1981 to treat such patients with poor prognosis to evaluate two questions: (1) Is L-17M (a protocol very useful for adult acute lymphocytic leukemia [ALL]) a good therapy for DHL? (2) What is the role of autologous stem cell transplantation (ASCT)

if done as part of frontline therapy as intensification? Initially, the above study was planned to be a randomized study, but patients refused to be randomized. In our previous publications, including ASCO abstracts of 1984 and the paper for this conference, we have clearly stated that patients refused randomization and that this is therefore a nonrandomized study.

Patients who agreed to have ASCT right after L-17M induction (arm II) often did not have a complete disappearance of bulky disease by x-ray examination; such patients were termed partial responders even though most of these patients are probably in CR. The patients who did not want ASCT unless they relapsed (arm I) were followed on the L-17M consolidation and maintenance arm. These patients only received ASCT if they showed clear-cut signs of progression. Usually these patients on arm I had longer time of observation before they received ASCT, and with longer follow-up when compared to arm II these patients had a higher tendency to show transient CR before they developed progressive disease. Considering the observation of CR or PR after L-17M induction had a high variability over time, the small patient number, and the dependence on x-ray evaluation, the differences of CR and PR between arm I and arm II are not statistically significant.

Our preliminary data suggest that L-17M induction if followed by ASCT is good therapy for DHL, but we feel that the consolidation and maintenance on the L-17M protocol is probably not the right therapy for DHL. Therefore, our new protocol is designed to compare M-BACOD versus L-17M induction followed immediately with ASCT.

Dr. Fisher: If I can summarize, we have some very exciting data in the diffuse aggressive lymphomas with long-term survival in some of these patients. We need to be very cautious about putting that data in the appropriate context of exactly what the previous treatment was and what the histology was and then try to report it in that fashion so that comparisons can be made by all of us. There may be less data in the indolent lymphomas, and I personally believe that they are harder to evaluate because the natural history is much more varied. Burkitt's lymphoma again carries a well-known natural history to use in making comparisons. In Hodgkin's disease we must again select our patients carefully.

III. Clinical Studies in Solid Tumors: Part One

The Natural History and In Vitro Biology of Small Cell Lung Cancer

D. N. Carney

INTRODUCTION

Small cell lung cancer (SCLC) will account for 20-25% of the 135,000 new cases of lung cancer that will occur in the United States in 1985 (1). Unlike the other forms of primary lung cancer including squamous cell (epidermoid) carcinoma, adenocarcinoma, and large cell lung cancer (collectively referred to as the non-small cell lung cancers [NSCLC]), SCLC is highly responsive to both intensive cytotoxic chemotherapy and radiation therapy (2-4). However, although clinical responses to cytotoxic therapy can be achieved in up to 90% of all patients, only 40-50% will achieve a complete remission (CR) and less than 10% of all patients will be cured of their disease (2). In most patients response durations are short, and the median survival for all patients treated is approximately 10 months.

Although many factors play a role in the responses observed including patient performance status, extent of disease at diagnosis, and sites of metastatic disease at diagnosis (including liver and the central nervous system [CNS]) (2,4,5), it is also possible that differences in therapeutic responses depend on biological properties inherent in the tumor cells themselves, such as the expression of drug or radiation resistance and genetic background. If continued advances are to be made in the treatment of SCLC, then there is a need to study the biological properties of this tumor both in vivo and in vitro.

The ability to establish continuous cell lines of SCLC from the majority of patients with this tumor, both before chemotherapy is given and after patients have relapsed from prior intensive combination therapy, and from different metastatic sites would greatly facilitate our efforts in understanding mechanisms of drug resistance and differences in clinical responses in this patient population. The recent development and characterization of large panels of human SCLC cell lines have clearly indicated the wide degree of heterogeneity in tumors arising from SCLC patients. The biological properties of these SCLC tumors may also be of prognostic importance. In this chapter the natural history of SCLC will be discussed and the establishment, characterization, and clinical correlates of established SCLC cell lines will be outlined.

NATURAL HISTORY AND THERAPEUTIC RESPONSES IN SMALL CELL LUNG CANCER

Clinically, SCLC is characterized by a short duration of symptoms, a rapid doubling time, and most important, a propensity for early widespread dissemination. Few patients present with localized disease, and so surgical resection for "cure," which is essential if patients with NSCLC are to achieve long-term survival, is rarely a consideration in SCLC. Without treatment, the median survival time from diagnosis is approximately 3 months (2,4). During the past decade, combination chemotherapy, with or without radiation therapy, has had a major impact on the outlook of patients with SCLC. Tumor regression is observed in almost 90% of all patients treated, and some patients can now be cured of their disease (2).

The response to cytotoxic therapy and survival is influenced by a number of factors, most notably the initial performance status (ambulatory status) of the patient. Patients with disease confined to the chest and draining lymph nodes (limited-stage disease) have a significantly higher overall response rate and CR rate and longer survival than those patients with more disseminated disease (extensive-stage disease) (2,4). Other factors, including the initial response to therapy and the presence of tumor in certain metastatic sites including liver and the CNS, may independently confer a poor outlook and response (5). Whereas most investigators would agree that intensive combination chemotherapy of two or more cytotoxic drugs is appropriate therapy for SCLC patients, there is

disagreement on the role of additional chest radiation therapy in those patients who have limited-stage disease. In several studies of patients with limited-stage disease no differences were observed in the overall response and median survival time between those treated with or without chest radiation. However, in the analysis of a large number of studies (2), it appears that the overall 2-year, disease-free survival ("cure") is higher among patients receiving chest radiation (4). Prospective, randomized studies under way will clarify the role of additional chest radiation in the treatment of limited-stage SCLC.

The role of prophylactic cranial irradiation (PCI) in the management of SCLC patients has also been questioned in recent years. At diagnosis, approximately 10% of patients will have evidence of CNS metastases. However, for those patients who survive initial therapy, the probability of developing CNS metastases increases significantly such that at 2 years from diagnosis this probability rises to 80% (2). The use of PCI in SCLC patients has reduced the risk of CNS relapse (4). However, as the benefit in survival appears to be restricted to those patients who achieve a CR elsewhere (i.e., in those in whom systemic disease is controlled), present recommendation for PCI is that it should only be used in patients who achieve a CR from initial induction systemic chemotherapy (6).

Finally, the role of surgical resection of the primary tumor and the role of even more intensive chemotherapy with or without bone marrow transplantation are under evaluation in several centers (7,8). Although surgical resection appears to have a minimal role as a primary therapeutic modality, studies evaluating this procedure in association with intensive cytotoxic therapy suggest that in those few patients with "very limited" stage disease the addition of surgery may increase the overall long-term disease-free survival. However, it must be acknowledged that only a very small percentage of SCLC patients would be eligible for these surgical procedures (7). Because SCLC is responsive to a wide range of different cytotoxic agents, several centers are evaluating the role of high-dose combination therapy with bone marrow support in the treatment of this disease (8). Reports from these studies are elsewhere in this volume.

THE BIOLOGY OF SCLC

Over the past 5-10 years, major advances have been made in the in vitro growth of tumor cells from clinical specimens of SCLC (9-13). Two culture techniques have been used including the soft agarose clonogenic assay (9,10) and mass culture techniques (11-13), but only limited success has been obtained in culturing continuous cell lines using the clonogenic assay for the initial culture (10). For this reason most studies on the biological properties of SCLC have come from studies of established SCLC cell lines isolated initially in liquid culture. Although initial attempts to establish SCLC lines met with limited success such that continuous lines could be established from only 10-20% of all specimens received (11), in recent years the use of a serum-free chemically defined medium has significantly improved our ability to establish SCLC lines. Using a defined medium containing hydrocortisone, insulin transferrin, 17 B estradiol, and selenium added to RPMI 1640 medium (HITES medium) (13), continuous SCLC cell lines can now be reproducibly established from 75% of all clinical specimens containing SCLC cells. Cell lines have been established with similar success from newly diagnosed previously untreated patients and from patients who have relapsed from prior therapy. In addition, cell lines have been established with equal success from a variety of different organ sites including lung, bone marrow, lymph node aspirates and biopsies, malignant pleural effusions, and other surgically resected tumor masses (14). Because no major differences have been observed in the in vitro biological properties of SCLC lines established from treated and untreated patients, or among lines established from different organ sites, in the remainder of this chapter the biological data from all lines, irrespective of origin, will be presented collectively.

In Vitro Growth Properties and Biomarkers of SCLC

Once established as continuous cell lines, the majority of SCLC lines grow as floating aggregates of tightly to loosely packed cells, frequently demonstrating areas of central necrosis. In contrast, cell lines of other forms of lung cancer usually grow as adherent monolayer cultures. Although substrate attachment is occasionally observed in SCLC cell lines, it is uncommon; these cells usually detach and float when confluence is reached. In general, SCLC lines have a relatively prolonged doubling time (50-130+ h) and a low colony-forming efficiency in soft agarose (1-5%), and form tumors with typical SCLC morphology in athymic nude mice after a latent period of 6-12 weeks following the subcutaneous inoculation of 1 x 10^6 cells. Cytologically and histologically, these cell lines and nude mouse tumors, in most instances, have the typical characteristics of SCLC, intermediate cell type (15). Exceptions to these features exist and may be of prognostic importance (*vide infra*).

The expression of a panel of biomarkers, including *l*-dopa decarboxylase (DDC)--the key amine precursor uptake and decarboxylation (APUD) enzyme--the peptide hormone bombesin (BLI), the APUD marker neuron-specific enolase (NSE), and the BB isozyme of creatine kinase (CK-BB), has been evaluated in a large panel of both SCLC and NSCLC cell lines. In previous studies of a small number of cell lines, these had been shown to be excellent markers for SCLC (16-21). The results of these studies, indicated in Table 1, clearly demonstrate that there is significant heterogeneity in the expression of these markers by SCLC. Based on these markers SCLC cell lines can be subdivided into two major categories: classic SCLC cell lines, which express elevated levels of all four biomarkers evaluated, and variant SCLC cell lines, which do not express DDC or BLI. These variant cell lines continue to express NSE, but at levels lower than those observed in classic SCLC lines, and also express elevated levels of CK-BB. The expression of NSE and CK-BB in variant lines confirms their SCLC lineage, their APUD cell origin, and clearly distinguishes them from cell lines of other forms of lung cancer.

Classic SCLC cell lines account for 70% of all cell lines established from SCLC tumors, and variant cell lines the remaining 30%. The differences in biomarker expression cannot be explained by either differences in the origin of the tumor or differences in the treatment status of the patient at the time the biopsy was obtained for culture.

A review of both the morphology and growth properties of variant cell lines indicates that these variant cell lines can be further subdivided into two categories: biochemical variant lines, which both morphologically and in their growth properties are identical to classic SCLC lines, and morphological variant lines, which are different in their growth rates, cloning efficiencies, and morphology. These morphologic variant lines account for 50% of all variant lines, and approximately 15% of all established SCLC lines.

Morphologic variant SCLC cell lines grow as much looser aggregates of floating cells than is observed in classic cell lines. These cells have more cytoplasm and more prominent nucleoli than classic SCLC lines and form tumors that histologically more closely resemble large cell carcinoma. The growth properties of these variant lines are significantly different from classic lines. Variant lines have a much higher colony-forming efficiency (15-30%) and a faster doubling time (24 h) than classic lines and have a much shorter latent period to tumor formation when inoculated into athymic nude mice. These growth properties and morphological appearances can be recognized in culture within several days after the clinical specimen is plated in culture medium.

Thus, SCLC cell lines demonstrate considerable heterogeneity in both their growth properties in vitro and their expression of a panel of biomarkers. Because major differences are observed between variant and classic lines with respect to growth rate, suggesting that variant lines may be "more malignant" than classic SCLC lines, the in vitro radiation biological properties and oncogene expression in a panel of classic and variant lines were evaluated.

Table 1. In Vitro Properties of Small Cell Lung Cancer Cell Lines

Characteristic	SCLC Cell Lines		Non-SCLC Lines
	Classic	Variant	
Morphology	Floating (Tight)	Floating (Loose)	Adherent
Cytology	SCLC	SCLC/LC	Non-SCLC
Nude Mouse Tumorigenicity	Yes	Yes	Yes
Cloning Efficiency	1-5%	10-30%	1-20%
Doubling time	35-136 h	20-24 h	20-75 h
Biomarkers			
DDC	Elevated	Absent	Absent
Bombesin	Elevated	Absent	Absent
NSE	Elevated	Elevated	Absent
CK-BB	Elevated	Elevated	Absent
Radiobiology	Sensitive	Resistant	ND
c-*myc* Oncogene amplification (DNA)	1/12	5/6	1/5

Note: *DDC, l-dopa decarboxylase; NSE, neuron-specific enolase; CK-BB, creatine kinase BB; ND, not determined.*

Radiobiological Properties and c-*myc* Oncogene Expression

The in vitro radiobiological properties of a panel of classic and variant SCLC cell lines have been studied using standard culture techniques (22-24). Cells were irradiated over a wide dose range and reproductive integrity was assayed using a soft agarose clonogenic assay (22). Results from these studies indicated that considerable differences exist in the radiobiological properties of classic and variant cell lines. The most striking feature observed is that classic cell lines have a very small shoulder compared to variant lines. Classic cell lines were characterized by D_0's ranging from .51-1.40 Gy and a low extrapolation number (n) ranging from 1-3. In contrast, although having similar D_0's, variant cell lines had extrapolation numbers (n) ranging from 5-11, indicating a greater ability of variant cell lines to accumulate sublethal radiation damage, and thus in a relative sense an increased radiation resistance in vitro compared to classic cell lines. In addition, for a clinically relevant dose of Gy, the surviving fraction of cells was significantly higher in variant lines than in classic cell lines.

The more aggressive in vitro growth properties of variant cell lines and their increased radiation resistance would suggest that variant lines may be amplified for one or more of the known oncogenes. The expression of the c-*myc* oncogene has been evaluated in a panel of lung cancer cell lines including classic and variant SCLC lines and NSCLC cell lines (25). Using the human c-*myc* probe, minimal amplification of c-*myc* DNA (5-fold) was observed in 1/12 classic cell lines. In contrast, significant c-*myc* DNA amplification (20- to 75-fold) was observed in 5/5 variant cell lines. The c-*myc* RNA expression was also greatly increased in these variant cell lines. With a single exception, no amplification or increased c-*myc* RNA expression was observed in NSCLC cell lines. Cytogenetic studies of these cell lines also revealed major differences between classic and variant lines. Studies of variant lines revealed one or more homogeneous staining

regions (HSRs) in each line studied, but studies of 8 classic cell lines did not reveal an HSR in any cell line (25,26).

Clinical Correlates of Classic and Variant Small Cell Lines

The major biological differences observed in vitro between classic and variant cell lines, including the more aggressive growth behavior in variant lines, their loss of APUD cell biomarkers, their increased radiation resistance, and the increased c-*myc* amplification and expression in these lines, suggests that patients with this phenotype of SCLC may have a worse prognosis. While many factors influence the response rate and survival of patients with SCLC, more recent data suggest that the histologic subtype may be important (27-29). Several histologic subtypes of SCLC have long been recognized, including the oat cell or lymphocyte variety and the intermediate cell types (27). In several studies, no differences in the clinical presentation or response to therapy or survival have been observed among patients with these two subtypes. However, in addition to these two major subtypes of SCLC, approximately 6% of patients at diagnosis will have histologic features of mixed small cell/large cell morphology (28,29). Although the clinical presentation and extent of disease among patients with this "mixed" morphology is similar to those with a pure SCLC morphology, the overall response rate, complete response rate, and survival are significantly worse than those observed in patients with pure SCLC, suggesting that these tumors are associated with a more aggressive behavior in vivo. As some of the variant cell lines have been derived from patients with a mixed small cell/large cell morphology, the data presented would suggest that variant cell lines of SCLC represent the in vitro model of this phenotype of SCLC.

DISCUSSION

The ability to establish SCLC cell lines has clearly indicated the degree of heterogeneity that exists in this tumor and suggests that rather than being a single disease entity, SCLC represents a spectrum of diseases with different behavior and prognosis. Although these cell lines have been used to generate monoclonal antibodies with different specificities, ones that may have value in the diagnosis of SCLC, the "purging" of bone marrow containing SCLC cells, and the evaluation of cytotoxic agents in vitro, the biological data generated suggest that in future studies the characterization of the properties of individual patient tumors may become an important part of staging and management of SCLC.

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A Review of the Use of Very High Dose Chemotherapy in Small Cell Carcinoma of the Lung

P. G. Harper, R. L. Souhami, S. G. Spiro, D. M. Geddes, and J. S. Tobias

INTRODUCTION

Combination chemotherapy has led to high remission rates in patients with small cell carcinoma of the lung (SCCL), though cure remains elusive and long-term survival (>2 years) is seen in only 4-16% of all patients. Cyclic alternation of chemotherapy, systemic or local radiation therapy, and surgery in a highly select group of patients with SCCL has not substantially altered these figures. Experimental evidence supports the concept of a dose-response relationship in very sensitive tumors (1), and this has been confirmed by SCCL human tumor xenografts. The development of autologous bone marrow transplantation (ABMT) has led to a number of studies utilizing very high dose chemotherapy, either alone or in combination. Out of necessity, the dosages and schedules used were chosen based on the experiences of the investigator and on the availability of cryopreservation rather than based on proven dose-response relationships or pharmacologic principles. Although ABMT has been used in a majority of the studies, it is possible that in many of them hematologic recovery is independent of marrow engraftment.

In reviewing published series, the following questions should be borne in mind: (*a*) Is there evidence that a steep dose-response relationship exists for each drug employed? (*b*) What are the optimum dosage and schedule for each drug? (*c*) What are the limiting toxicities of each agent? (*d*) Is it better to use a very high dose of a single agent or two agents rather than to compromise dosages when drugs are used in multiple combinations? (*e*) Is the approach practical for the patient with SCCL, bearing in mind age, usual smoking habits, and the presence of associated cardiovascular and other respiratory disease? (*f*) When autologous bone marrow support has been used, is there evidence that it is needed?

INTENSIVE THERAPY AT THE TIME OF RELAPSE

In the difficult situation of relapse, a variety of drug combinations used against drug-resistant tumors yielded nine responses (three complete, six partial or less) among the 11 patients studied (first three studies, Table 1). This result is interesting because the majority of the tumors previously had been treated with standard doses of the same drugs.

Pico et al (1) treated 10 patients with conventional therapy. A planned bone marrow harvest and cryopreservation took place prior to the first or second course of conventional therapy. At the time of relapse in four patients, this marrow was used to support very intensive therapy. All four patients responded (including two who had complete remissions [CRs]); however, the remissions lasted only 6-27 weeks and toxicity caused one early death at 15 days. Rushing et al (2) recently reported on the use of increasing doses of 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU) of patients with relapsed and refractory disease. There were four major responses but three treatment-related deaths (Table 1). The activity of high-dose therapy in resistant tumors has led to its use in more favorable groups of patients.

INTENSIVE THERAPY AS "INITIAL" TREATMENT FOR SCCL

We completed two sequential studies in which a very high dose of cyclophosphamide was given as initial treatment. In the first study, 25 patients (22 with limited and 3 with extensive disease) received a single

Table 1. Intensive Drug Therapy on Relapse in Small Cell Carcinoma of the Lung

Author (Reference)	Patients Entered (No. SCCL)	Previous Therapy	Intensive Therapy (Administration)	ABMT	Neutropenia Median Duration	Response	Median Duration	
							Remission	Survival
Gale[a]	9 (1)	Conventional	VB 1.0 mg/kg AD 120-200 mg CY 100-200 mg/kg TBI 8 Gy (4 pt)	Yes	19 d (0.5×10^9/l)	1 CR		4 mo
Spitzer (10)	19 (8 relapsed; 1 remission)	Aggressive	CY 4.5 g/m^2 (d 1-3) BCNU 300 mg/m^2 (d 1) VP-16-213 600 mg/m^2 (d 1-4)	Yes		1 CR 6 R	4-55 wk (18.5 wk)	
Douer et al (11)	14 (1)	Conventional	CY,VB,TBI	Yes		1 CR	8 wk	17 wk
Pico et al (1)	(10 LD) 4 relapsed to date	Conventional AD, VP-16-213, cisplatin, CP, RT	BCNU 300 mg/m^2 (d 1) PRCB 200 mg/m^2 (d 1-4); L-PAM 140 mg/m^2 (d 5)	Yes; harvest after 2 courses conventional therapy	6-11 d	2 CR 2 PR	-	15 d 6 wk 16 wk 27 wk
Rushing et al (2)	2 relapsed 6 refractory	C, AD, VC, VP-16-213	BCNU 600 mg/m^2 (1 pt) BCNU 900 mg/m^2 (5 pt) BCNU 1 g/m^2 (2 pt)	Yes	Platelets <50,000 (8-331 d)	1 CR 2 EG CR 1 PR 2 Some change 2 NR		3 toxic deaths

Note: *SCCL, small cell carcinoma of the lung; ABMT, autologous bone marrow transplantation; VB, vinblastine; AD, Adriamycin (doxorubicin); CY, cyclophosphamide; TBI, total body irradiation; CR, complete response; BCNU, 1,3, bis-(2 chloroethyl)-1-nitrosourea; VP-16-213, etoposide; R, response; LD, limited disease; CP,* Corynebacterium parvum; *RT, radiotherapy; PRCB, procarbazine; L-PAM, melphalan; VC, vincristine; NR, no response.*

[a] *In this group, four of nine treated with Adriamycin died of heart failure.*

Table 2. Intensive Chemotherapy at Presentation in Small Cell Carcinoma of the Lung

Author (Reference)	Patients (N)	Intensive Therapy	ABMT	Subsequent Therapy[a]	Response		Median Duration	
					Intensive	Subsequent	Remission	Survival
Souhami et al (15)	25	CY 40 mg/kg (d 1-4) (9 pt) CY 50 mg/kg (d 1-4) (16 pt) Mesna for urothelial protection	Yes + sequential delay	DXRT to 1^{O} site (d 40) 40 Gy in 20 fractions At relapse cisplatin 60 mg/m² (d 1), VP-16-213 120 mg/m² (d 1-3)	14 CR (56%) 6 PR (24%)	At relapse 60% R	49 wk	66 wk
Souhami et al (16)	25 LD	CY 50 mg/kg (d 1-4) (18 pt) for 2 courses CY 40 mg/kg (d 1-4) (8 pt) VP-16-213 120 g/m² (d 1-3)	Yes + sequential delay	DXRT 40 Gy in 20 fractions PCI At relapse cisplatin 60 mg/m² (d 1), VP-16-213 120 mg/m² (d 1-3)	13 CR (50%) 8 PR (31%) 3 deaths		49 wk	54 wk
Spitzer et al (8)	14 LD	CY 1.5 m/m² (d 1-3) VP-16-213 200 mg/kg (d 1-3) VC 2 mg (d 1,3) AD 80 mg/m² (d 1) (8 pt)	Yes	Maintenance: 4 courses at lower doses PCI to all DXRT to 1^{O} site for all in PR and half in CR	7 CR 6 PR		(CR 42 wk) 36 wk	(CR 74 wk) 56 wk
Wolff et al (3)	13 ED	VP-16-213 400 mg/m² (d 1-3) for 2 cycles	Not needed	CY, AD, VC Standard dose x 6; CR consolidated with VP-16-213 intensification; PR DXRT to distant sites	4 CR 4 PR 2 NR 3 deaths	4 CR 4 PR		(CR 11 mo) 7.5 mo

Note: *ABMT, autologous bone marrow transplantation; CY, cyclophosphamide; DXRT, deep X-ray therapy; VP-16-213, etoposide; CR, complete remission; PR, partial remission; R, response; LD, limited disease; PCI, prophylactic cranial irradiation; VC, vincristine; AD, Adriamycin (doxorubicin); ED, extensive disease; NR, no response.*

[a] *In the first group (Souhami et al [15]), pulmonary fibrosis occurred in 79% of patients and was symptomatic in 74%.*

Table 3. Late Intensification in Small Cell Carcinoma of the Lung

Author (Reference)	Induction Therapy	Late Intensification Therapy	ABMT	Patients		Response		Median		Comment
				Induction	Intensification	Induction	Intensification	Remission	Survival	
Smith et al (5)	4 courses VP-16-213 100 mg/m² (d 1-3) AD 40 mg/m² (d 1) VC 1.4 mg/m² (d 1)	CY 7 g/m² (12 h) (Priming)	Yes and No		27 (16 LD; 11 ED)	12 CR 10 PR 5 PD	12 CR 4 CR 6 PR 1 death	45 wk 10+ wk	52 wk 36 wk	PCI ABMT not necessary
Banham et al (6)	3 to 4 courses CY, AD, VC, PRED; subsequently CY, AD, VP-16-213 with VC and MTX	CY 200 mg/kg (12 h) VP-16-213 200 mg/m²	Yes	67 36 LD 31 ED	19/36 3/31	LD 20/36 CR ED 5/31 CR		LD 9 mo	CR 1 yr	
Stewart et al (13)	3 courses CY, AD, VP-16-213 VC, MTX (8 pt) + CCNU (2 pt)	CY 60 mg/kg (d 1-2) TBI 8-10 Gy + BCNU 250-300 mg x 2 (5 pt) or CCNU 150 mg/m² (1 d) (1 pt)	Yes	28	10	LD 2 CR 1 NR ED 1 CR 6 PR	LD 2 CR 1 PR ED 2 CR 5 PR		LD 18.5 mo ED 9 mo	Deaths 1. Interstitial pneumonitis 2. Radiation liver failure 3. Progressive disease
Sculier et al (14)	3 courses CY, AD, VP-16-213 Cisplatin	AD 135 mg/m² (d 1) (CR only) CY 3 g/m² (d 2,3) escalated (7 pt) VP-16-213 240 mg/m² (d 2,3)	Yes		10	3 CR 6 PR 1 NC	3 CR 4 PR	8,8,11 mo 14+,10, 64 mo	19,8,21+ mo 14+,12+, 6+ mo	PCI all
Farha et al (7)	3 courses VC, AD + IFOS (10 pt) + CY (11 pt)	CY 1.5 g/m² (d 1-3) VP-16-213 200 mg/m² (d 1,3) VC 1.5 mg/m² (d 1,3) MTX 1.5 gm (11 pt)	Yes	21 LD		9 CR 11 R	9 CR 5 CR 6 R		CR 16 mo <CR 10 mo	PCI; primary irradiation for all in PR and one half in CR consolidation

Ihde et al (9)	CY, MTX, CCNU x 2 VC, AD, Proc x 2	DXRT to initial sites CY 60 mg/kg (d 1,2)	Yes	27 ED	8/10	3 CR 5 PR	3 CR 1 CR 2 PR 2 died	4,8,15 mo 8 mo 2, 2 mo		No benefit
Klastersky et al (12)	2 or 3 courses cisplatin, AD, VP-16-213	Cisplatin 60 mg/m² (d 1) AD 45 mg/m² (d 1) VP-16-213 120 mg/m² (d 1-3) Double dose x 1 (7 pt) Double dose x 2 (3 pt) Triple dose x 1 (3 pt)	Yes and No	36	13	5 CR 7 PR	1 died 4 CR 1 died 2 CR 3 PR 1 PD	7,11,17+ mo 104 mo 8,? mo 6,5,10 mo 3	22,13,5 mo 17+,10+ mo 8,8, mo 11.5,15 mo 7	
Spitzer et al (8)	3 courses VC, AD, IFOS (19 pt) VC, AD, CY (10 pt)	CY 4.5 g VP-16-213 600 mg VC 2 mg MTX 500-1200 mg (9 pt) AD 40-80 mg/m² (4 pt)	Yes		29	10 CR 19 PR	10 CR 11 CR 8 PR No deaths		Overall 48 wk PR 46 wk	PCI DXRT 1^0 all (50 Gy)

Note: *ABMT, autologous bone marrow transplantation; AD, Adriamycin (doxorubicin); VC, vincristine; CY, cyclophosphamide; CR, complete remission; PR, partial remission; PD, progressive disease; PCI, prophylactic cranial irradiation; PRED, prednisone; MTX, methotrexate; LD, limited disease; ED, extensive disease; CCNU, 1-(2 chloroethyl)-3-cyclohexyl-1-nitrosourea; TBI, total body irradiation; BCNU, 1,3, bis-(2 chloroethyl)-1-nitrosourea; NR, no response; NC, no change; IFOS, ifosfamide; R, response; DXRT, deep X-ray therapy.*

cycle of high-dose cyclophosphamide (160 mg/kg in 9 patients, 200 mg/kg in 16 patients) given over 4 days. To assess the need for autologous bone marrow support in this situation, we returned marrow harvested prior to the intensive chemotherapy with sequential delays. No substantial delay in the return of normal blood counts was seen; however, there was a trend suggesting that bone marrow support hastened recovery. Following hematologic recovery, radiation was given to the mediastinum and site of the primary lesion, after which no further treatment was given until relapse, when VP-16-213 and cisplatin were used. The patient response rate was 80%, and 56% of those responses were complete. The median duration of remission was 49 weeks, and the median survival was 66 weeks.

In the second study, 16 patients were treated with cyclophosphamide (200 mg/kg over 4 days) and 9 received cyclophosphamide (160 mg/kg over 4 days) plus VP-16-213 (200 mg/m^2 for 3 successive days). This intensive therapy was repeated after 1 month following hematologic recovery. Subsequently, radiotherapy was given as in the first study, and no further treatment was given until relapse, when the same relapse therapy was again used. This treatment schedule proved more toxic than the previous schedule, and there were three treatment-related deaths. The overall response rate was 92%; 50% of these responses were complete. The median disease-free intervals for both studies were identical, but the median survival rate was lower for the second study, in which hematologic toxicity prevented adequate treatment at relapse. These results are reported separately in this volume.

Wolff et al (3) twice treated 13 patients who had extensive SCCL with a dose of 1200 mg/m^2 VP-16-213 over 3 days. Ten patients responded, including four who had CRs. There were three treatment-related deaths in patients with poor performance scores. Considerable additional chemotherapy was then given, including six courses of chemotherapy at standard doses, the patients in CR having a final consolidation with high-dose VP-16-213 and the patients in partial remission (PR) having radiotherapy to all remaining sites of disease. The median survival was 7.5 months overall, and it was 11 months for complete responders (Table 2). In 14 patients, Spitzer et al (4) used intensive combination chemotherapy followed by 4 courses of maintenance therapy with the same drugs at lower doses (Table 2). Prophylactic cranial irradiation was undergone by all patients, those in PR and half those in CR having radiotherapy to the primary site. The median disease-free interval was 36 weeks, and median survival was 56 weeks overall (74 weeks for patients who had CRs).

INTENSIVE CHEMOTHERAPY AS A POLICY OF LATE INTENSIFICATION

The results of this method of treatment are summarized in Table 3. In essence, the structure of the studies is such that conventional therapy is given for a variable number of courses, and, then when the tumor is smallest, very intensive treatment is given. Theoretically, this has many advantages. In practice, however, this method of treatment is fraught with many difficulties. First, the response rate to the high-dose therapy can be assessed only in those whose tumor remains after initial treatment--a group unlikely to benefit. Second, in complete responders the value of the late intensification is only measurable in terms of survival. Unless this effect is dramatic, it can only be shown by a randomized prospective study comparing the late intensification protocol with conventional therapy. Third, in the design of the studies a decision must be made about whether effective drugs should be reserved for use only in the intensification regimen. Finally, only a proportion of patients starting the protocol will be eligible for late intensification, and these patients will be a select population. In the majority of the studies, it is only one-third of patients who have come through to the intensification phase.

The results are summarized in Table 3, and we will comment only on particular instances. Smith et al (5) reserved cyclophosphamide in a dose of 7 g/m^2 over 12 h for the high-dose phase of treatment. Of 27 patients who began intensification therapy, 12 were already in CR and no further comment on response can be made. Of 15 who were already in PR, CR was

obtained in 4 and a further shrinkage of tumor in 6. However, the median duration of unmaintained remission was only 10 weeks. Autologous bone marrow support was shown not to be essential.

In the study of Banham et al (6), of 67 patients entered into induction therapy, 22 entered intensification therapy. Cyclophosphamide was used in both phases of treatment. Of those patients in induction therapy, 19 of 36 with limited disease and 3 of 31 with extensive disease entered the consolidation phase; results were no better than those achieved with conventional therapy. The results of Farha et al (7) and Spitzer et al (8) are reported in Table 3. Ihde et al (9) showed clearly the difficulties of interpreting data from late intensification protocols. Of 27 patients with extensive disease entered in their study, only 10 were suitable for intensification therapy. Two of these refused treatment, so only 8 of 27 received the high-dose therapy. Of the five patients in PR who were entered in the study, one CR and two further responses were obtained, but the duration of these responses was short. The fact that the induction regimen used was identical to that of their previous studies allowed a direct comparison between the protocols. Ihde et al concluded that no benefit had been obtained by the process of late intensification.

SUMMARY

Very high dose chemotherapy has theoretical advantages for a sensitive tumor such as SCCL. The results reported to date have confirmed that further remission can be obtained in many patients, but as yet these have not been translated into a survival advantage.

The need for autologous bone marrow support has not been determined in the majority of schedules. It is important that no patient be put at great risk by withholding bone marrow support; thus, we have suggested that a reasonable method of determining its usefulness might be to delay the day of return of the bone marrow in a sequential fashion. In this way any patient would risk a delay in return of useful marrow function for only a few days.

Intensive chemotherapy schedules are necessarily costly in terms of days spent in the hospital on treatment, and shortening total treatment time may yet increase time spent off treatment and at home. However, patient numbers in all studies to date are relatively small, and where many changes in induction or consolidation therapy have taken place, it is difficult to interpret the studies' true usefulness.

Bone marrow metastases in SCCL are of course common. Although all the studies discussed here used the absence of gross bone marrow contamination as an entry criterion, it is suspected that tumor cells were also returned at the time of marrow reinfusion. Whether this would make a substantial difference in results has not been determined. As in other diseases, methods of "cleaning up" the bone marrow are being devised.

The series reviewed here show that very intensive chemotherapy can lead to an increased remission rate and, indeed, to more CRs. However, as yet this finding has not been reflected by improvements in survival rates, and additional studies are needed to evaluate high-dose chemotherapy's true role.

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Very High Dose Cyclophosphamide as Initial Treatment for Small Cell Lung Cancer: Intrinsic and Acquired Drug Resistance

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INTRODUCTION

The results of treatment of small cell cancer of the lung (SCCL) are still very disappointing. Among unselected patients treated with cyclic combination chemotherapy, only 10% are alive at 2 years. Recent technical advances in the management of bone marrow aplasia have made it possible to give very high doses of cytotoxic agents relatively safely. Understandably, therefore, considerable interest has been generated in using high-dose chemotherapy for patients with SCCL. However, the clinical evidence that high-dose chemotherapy might be of value against any solid tumor is meager. A dose-response relationship has been suggested for alkylating agents and melanoma (1) and, less convincingly, for etoposide (2) and other tumors (3).

Most studies of SCCL have used high-dose therapy in an attempt to consolidate a complete or partial response following conventional combination therapy (4,5). This strategy assumes that a dose increase is beneficial and that drug resistance acquired during induction therapy does not reduce the efficacy of high-dose therapy to the point where the additional tumoricidal effect is small and does not justify the risks and toxicity of treatment.

In an attempt to examine the value of high-dose therapy for SCCL we have used a single agent, cyclosphosphamide, as the initial treatment in a series of sequential studies. In the first study our intention was to evaluate response rate and disease-free interval following a single cycle of high-dose therapy and to determine if the tumors were sensitive to other agents when the patient relapsed. In the second study, encouraged by the results of the first, we administered two cycles of the drug in an attempt to produce cure. In this study we made careful measurements of tumor volume reduction, using thoracic computed tomography (CT) in order to show whether tumors still present after the first cycle of treatment would respond to a second exposure to cyclophosphamide.

PATIENTS

All patients were untreated and had histologically or cytologically proven SCCL. Staging investigations included chest x-ray, bronchoscopy, full blood count, tests of renal and hepatic function, isotope bone scan, and liver ultrasound. Patients had no marrow involvement, as judged by bone marrow aspiration and biopsy. Table 1 provides further details. Before treatment began, bone marrow was harvested (while the patient was under general anesthesia) and cryopreserved.

The plan of the two studies is shown in Fig 1. In study 1, patients received 40-50 mg/kg cyclophosphamide on each of 4 successive days (total 200 mg/kg for 16 patients, 160 mg/kg for nine). When the blood count had recovered, the patients were assessed for response by chest x-ray and bronchoscopy and then received 40 Gy of thoracic radiation in 20 daily fractions. No further treatment was given until they relapsed; then they were treated with cisplatin 60 mg/m^2 and etoposide 120 mg/m^2 x 3.

In study 2, patients received two cycles of chemotherapy. For 18 patients this consisted of cyclophosphamide alone, 200 mg/kg alone as in study 1, but eight patients were given etoposide 600 mg/m^2 by infusion in addition. Since these patients showed no differences in response rates or relapse pattern, they are included in the analysis below. Response was assessed by chest x-ray and bronchoscopy after *each* cycle of chemotherapy. Chemotherapy administered at relapse was the same as in study 1.

Table 1. Patient Characteristics

Variable	Study 1	Study 2
Number	25	26
Male/female	11/14	18/8
Age range in years (mean)	32-69 (52)	37-65 (52)
LD/ED[a]	22/3	26/0

[a] *LD, limited disease; ED, extensive disease.*

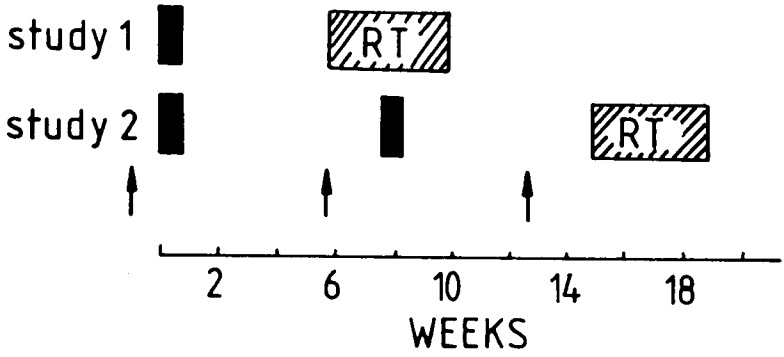

Figure 1. Plan of studies 1 and 2. Solid black blocks, *cyclophosphamide 200 mg/kg*; RT, *radiotherapy 40 Gy in 20 fractions*; arrows, *timing of CT scans.*

The cryopreserved bone marrow was returned to the patients 2 days after chemotherapy. In study 2, it was divided into two equal aliquots and half was returned after each cycle of chemotherapy.

In study 2, the tumor volume was calculated from the CT scans. The volumes were derived from the proportion of tumor that could be clearly defined on each of the three scans. A mathematical model was used to calculate the proportion of the tumor that was clinically drug resistant (6). The following parameters can be derived:

- K^* The fraction of sensitive cells killed by each treatment.
- R_0 The proportion of cells intrinsically resistant.
- R_1 The proportion of cells resistant after the first cycle.
- K_0 The percentage of cell kill on the whole tumor with the first cycle.
- K_1 The percentage of cell kill on the whole tumor with the second cycle.

RESULTS

The response rates in studies 1 and 2 are shown in Table 2. Complete response means no definite tumor seen on x-ray or bronchoscopy and a negative bronchial biopsy and cytology. The overall response rate was the same in both studies. In study 2 the second cycle of chemotherapy converted five partial responses to a complete response.

The survival rates of the two groups are shown in Fig 2. The overall survival rate was worse in study 2. Figure 3 shows that the disease-free survival rate was the same in the two studies. The reason for the inferior survival rate of study 2 lies with the survival rate after relapse, which was worse in study 2 (Fig 4). This is because the response rates to second-line chemotherapy were lower in study 2 (Table 3), due in part to

Table 2. Response Rates for Studies 1 and 2

Study	Total No.	CR No.	CR %	PR No.	PR %	NR No.	NR %	Deaths No.	Deaths %
Study 1									
All patients	25	14	56	7	28	4	16	0	
Study 2									
All patients									
Overall	26	13	50	8	31	2	8	3	11
1st cycle	26	8	30	15	57	1	4	2	8
2nd cycle	23	13	56.5	8	35	1	4.5	1	11
CPM alone									
Overall	18	10	56	4	22	2	11	2	11
1st cycle	18	7	39	8	44	1	5.5	2	11
2nd cycle	15	10	67	4	27	1	7		

Note: *For study 2, response rates are listed separately for all patients and for those receiving cyclophosphamide (CPM) alone. CR, complete response; PR, partial response; NR, no response.*

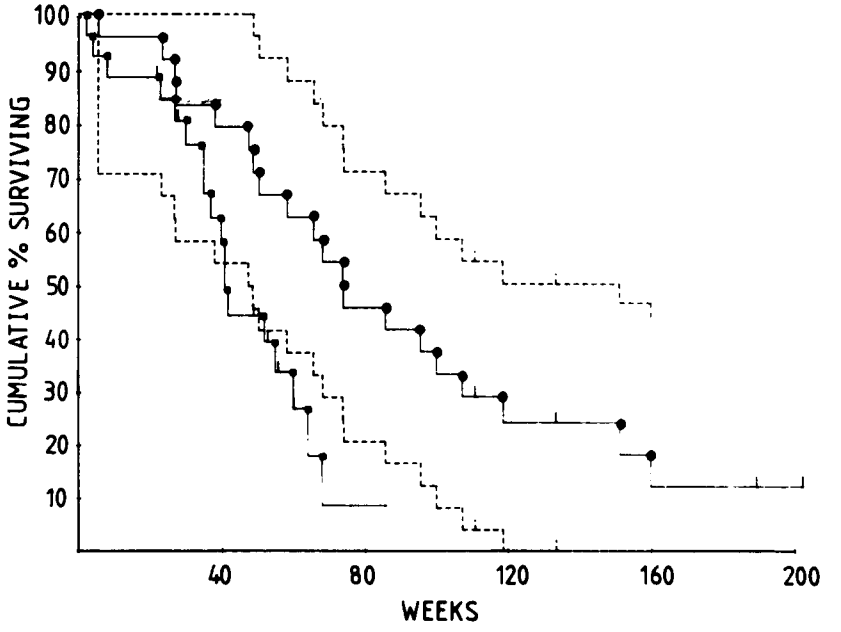

Figure 2. Survival rates in studies 1 and 2. Circles, *study 1*; squares, *study 2*; dashed lines, *95% confidence limits for study 1.*

considerable difficulty in giving further treatment because of hematologic toxicity.

Estimates of K*, R_0, R_1, K_0, and K_1 are shown in Table 4 for assumed volume-doubling times of 30 or 50 days. Mean tumor volume was reduced from 99.16 cm^3 (± 47.6) to 20.9 cm^3 (± 11.8) with the first cycle, but only to 14.2 cm^3 (± 13.1) with the second. The calculated proportion of

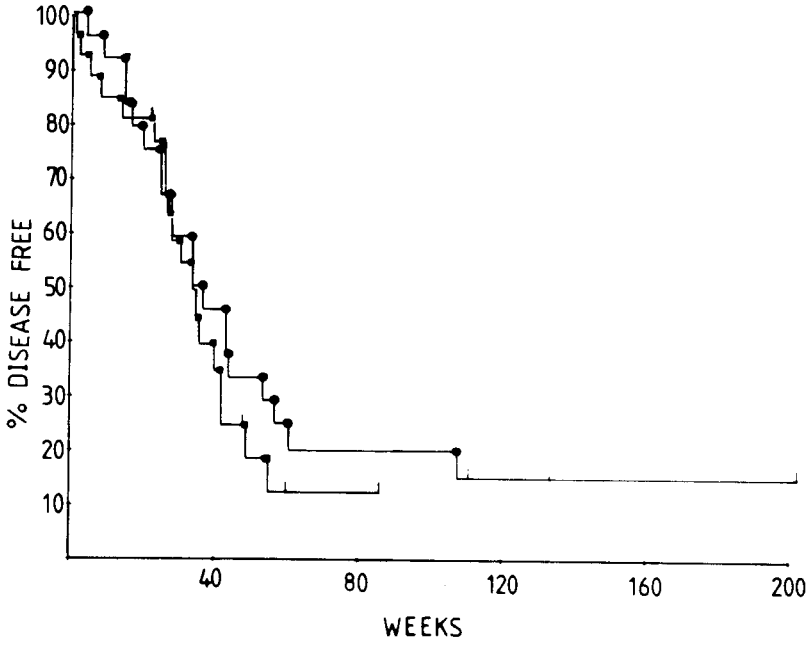

Figure 3. Disease-free survival rates in studies 1 and 2. Circles, *study 1*; squares, *study 2.*

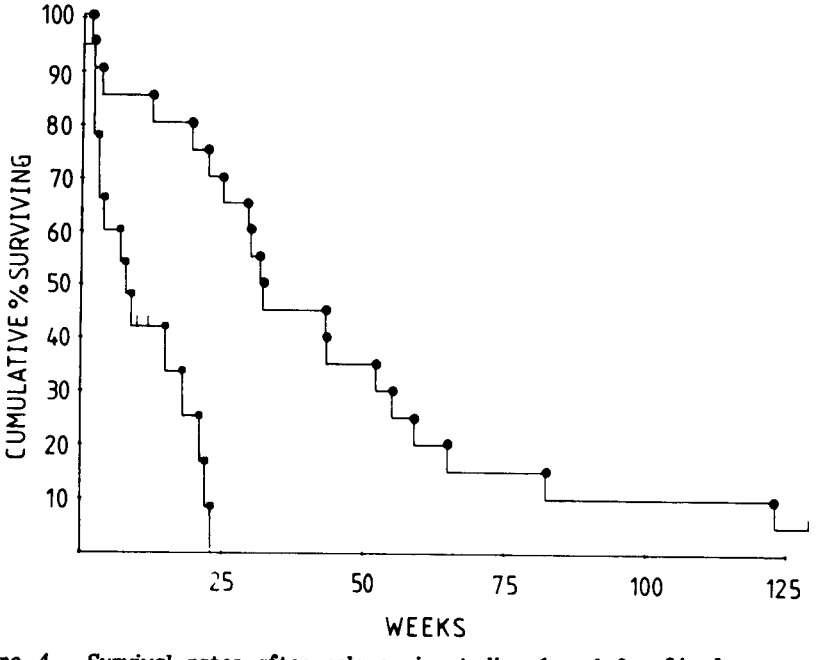

Figure 4. Survival rates after relapse in studies 1 and 2. Circles, *study 1*; squares, *study 2.*

resistant tumor increased by 10- to 16-fold after the first cycle, depending on the assumption made about volume-doubling time. These results indicate a varying but sometimes considerable degree of intrinsic

Table 3. Response to Chemotherapy after Relapse

Patients	Study 1	Study 2
Number	16	11
Complete response	4	0
Partial response	5	5
No response	7	6

resistance before treatment and a large increase in cyclophosphamide resistance after the first cycle.

DISCUSSION

These studies show that a very high response rate can be achieved with a single cycle of high-dose cyclophosphamide in patients with untreated SCCL, thus providing direct evidence that a dose-response relationship for alkylating agents exists in this disease. The addition of a second cycle of high-dose therapy did not improve the survival rate or prevent local relapse, and caused unsubstantial further tumor reduction as seen on CT scan. Only five of 15 partial responders were converted to complete responders by a second cycle of treatment.

These disappointing clinical results are reflected in the mathematical assessments of clinical drug resistance: a significant proportion of tumor is resistant before treatment and the proportion rises sharply after a single cycle. The mathematical model is based on many assumptions about tumor growth and on the nature of the abnormalities seen on the CT scan after treatment, but if the clinical and tumor volume data are taken together they indicate that a substantial degree of cyclophosphamide resistance is present very early in treatment.

Table 4. Estimates (Mean Percentage ± SD) of Drug-Resistant Portions of Tumors

Assumed volume-doubling time (days)	Drug Resistance Parameters				
	K*	R_0	R_1	K_0	K_1
30	94.6 (±4.2)	1.2 (±1.0)	17 (±11)	93.8 (±5.0)	78.3 (±11.2)
50	92.6 (±5.3)	3.3 (±3.0)	30 (±20.0)	89.6 (±7.6)	64.2 (±19.5)

Note: *K*, the fraction of sensitive cells killed by each treatment; R_0, the proportion of cells intrinsically resistant; R_1, the proportion of cells resistant after first cycle; K_0, the percentage of cell kill on the whole tumor with first cycle; K_1, the percentage of cell kill on the whole tumor with the second kill.*

If drug resistance develops to a comparable degree after conventional therapy, then late intensification strategies using the same drugs as in the induction phase are likely to fail, even if some additional responses are seen.

In studies now in progress, cyclophosphamide is administered after non-cross-resistant chemotherapy in an attempt to find out if there are grounds for believing that acquisition of clinical resistance to one drug is not accompanied by significant resistance to another. SCCL is a tumor in which it is possible to make such assessments, which will be important in planning high-dose strategies for this and other cancers.

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High-Dose Chemotherapy Intensification with Autologous Bone Marrow Support in Limited Small Cell Bronchogenic Carcinoma

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INTRODUCTION

Small cell bronchogenic carcinoma (SCBC) confined to the thorax (limited disease) has a high response rate (approximately 90%), complete remission (CR) rates of approximately 50%, and median response durations approximating 12 months, but only a limited number of long-term survivors (12% disease free at 3 years). The low number of long-term disease-free survivors very much resembles the CR statistics of patients with acute myelogenous leukemia (AML). A number of the active drugs used against SCBC have marrow toxicity as a limiting toxicity. However, in comparison with patients with AML in CR, patients with SCBC who are eligible for high-dose chemotherapy and bone marrow transplantation are usually older and often have associated cardiac and pulmonary problems. With autologous bone marrow transplantation (ABMT), there is the extra potential problem of bone marrow involvement in SCBC.

With these considerations in mind, 4 years ago we began pilot studies of intensification therapy in limited SCBC. The objectives were to identify the correct drugs to add to high-dose cyclophosphamide and 4'-demethylepipodophyllotoxin-D-ethylidene glucoside (VP-16-213), a high-dose drug combination with which we had previously had considerable experience (1, 2), and to identify the patient group most likely to benefit from such an approach.

In this chapter we will describe the outcome of 32 patients with limited SCBC who had intensive treatment with combinations of high-dose Cytoxan (CYT) and VP-16-213 with ABMT support after three or four courses of induction chemotherapy.

MATERIALS AND METHODS

Thirty-two patients with histologically proven SCBC were included in the study. There were 17 men and 15 women, ranging between 35 and 68 years of age, with a median age of 59 years.

All patients received three or four courses of induction therapy as outpatients at 3- to 4-week intervals. The patients were then placed in a protected environment where they were given two courses of intensive chemotherapy and ABMT.

Ten of the 32 patients received induction therapy with vincristine (VCR), ifosfamide (If), and Adriamycin (Ad), which was intensified two times with CYT, VP-16-213, and VCR with ABMT. The remaining 22 patients received epipodophyllotoxin, CYT, hydroxydaunorubicin, and Oncovin (ECHO) as induction therapy. This regimen was intensified with the same doses of CYT and VP-16-213. However, nine patients who received ECHO induction also received high-dose methotrexate (MTX), four also received Ad, and two patients also received both Ad and MTX. The other seven patients who received ECHO induction therapy in this study had Ad and MTX deleted from their therapy because of gastrointestinal toxicity.

For the intensified regimen, the dose of CYT was 1.5 g/m^2 for 3 consecutive days in each of the two courses of intensification therapy (a total of 4.5 g/m^2 per course). Most patients received a 200 mg/m^2 dose of VP-16-213 for 3 consecutive days (a total of 600 mg/m^2 in each intensification course), and most patients also received 1.5 mg/m^2 of VCR (maximum 2 mg/dl). In the second study in which the patients received ECHO inductions, attempts were made to increase the cytoreduction intensity of high-dose CYT and VP-16-213 by including MTX at doses of 0.5-1.5 g/m^2 with

Table 1. Survival with High-Dose Intensification

Group	Response		N	Survival		Patients Disease Free at Last Follow-Up (Months Disease Free)
	Induction	Intensification		Median*	Range	
1	CR	CR	13	23	6-47+	8, 21, 36, 39, 46, 47
2	PR	CR	9	14	6-25+	25
3	PR	PR	10	9	5-28	---
Overall			32	16	6-47+	

*Kaplan-Meier estimates.

citrovorum factor just prior to starting therapy and Ad at doses of 40-60 mg/m^2 by continuous infusion on day 4 of therapy. Bone marrow was usually collected at the end of the second and third courses of induction therapy and infused 24-48 h after the last dose of chemotherapy during each of the intensification courses. Two to three weeks following discharge from the protected environment, chest irradiation (50 Gy at 2 Gy/day over 5 weeks) and prophylactic brain irradiation (30 Gy at 3 Gy/day over 2 weeks) were commenced on an outpatient basis. A small number of patients in the ECHO-induction phase of the study received maintenance ECHO therapy at approximately a 20% reduction in intensity after completion of the radiation therapy. In the last stage of the study, maintenance chemotherapy was given for two courses after intensification but before radiation.

RESULTS

Thirteen patients achieved CR (41%), 17 achieved partial remission (PR) (53%), and two achieved less-than-partial remission (6%) at the end of induction therapy. After intensification therapy with ABMT support, an additional nine patients achieved CR. Therefore, after intensification therapy, 22 patients achieved CR (69%), and the remaining 10 patients had achieved PR, for a response rate of 100%.

Survival is summarized in Table 1. Patients are subdivided into those who achieved CR through induction therapy before intensification (group 1), those who achieved CR after intensification (group 2), and those who never achieved CR (group 3). The overall median survival time is 16 months, ranging from 6 to 47+ months. The projected median survival for those patients achieving a CR after induction therapy (group 1) is 23 months. Six of these patients remain disease free at present; four of these six have been so for 3 years or longer. On the other hand, only one of the nine patients to achieve CR with intensification therapy (group 2) is disease free. The median survival is only 9 months for those patients who never achieved CR (group 3). Two of the five patients in group 1 who have relapsed received only one course of intensification therapy because of an inadequate number of bone marrow cells, and one also did not receive chest irradiation within the first few months after intensification therapy. Another patient who died without evidence of disease had received only one course of intensification therapy because of the development of possible serum hepatitis. Therefore, of the ten patients in CR after induction therapy who received the complete protocol (two courses of intensification therapy followed by chest irradiation within 3 months), six remain free of disease, and four have relapsed. All four relapses were owed to disease found outside the chest or to disease that occurred simultaneously in other sites besides the chest.

Analysis of the cause of death in patients who achieved CR (group 1 and group 2) was evaluated. Most important, of the 11 relapses in these 22 patients, only one was owed to disease in the lung alone, and this patient received only one course of intensification therapy and no thoracic irradiation. Four relapses were owed to brain disease, and all were in patients who had already received prophylactic brain irradiation.

The neutrophil count routinely fell to 0/mm^3 and was less than 500/mm^3 for a median of 11 days. The neutropenia was no more severe in the second course of therapy than in the first. Platelets fell below 20,000/mm^3 in most of the patients (75%), but only for the short duration of 1-2 days.

Patients who received a VIA (VCR, If, Ad) induction treatment and no Ad or MTX during intensification therapy had a low frequency of fever (35%) and no documented infection. They also had only a 25% incidence of mild to moderate mucositis and no incidence of severe mucositis. However, 80% of patients who received Ad or MTX for intensification experienced fever. Twenty-six percent of these instances were documented infections. Severe mucositis occurred in 26% of courses, and this therapy was also associated with a 41% instance of skin rashes.

DISCUSSION

High-dose CYT and VP-16-213 combination therapy with ABMT can be given with acceptable toxicity to patients who have limited SCBC. This therapy is associated with increased tumor response, but the only patients likely to benefit from this study's approach were those patients who received intensified therapy while in CR (having minimal disease). Interestingly enough, the relapses were systemic, a number of them owed to disease occurring in the brain after prophylactic brain irradiation. This suggests that chemotherapy still needs to be more aggressive. Therefore, studies of high-dose chemotherapy with unmanipulated autologous bone marrow should be confined to those patients with complete response after initial induction therapy.

To ensure that this group of patients (those having minimal disease) worthy of further study was not a selected group, we examined the response durations and survival times of approximately 50 patients with limited disease who experienced CR during the last 5 years of combination therapy studies at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston. We found no differences in response duration or survival in those patients who achieved early or late CR (Figs 1 & 2). The unfavorable outcome of high-dose chemotherapy and ABMT in patients with PR at the time of intensification also has been documented by other groups (3-5).

The next question is how to further intensify the chemotherapy. Our studies show that the introduction of drugs that induce mucositis, such as Ad or MTX, appears unsuitable because they cause gastrointestinal toxicity and are associated with a higher frequency of documented infections when the intestinal mucosal barrier has been damaged. A number of studies have suggested significant clinical synergism of VP-16-213 and *cis*-diamminedichloroplatinum (cisplatin) in both untreated and relapsed SCBC (6,7). An obvious choice is to add cisplatin at maximum tolerated doses (120 mg/m^2) to CYT, VP-16-213, and VCR and possibly escalate the VP-16-213 to a higher dose. This is in fact what we propose to do. The drug doses to be used in our future studies are documented in Table 2.

In partially responding patients we intend to initiate a pilot study without ABMT with doses at 60% of those used with ABMT (Table 3). We hope from these studies to gain some insight into whether marrow infusion is necessary for hematopoietic recovery. If these patients do exceptionally well, it may become clear that contamination of the infused bone marrow was responsible for the poor clinical results in PR patients in this study.

These studies do suggest the need to investigate high-dose chemotherapy further in those patients with minimal disease. However, as a final point, Table 4 shows how difficult it would be to prove increased therapeutic efficiency of high-dose chemotherapy and ABMT in limited

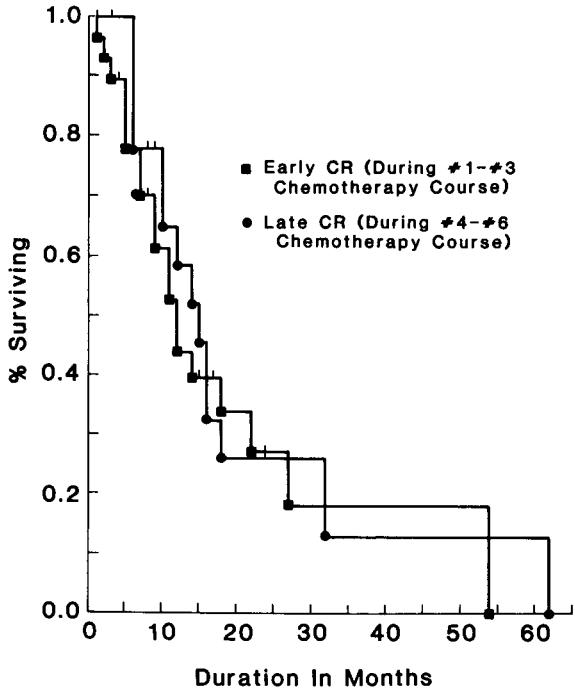

Figure 1. Survival duration of patients with limited small cell bronchogenic carcinoma. Comparison of patients who achieve complete remission early (during the first three chemotherapy courses) and those who achieve complete remission later.

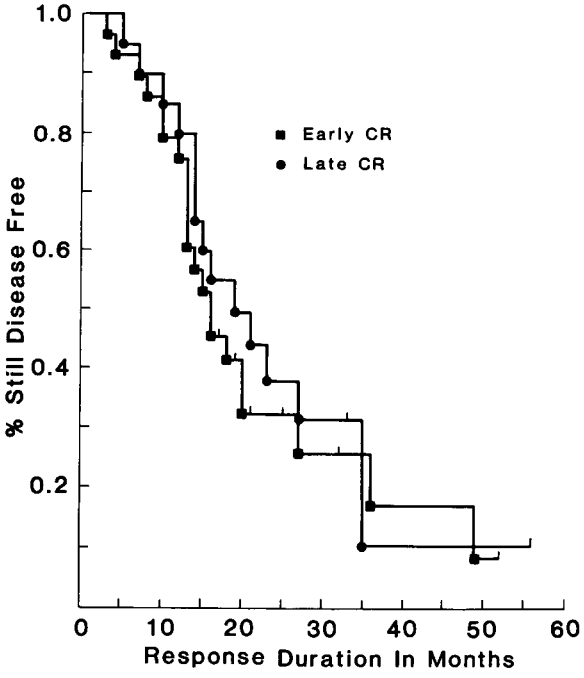

Figure 2. Response duration of patients with limited small cell bronchogenic carcinoma. Comparison of patients who achieve complete remission early (during the first three chemotherapy courses) and those who achieve complete remission later.

Table 2. Intensification Therapy

Drug	Dose	Day
Cytoxan	1.5 g/m^2	1, 2, 3
VP-16-213	250 mg/m^2	1, 2, 3
Vincristine	2.0 mg/m^2	1, 3
Cisplatin	120 mg/m^2	1

Note: Bone marrow will be infused on day 5.

Table 3. Pilot Study to Determine Myelosuppression without Autologous Marrow

Dose Level	Induction as Before			Fraction of ABMT Program
	Cytoxan (g/m^2, Days 1, 2, 3)	VP-16-213 (mg/m^2, Days 1, 2, 3)	Cisplatin (mg/m^2, Day 1)	
1	0.9	150	80	60%
2	1.2	200	100	80%
3	1.5	250	120	100%

ABMT, autologous bone marrow transplantation.

Table 4. Statistical Considerations

Survival	Historical Control	Transplant	Power*
Disease free			
Two years	25%	50%	0.88
Three years	12%	30%	0.75
Survival			
Two years	40%	60%	0.68
Three years	20%	40%	0.75

*Sixty-five patients evaluated using a two-sample, one-sided binomial test.

disease. Using historical data as a control group and a two-way statistical test for analysis, one would need 65 patients for the study if the positive outcomes shown on Table 4 were achieved. Obviously, if our pilot studies continue to be positive, we need collaboration between multiple institutions to gain the numbers needed for the definitive answer.

ACKNOWLEDGMENT

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Treatment of Small Cell Lung Cancer with Non-Cross-Resistant Induction and Intensive Consolidation Chemotherapy and Autologous Marrow Transplantation: A Randomized Study

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INTRODUCTION

The present problem in small cell lung carcinoma (SCLC) is early relapse following induction therapy, an effect probably owed to the insufficient killing of tumor cells by inadequate induction chemotherapy. The current combination chemotherapy regimens result in a median survival time of $\geq$ 7 months for patients with extensive disease (ED) and $\geq$ 14 months for patients with limited disease (LD) (1).

We initiated a study of intensive consolidation chemotherapy (IC) combined with autologous bone marrow transplantation (ABMT) for the treatment of a random selection of SCLC patients who respond to chemotherapy. The rationale behind this approach comes from recent evidence that there is a steep dose-response curve to chemotherapy in SCLC (2) and from a mathematical model that suggests that the dose-response curve is even sharper for minimal residual disease and that therapy subsequent to induction may need to be intensified (3). The objective of this study was to evaluate the effectiveness of IC and ABMT treatment on tumor progression and duration of survival.

PATIENTS AND METHODS

All of the patients entered in the study had histologically confirmed SCLC. Initially, each patient received three courses of vincristine (1.5 mg/m^2), cyclophosphamide (600 mg/m^2), doxorubicin (60 mg/m^2), and methotrexate (40 mg/m^2), which were administered at 3-week intervals. This was followed by prophylactic brain irradiation combined with two courses of cisplatin (80 mg/m^2) and etoposide (120 mg/m^2 x 3). Reevaluation consisted of repeating all tests with previously positive or suspicious results, computed tomography (CT) chest scan, bronchoscopy, isotopic liver and bone scans, CT brain scan, liver needle biopsy, and iliac bone marrow aspiration and biopsy.

Upon the second evaluation, providing there was a Karnofsky index of more than 50, no cardiac or kidney failure, and informed consent, LD patients with complete or partial response and ED patients in complete remission (CR) were randomly treated in one of two ways: intensive consolidation therapy (4) was given by administration of a total dose of 6 g/m^2 cyclophosphamide over a period of 4 days, etoposide (500 mg/m^2) over 4 days, and 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU) (300 mg/m^2) followed by ABMT or one additional cycle of the former drugs at a conventional dosage (cyclophosphamide [750 mg/m^2], etoposide [120 mg/m^2] by mouth x 5 days, and BCNU [60 mg/m^2]).

Marrow was aspirated from the posterior iliac crest after the second evaluation (i.e., after 18 weeks of treatment). Mononucleated cells were isolated with a Haemonetics 30, cryopreserved in 10% dimethyl sulfoxide (5), and stored in liquid nitrogen until the autograft was performed.

CR was defined as total disappearance of the disease for at least 3 weeks. A partial remission (PR) was defined as a 50% or greater reduction in tumor size (5). The disease-free interval was measured as the time between randomization and relapse, and survival was calculated from the onset of chemotherapy to death or the most recent follow-up examination. The actuarial method of Kaplan and Meier was used to plot the survival curves (6). Comparisons were made using the Log-Rank test (3).

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS WHO RANDOMLY RECEIVED EITHER INTENSIVE CONSOLIDATION OR CONVENTIONAL TREATMENT.

	INTENSIVE CONSOLIDATION	CONVENTIONAL TREATMENT
Number of patients	18	20
Median age (range)	51 (41-61)	54 (48-61)
Male/Female	17/1	20/0
Karnofsky index		
≥ 70 : < 70	16/2	17/3
Limited/extended	12/6	15/5

RESULTS

Induction Therapy

Of the 98 patients in this study, 83 completed the induction program. The overall response estimated by clinical, radiological, fibroscopic, and pathological assessment was 77%. Thirty-three percent of LD and 18% of ED patients achieved a CR, and 44% of LD and 60% of ED patients achieved a PR. Based on our criteria, 46% of the patients were eligible for randomization. Table 1 lists the characteristics of these patients. The two groups were matched by median age, performance status, and the extent of disease.

Toxicity of Intensive Consolidation and ABMT

Three of the six ED patients died of septicemia and acute respiratory distress syndrome before engraftment could be documented. Of the remaining patients, bacterial sepsis and fungal infections appeared despite their seclusion in an aseptic unit, as previously described (7). All of the patients required antibiotics during the period of neutropenia following transplantation. The median recovery time for granulocytes to above 500/µl was 16 days (range, 8-22 days) and for platelets to reach 50,000/µl was 19 days (range, 8-32 days). All patients experienced nausea, vomiting, and anorexia. Discrete liver dysfunction in 30% and skin rash in 20% of the patients was observed.

Response and Survival

Table 2 shows the response following IC. All seven LD patients with a PR prior to IC subsequently achieved a CR. The two ED patients evaluated as CR at the time of randomization, but defined as PR after review of the x-ray documents by two independent investigators, achieved CR after IC. The median response time for ED patients submitted to IC was 34 weeks compared with 10 weeks for controls. For the same groups, the median survival was 42 weeks and 40 weeks, respectively. Of the four LD patients who received IC in CR, two are still alive and disease free at 104 and 168 weeks after the onset of IC. Of the seven LD patients who received IC in PR who achieved CR, four are still disease free at 31, 35, 43, and 138 weeks. Figures 1 and 2 compare treatment methods, with

TABLE 2. RESULTS OF INTENSIVE CONSOLIDATION AND AUTOLOGOUS BONE MARROW TRANSPLANTATION

PATIENT	AGE	SITES OF DISEASE AT DIAGNOSIS	RESPONSE AFTER INDUCTION THERAPY	RESPONSE AFTER CONSOLIDATION	DURATION OF RESPONSE (days)*	SITES OF RELAPSE
			EXTENSIVE DISEASE			
MAT	61	Chest, pericarditis	CR	Death	-	-
DRI	56	Chest, liver	CR	CR	304	Chest
VER	47	Chest, CNS	CR	Death	-	-
PAU	44	Chest, nodes	PR+	CR	249	Chest, nodes
CAS	41	Chest, nodes	PR+	CR	111	Chest
CER	51	Chest, bone	PR+	Death	-	-
			LIMITED DISEASE			
VDB	57	Chest	CR	CR	1038+	-
BIL	48	Chest	CR	CR	532+	-
WAU	46	Chest	CR	CR	198	Liver
COP	53	Chest	CR	CR	157	Chest
BAR	46	Chest	PR	CR	538	Chest
HUT	49	Chest	PR	CR	314	Chest, marrow
VDG	59	Chest	PR	CR	199	Chest
MAR	60	Chest	PR	CR	277+	-
MAN	55	Chest	PR	CR	155+	-
MAT	58	Chest	PR**	CR	100+	-
DUR	50	Chest	PR**	CR	80+	-
FIF	51	Chest	PR	$	9+	-

* Measured after randomization (i.e. after 18 weeks of induction treatment)
** Relapsed at time of consolidation
$ Not yet evaluated

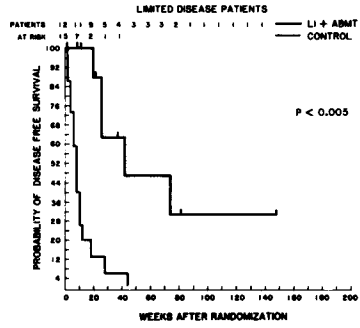

Figure 1. Actuarial disease-free interval from randomization for patients with limited disease treated with intensive consolidation or conventional chemotherapy.

respect to disease-free interval (DFI) and survival of LD patients. Preliminary results from the Log-Rank test indicate that intensive consolidation resulted in longer DFI ($P<.005$) and longer survival period ($P<.05$). Median DFI for LD patients undergoing intensive consolidation was 42 weeks in comparison with 8 weeks for LD control patients, and median survival was 104 and 62 weeks, respectively.

DISCUSSION

Our results are both encouraging and disappointing. Increased tumor kill was achieved in nine patients (two ED, seven LD) using high-dose chemotherapy, as well as prolonged DFI and survival period for the LD patients responding to IC (Figs 1 & 2). The hematological and nonhematological toxicity of the high-dose chemotherapy was acceptable. The bone marrow used for the graft was taken after 18 weeks of treatment (five cycles of chemotherapy), and produced the same hematological recovery rate as bone marrow collected prior to chemotherapy, as reported by others (8). Nevertheless, all of the ED patients who survived the IC

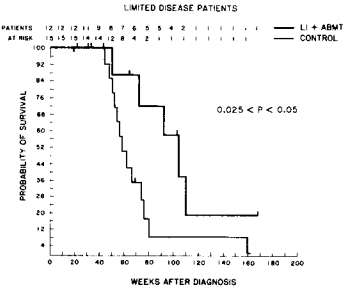

Figure 2. Actuarial survival time according to treatment type for patients with limited disease.

relapsed and eventually died. Among the 12 autografted LD patients, five have relapsed whereas two are potentially cured since they remain disease free at approximately 2 and 3 years. It is worth mentioning that these two patients were in CR before IC. It is interesting to note that in the eight patients in relapse, seven recurrences were at the primary site and only two at metastatic sites (Table 2).

It seems that high-dose chemotherapy, as practiced in our study, may lengthen the DFI and survival period for a significant number of individuals; however, it constitutes a cure in only a small number of cases. Our results are very similar to those of Spitzer et al (9). In their study, patients received three courses of induction therapy using vincristine, isophosphamide (ifosfamide), and doxorubicin (Adriamycin), and were then given two cycles of IC using cyclophosphamide, VP-16-213, and vincristine with or without methotrexate and Adriamycin. Despite the two cures after IC and ABMT, the proportion of patients with long-term disease-free survival was similar in both studies.

To improve our results, our next study will include chest radiotherapy, a shorter induction regimen (three courses), a higher degree of intensification using an alkylating agent that is not included in the induction regimen, and because of the potential danger of bone marrow contamination, in vitro treatment of the marrow with drugs or monoclonal antibodies plus complement, prior to transplantation.

ACKNOWLEDGMENT

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Treatment of Advanced Metastatic Neuroblastoma with Supralethal Chemotherapy, Total Body Irradiation, and Reconstitution with Autologous Bone Marrow

C. S. August, W. L. Elkins, E. Burkey, G. J. D'Angio, and A. E. Evans

INTRODUCTION

We have recently reported the initial results of our attempts to treat recurrent, metastatic neuroblastoma with supralethal chemotherapy and total body irradiation (TBI), followed by rescue with allogeneic or autologous bone marrow (1). The purpose of this communication is to update the previous results and to describe our experience with 10 additional patients. Thus, we will focus on 15 patients treated with autologous marrow and followed for 1-43 months. Our results, to date, suggest that this approach is capable of inducing long-term remissions in some children with end-stage neuroblastoma.

PATIENTS AND METHODS

Between December 1980 and June 1984, we treated 15 children (11 boys and 4 girls) from one and one-half to 19 years of age. Prior to undergoing our protocol, all patients had metastatic disease or recurrent local disease. The clinical characteristics of each child at the time of referral are presented in Table 1. Patients with rapidly progressing disease were excluded if an index tumor mass failed to regress after treatment with either 10 Gy (prior to February 1, 1984) or 20 Gy local radiation therapy (LRT) given in four or five daily fractions. Harvests were postponed if bilateral bone marrow aspirates and biopsies of the intended harvest sites performed 3-5 days prior to the scheduled harvest disclosed neuroblastoma. To select the most compatible donors for platelet transfusions, human leukocyte antigen (HLA) typing was performed on all patients.

Laboratory methods, harvest, cryopreservation and storage of marrow, administration of TBI, and patient management have been previously described (1). Tumor was sought in bone marrow specimens by routine cytologic and histopathologic techniques. In addition, aspirated marrow was centrifuged over Ficoll-Hypaque and restudied since this procedure tended to concentrate tumor cells. More recently, the soybean lectin method has been used to concentrate neuroblastoma cells (2). All patients were given oral vancomycin, gentamycin, and nystatin (3) and maintained in protected environments that involved conventional isolation rooms for the first 11 patients (4) and laminar air-flow rooms (Sci-Med, Minneapolis, MN) for the remaining 4.

The patients first underwent surgery or LRT or both to debulk tumors prior to starting chemotherapy. Thus, five children had surgery and 14 received LRT (10 Gy) to involved bones and to tumors measuring 5 cm or more in greatest diameter. Only patient 14 received no debulking therapy. We then attempted to treat patients uniformly with VM-26 (180 mg/m^2) and doxorubicin (45 mg/m^2) 7 and 5 days prior to marrow infusion. Melphalan was administered at a dose of 140 mg/m^2 on the sixth day and at a dose of 70 mg/m^2 on the fourth day before marrow infusion. The children then received TBI (3.33 Gy) days 3, 2, and 1 before infusing thawed, cryopreserved marrow cells. However, four children received no doxorubicin because of high doses received previously (>300 mg/m^2) or cardiotoxicity or both. One child received no VM-26 because of a previous anaphylactoid reaction to the drug and patient 25 received a one-third reduction in doses of all drugs and TBI because of his young age. No maintenance chemotherapy was given to any patient thereafter, and no child received a

Table 1. Autologous Bone Marrow Transplantation in 15 Children with Advanced Neuroblastoma

Unique Patient No.	Age/Sex	Prior Treatment	Involvement before ABMT
5	4/M	V, C, D, DOX, VM-26, CPDD	LN, R, acetabulum
7	9/M	V, C, D, CPDD, VM-26	Bones x 4
8	6/M	V, C, D, LDTBI	LN x 5 (bulky)
9	4/M	V, C, D, DOX, VM-26, CPDD	LN x 2 (bulky)
11	5/M	V, C, D, LRT, DOX	LN
12	19/M	V, C, DOX	Abdomen, bone, BM
13	4/F	V, C, D, DOX, VM-26, CPDD	Abdomen, chest wall, bones & BM
14	3/F	LRT, V, C, VM-26	Lungs
15	4/M	V, C, D	LN
16	4/F	V, C, D, DOX, VM-26	Skull, retroperitoneum, LN, BM
17	4/M	V, C, D, DOX, LRT	Bones x 3
21	5/F	CPDD, VM-26, C, DOX	Retroperitoneum, bone
23	9/M	V, C, D	Retroperitoneum, bone, LN
25	1.5/M	V, C, D, CPDD, DOX	Bones, BM, LN, abdomen, retroperitoneum
26	4/M	V, C, D, LRT	Retroperitoneum, LN

Note: *ABMT, autologous bone marrow transplantation; V, vincristine; C, cyclophosphamide; D, decarbazine (DTIC); DOX, doxorubicin; CPPD, cisplatin; LN, lymph nodes; R, right; LDTBI, low-dose total body irradiation (4 Gy); BM, bone marrow; LRT, local radiation therapy.*

second infusion of autologous marrow. All relapses were histopathologically shown at biopsy or autopsy to represent recurrent neuroblastoma.

RESULTS

All patients experienced severe mucositis, especially of the tongue and buccal mucous membranes. Anorexia frequently persisted for 1-2 months after mouth ulcers healed. Three children died in the first posttransplant month with mucositis, hemorrhagic enteritis, *Aspergillus* infection

(one), and cardiorespiratory arrests of uncertain cause (two). A fourth patient (patient 26), who had comparably severe mucositis, developed interstitial pneumonia and respiratory failure, presumably because of cytomegalovirus. Two patients developed extensive bullous skin reactions, and later, pericardial effusions. No patient developed idiopathic interstitial pneumonia and none has had severe hemorrhagic cystitis.

The hematologic recovery is summarized in Table 2. All patients who survived the first posttransplant month achieved absolute neutrophil counts of greater than 1000/mm^3 between 10 and 125 days after marrow infusion. Recovery of erythrocyte and platelet production was variable; five children had not achieved reticulocyte counts of greater than 1%, and six children had not freed themselves from their need for platelet transfusions at the time of this writing. Data from individual patients (not shown) suggest that children who received more than 1.0 x 10^8 nucleated marrow cells/kg after cryopreservation and thawing tended to recover hematopoiesis faster than those who did not.

The effects of the treatments given to each patient are summarized in Table 3. Five patients achieved complete clinical remissions that, as of August 1, 1984, have lasted for more than 43, 30, 17, 13, and 4 months. Four children achieved complete remissions (CRs) within 3 months but relapsed after 3, 3, 5, and 7 months. None of these patients survived for more than 9 months after autologous marrow transplantation. Three of the four patients who relapsed did so at sites of original disease. The fourth patient, who had extensive bone disease prior to starting treatment, relapsed with a solitary lesion in the skull. In no patient did miliary seeding of lungs, liver, spleen, or other reticuloendothelial organ occur.

Two patients experienced no perceptible changes in bulky disease in spite of double-dose LRT (20 Gy), which was given to patient 23. Two patients died within the first month and were not evaluable. Patient 26 was autografted so recently that his response was also not evaluable. Thus, of 12 evaluable patients, 9 achieved CRs with 4 being sustained beyond 1 year. The four relapses occurred 7 months or less after autografting, and the relapsed patients all died 2-4 months after their recurrences were discovered. Two clear treatment failures indicated that our protocol failed in half of the evaluable patients.

DISCUSSION

The 15 children described in this report were given lethal doses of chemotherapy and TBI in an attempt to cure otherwise fatal illnesses. At the outset, we estimated their life expectancies to be only a few months. Thus, the 13- to 43-month survival of four patients, none of whom receives any maintenance chemotherapy, is encouraging.

Using melphalan as the principal alkylating agent, in combination with TBI, was an attempt to employ a drug to which our patients' tumors had never been exposed and to avoid hemorrhagic cystitis, which had already occurred in some of our patients (1). Indeed these results, coupled with survivals of more than 62 and 44 months in two of five recipients of allogeneic marrow treated identically, suggest that this protocol is worth studying further, particularly earlier in those children whose prognoses are unfavorable at diagnosis. It has also been encouraging that neither severe hemorrhagic cystitis nor idiopathic interstitial pneumonia occurred in this group of patients.

Incomplete hematopoietic recovery is a problem in our patients. Virtually all of the patients undergoing marrow harvests were exposed to a minimum of three chemotherapeutic agents (vincristine, cyclophosphamide, and decarbazine--most commonly) and some, as many as six. Whether the number of drugs or the total cumulative doses play roles in determining the quantity or quality of the harvested, cryopreserved stem cells and whether our therapy damages the marrow's microenvironment await analysis. Our present approach is both to use available prognostic indicators to identify patients who have a more than 75% risk of dying of their disease and to harvest large volumes of marrow (20 ml/kg) as early as possible. Whenever possible, a second harvest of equal volume is carried out.

Table 2. Hematologic Recovery
in 15 Children Autografted for Advanced Neuroblastoma

Laboratory Data	Median	Range	(N)
Cell dose (nucleated marrow cells x 10^8/kg)	0.9	0.1-9.0	(15)
Time (days) until			
Absolute neutrophil count >1000/mm^3	31	10-125	(13)[a]
Reticulocyte count >1%	41	12-42	(10)[a]
Last platelet transfusion	46	10-56	(9)[a]

[a]*Number of patients fulfilling the criterion.*

Table 3. Tumor Responses in 15 Children
Autografted for Advanced Neuroblastoma

Results[a]	No. of Patients	Survival Time (mo)
Complete clinical remission sustained	5	43+, 30+, 17+, 13+, 4+
Complete clinical remission with relapse	4	9, 6, 9, 9
Partial remission	1	1+
No effect or progression	2	1, 2+
Not evaluable	3	1, 1, 1+

[a]*Results achieved within 3 months of therapy.*

Final questions that remain are whether tumor cells exist in the harvested marrow, whether these cells survive freezing and thawing, and whether they may be responsible for some relapses. The existence of a number of long-term survivors suggests that our early techniques that have been used to evaluate marrow for the presence of tumors were, for practical purposes, adequate. We have also assumed that reinfused tumor cells would induce disease whose distribution would be quite different from the original (i.e., diffusely involving lungs and the major reticuloendothelial organs). To date, this has not occurred in any of our patients, and at this time, the number of relapses is too small and our total experience is too limited to reach meaningful conclusions.

Finally, it must be emphasized that we restricted the application of autologous marrow rescue to patients whose marrows were deemed free of tumor. This excluded many children who might have benefited from the treatment. Methods of removing tumor cells from bone marrow, as described by others, may widen the applicability of this form of treatment to more children with neuroblastoma and, perhaps, provide insight into the pathologic questions raised in the preceding paragraph.

ACKNOWLEDGMENTS

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Autologous Bone Marrow Transplantation for Patients with Neuroblastoma

J. Graham-Pole

Neuroblastoma is the most common extracranial solid tumor of childhood, accounting for about 10% of pediatric cancers. The outcome for patients with neuroblastoma is clearly linked to age and clinical stage at diagnosis (1,2). Infants under 1 year old and children with disease that is at an early stage have an excellent prognosis with limited treatment. Unfortunately, at least 50% of children with neuroblastoma present with advanced disease. For them the outcome remains poor, in spite of the use of many different regimens combining chemotherapy, radiation, and surgery. Table 1 shows the past 5 years' cumulative experience of several centers treating advanced neuroblastoma. A small (10-15%) proportion of such children are reported alive and disease free at 2 years, although in the past 15 years I have known no child over 1 year old with disseminated disease to be cured.

EARLY STUDIES WITH MARROW-ABLATIVE CHEMOTHERAPY

In the past 7 years, several investigators have explored the use of autologous and allogeneic marrow infusions following bone marrow-ablative treatment of patients with disseminated neuroblastoma. In 1982, we reported our experience in London of treating 12 children with intravenous melphalan (L-PAM) in a single dose of 140 mg/m^2, followed by reinfusion of harvested noncryopreserved autologous marrow (3). These children all had advanced disease and were all in their initial clinical remission. Seven showed radiological evidence of further tumor shrinkage, and three became long-term survivors (18-35 months after completing treatment).

We chose L-PAM because of the sensitivity of neuroblastoma to other nitrogen mustards, its rapid elimination, and its apparent lack of major extramedullary toxicity. In in vitro experiments with fresh and cultured neuroblastoma cells in a clonogenic assay (4), we found a linear dose response to L-PAM up to a high drug concentration (Fig 1), which further increased with longer exposure duration.

We later reported (5,6) the results of phase I-II studies performed in Cleveland on adult and pediatric patients with various cancers. We gave L-PAM in three divided doses ranging from 120 to 225 mg/m^2 total dose and concluded that gut toxicity limited the maximal dosage to 180 mg/m^2 (5). Of 10 children with neuroblastoma refractory to other treatment, 7 had complete or partial responses of 6 months' median duration, but all patients eventually relapsed (6).

A recent study from Marseilles (7) supports the apparent benefit of incorporating such treatment early in the disease course before the patient becomes refractory. Of eight children treated in complete remission or with minimal evidence of disease, five are reported in continuing remission at from 14 to 38 months, while only three of seven with refractory disease are alive, all with active disease.

STUDIES WITH HIGH-DOSE CHEMOTHERAPY AND TOTAL BODY IRRADIATION

Based upon protocols developed from allogeneic bone marrow transplantation (BMT) and upon the known radiosensitivity of neuroblastoma, several investigators have extended the above observations to examine the combined use of L-PAM and total body irradiation (TBI). August (8) at the Children's Hospital of Philadelphia has recently published the results of treating 10 children with refractory neuroblastoma by a regimen using L-PAM (180 mg/m^2), doxorubicin, VM-26, and TBI (3.3 Gy daily x 3). Six children were "rescued" with autologous and four with allogeneic marrow

Table 1. Metastatic Neuroblastoma > 1 Year at Diagnosis: Recent Survival Data (>2 Years) with Combination Chemotherapy

Institution	No. of Patients	Survival (%)	Reporter/Year
CCSG	75	9 (12)	Finkelstein/1979
GOS, London	61	5 (8)	Ninane/1981
St. Jude	145	22 (15)	Hayes/1983
SWOG	21	2 (10)	Nitschke/1984
Dana-Farber	46	4 (9)	Rosen/1984
Overall	348	42 (12)	
Personal Experience	~100	0	1969-1984

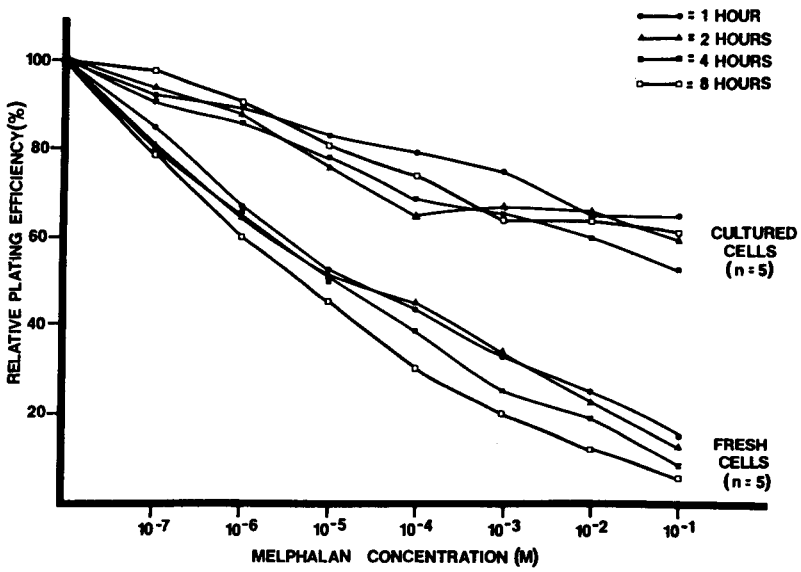

Figure 1. Comparative inhibition by melphalan of fresh and cultured neuroblastoma cell colonies.

infusions. Four of these 10 remain in remission 22-54 months later, 3 died of toxic reactions, and 3 relapsed. Two long-term survivors received autologous marrow, and two received allogeneic marrow.

Helson (9) at Memorial Hospital, New York, has also reported promising results, although with shorter follow-up. Eleven children with advanced neuroblastoma in relapse and six in remission received L-PAM (dose up to 240 mg/m^2) plus dianhydrogalactitol (240 mg/m^2) with or without TBI (4.5-6 Gy, single fraction). With a median follow-up of 12 months, there are five survivors among the relapsed patients and four among the remission patients.

The Pediatric Oncology Group (Protocol 8340, 1983) is currently comparing the effects of treating disseminated neuroblastoma either in initial clinical remission or after relapse, using either autologous or allogeneic marrow rescue. We are using L-PAM (180 mg/m^2) in three divided daily doses, followed by TBI (1.5 Gy x 6 doses), with or without local irradiation to sites of bulky disease. Our pilot studies on 10 patients, all with refractory disease, are summarized in Table 2 and show that, of

Table 2. Refractory Metastatic Neuroblastoma: Results of Treatment with L-PAM, TBI, and BMT

Patient	BMT	Disease Status	Outcome
1	Auto	Rel	Died 4 wk IP
2	Auto	Rel	CR → Rel 12 mo
3	Auto	2nd Rem	Died 8 wk *Aspergillus*
4	Auto	2nd Rem	CCR 5 mo
5	Auto	Rel	Too early
6	Auto	Rel	PR → Rel 12 mo
7	Syn	5th Rem	CCR 39 mo
8	Allo	Rel	CR → Rel 12 mo
9	Allo	Rel	CR → Rel 5 mo
10	Allo	Rel	Died 6 wk IP

Note: *CR, complete remission; CCR, complete clinical remission; PR, partial remission; Rel, relapse; IP, interstitial pneumonia.*

the nine evaluable patients, three died of early toxic reactions, six had objective responses, four relapsed, and two survived in remission 39 and 5 months later.

Finally, Fig 2 shows the overall experience of four centers in the Children's Cancer Study Group (10). They used variable regimens for 47 children with refractory neuroblastoma, having in common the inclusion of L-PAM in high dosage and BMT. They report an actuarial survival of 25% at 2 years; about 25% died early of toxic reactions, and 50% relapsed within 12 months of BMT.

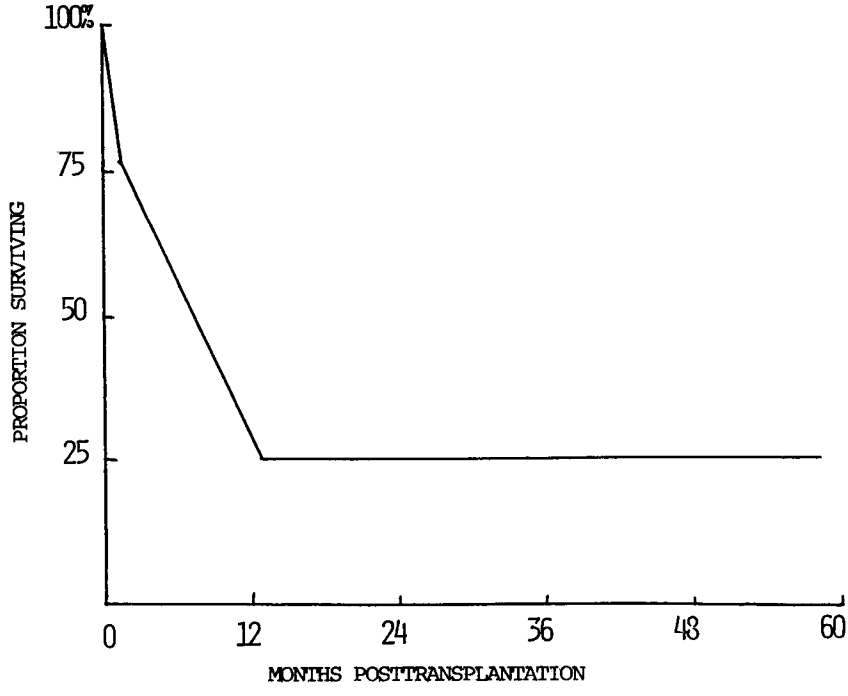

Figure 2. High-dose chemotherapy and bone marrow transplantation for refractory neuroblastoma in the Children's Cancer Study Group.

CONCLUSIONS

I have reached four major conclusions from these early results of ablative treatment and bone marrow infusions in advanced neuroblastoma: (*a*) conventional treatment is very rarely curative for children over 1 year old with advanced disease at diagnosis; (*b*) bone marrow ablation with chemotherapy with or without TBI may cure more than 30% of such children; (*c*) no advantage has yet appeared between allogeneic and autologous marrow rescue; (*d*) we should treat such children in first clinical remission and not await an initial relapse, since the disease is by this time extremely refractory.

Simone (11) recently summarized the current treatment of neuroblastoma as cause for excellent research opportunities and cautious optimism. He pointed out that the armamentarium of effective chemotherapy is considerable, and the best regimen has not yet been devised. Autologous marrow rescue remains in its infancy, and there are exciting studies of effective in vitro elimination of neoplastic cells from the harvested marrow of these patients (12). Neuroblastoma is a biologically seductive neoplasm, and we can expect both conceptual and practical help from the laboratory (11).

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Panel Discussion: Session III

R. L. Souhami and G. Spitzer, Moderators

Dr. Robert Souhami: Dr. Carney, in view of the tremendous proliferative thrust of the variant lines, one would expect that, as the disease progresses, the variant line would become ascendent. Is that true, and how do you define the variant line? Do you have to have any number of variant cells before you call it variant, or is it an all-or-none phenomenon?

Dr. Desmond Carney: Well, to answer your second question first, which is the easiest: The variant phenotype is defined when one of the amine precursor uptake decarboxylase (APUD) markers, dopa decarboxylase, is expressed. In the patient from whom variant lines are derived, one sees histological evidence of mixed small cells-large cells. Patients with this phenotype as reported by Rattice et al and Hirsch et al have a much worse prognosis than patients with pure small cell lung cancer (SCLC).

Dr. Emil Frei: So if there are any variant cells in the patient, you call the line variant?

Dr. Carney: As most variant cell lines are derived from patients with a mixed small cell-large cell morphology, we now believe that this histologic type is the in vivo correlate of variant lines.

Dr. Frei: And the classical is purely SCLC?

Dr. Carney: Yes, either lymphocyte or intermediate cell type.

Dr. Robert Gale: How about the evolution of this?

Dr. Carney: Well, as you may have already gathered, most of these data are from a retrospective evaluation of our laboratory studies. If you look at patients diagnosed with SCLC, approximately 6% will have this histologic type that we now call variant. However, following treatment and at autopsy, approximately 40% have this phenotype. This suggests that with cytotoxic therapy the variant phenotype appears. It may be missed in the initial biopsy specimen because of the small volume of tissue examined. It may be a process of evolution. In culture, however uncommon, two of our cell lines have undergone conversion with time from a classic cell to a variant cell line.

Dr. Gale: Is that selection, or is that actual clonal evolution?

Dr. Carney: I think it is clonal evolution. The relationship between c-*myc* oncogene and the variant phenotype will be studied by inserting c-*myc* into classic cell lines. This may answer the question whether insertion of c-*myc* changes classic cell lines into the variant lines.

If I could just add one other point as related to the oncogenes and maybe Dr. John Graham-Pole might want to comment on this later. I think we are all interested in the data on N-*myc* amplification in neuroblastoma and its relationship to poor prognosis and poor survival. We have now looked at N-*myc* in src cell lines. Several lines are amplified for N-*myc*. They don't fit into any certain class. As yet we don't know exactly what its relationship is to prognosis. This means in more than 50% of our SCLC lines, there is amplification of an *myc*-related sequence, which strongly suggests that the *myc*-related oncogenes have a role in the malignant behavior of SCLC.

Dr. Gary Spitzer: Have you ever attempted to do anything similar to what Dr. Thierry Philip has described in Burkitt's lymphoma, that is, to grow cell lines from histologically negative bone marrow and study the potential frequency of bone marrow contamination, particularly in limited disease?

Dr. Carney: Over the last 5 years, we have handled over 300 bone marrow samples from patients with SCLC. Two hundred fifty cell lines were unequivocally pathologically negative by all our criteria, including routine pathology and cytospin preparation. We've never grown anything out of those. In six specimens tumor cells were observed only in the cytospin preparation. We have cultured lines out of two of those. Based on our own data, the most sensitive detection method at present is the cytospin. It is fairly sensitive and early. Others have reported that a clonogenic assay may be useful for the detection of tumor cells. In our hands, in 400 assays, we have not found it useful.

Dr. Spitzer: So your feelings on limited disease are, if you don't detect tumor cells on Ficoll-Hypaque preparation from marrow, bone marrow involvement is unlikely.

Dr. Carney: In 5 years we have actually changed anything in only three patients based on the cytospin preparation results. Two of those were in the preparation for bone marrow transplants in which we found tumor cells in the cytospin preparation and the third was a patient with otherwise limited stage disease. I think it is a very rare occurrence. However, it may be just that our detection techniques are really not that sophisticated.

Dr. Spitzer: Dr. Souhami will now present more data on late intensification and Dr. Michel Symann will follow.

Dr. Souhami: I want to amplify, briefly, some of the data from the double-high-dose cyclophosphamide study. We have been using the computed tomography (CT) scan to give a measure of resistance between one cycle and the next. As Dr. Harper has pointed out, we carry out CT scans before the first cycle of cyclophosphamide, before the second cycle of cyclophosphamide, and after the second cycle. We were able, in 11 out of the 26, to get very good visualization of the tumor, without any collapse or consolidation, on all three scans. It is the data from these 11 that I am going to discuss.

The scans are not taken completely synchronously with the administration of cyclophosphamide. However, we can use these data to make a mathemathical model in which a resistant proportion of cells is assumed to be present in the tumor before the first cycle. With the first cycle, the sensitive proportion diminishes and then regrows. There is then a further cycle of treatment. We are able to compute roughly what the resistant proportion is before the first and second cycle. If we assume a volume doubling time of 30 days, the resistant proportion before the first cycle is about 1% of all the tumor. This rises to 17% before the second cycle. If you assume a volume-doubling time of 50 days, it rises from 3% to 30%. Now, of course, these are only mathematical estimates of what is actually going on, because the shadows on the CT scan are not entirely tumor.

The mean tumor volume before the first cycle of treatment was 100 cm^3 and the mean volume before the second cycle of treatment was only 20 cm^3--a four-fifths reduction. After the second cycle the mean tumor volume was essentially unchanged, being 14 cm^3. In other words there was no further reduction in tumor mass, judged on the CT scan, in spite of a further 200 mg/kg of cyclophosphamide. These data, in conjunction with the failure to improve local relapse, the failure to improve response, and the failure to improve disease-free interval (DFI), indicate that after one cycle of cyclophosphamide at high dose, there is no further advantage or possibility of benefit in giving more. Resistance to cyclophosphamide is complete, or virtually complete, after only one high-dose administration.

If one gives pulsed chemotherapy in conventional dosage and there is a reduction in tumor volume, will we also be acquiring resistance after three or four cycles of conventional-dose chemotherapy? How quickly will resistance be acquired? Obviously, the early acquisition of drug resistance both in SCLC and in lymphoma would mean that although you might see further responses with high-dose chemotherapy, after three or four cycles of treatment, such responses will be transient and not translated into clinical benefit for the patient. What will be the optimal timing for high-dose administration if there is prior chemotherapy both in this disease and in others? If you have resistance to drug A developing, so that the rate of tumor shrinkage is slowing down, how quickly is resistance to drug B developing? We need to know the answer to this question because late intensification might fail when given after three or four cycles but might not if given earlier.

In our present study we shall try to answer this question in SCLC. We give three drugs (Adriamycin, vincristine, and etoposide) on two occasions and then give a single cycle of high-dose cyclophosphamide. We measure the tumor volume to see if this initial chemotherapy has abrogated the response to cyclophosphamide even though the patients have not been previously treated with that drug.

Dr. Michel Symann: I wish to show you a few results both encouraging and disappointing concerning high-dose chemotherapy in the setting of late consolidation. In this randomized study, patients were first subjected to three cycles of debulking chemotherapy combining methotrexate (40 mg/m^2), vincristine (Oncovin) (1.5 g/m^2), cyclophosphamide (600 mg/m^2), and Adriamycin (60 mg/m^2). They then received prophylactic whole-brain irradiation and two runs of a so-called non-cross-resistant consolidation regimen with etoposide (VP-16-213) and cisplatin (platinol). After this, they were fully restaged and the patients with limited disease either in partial or complete remission and the patients with extensive disease in pathologically proven complete remission were then randomized either to late intensification plus autologous bone marrow transplantation (ABMT) or to a last run of chemotherapy at conventional dosage. We used as intensification the cyclophosphamide, 1,3-bis-(2 chloroethyl)-1-nitrosourea, and vincristine (CBV) program of Houston.

At this time 98 patients have been included in the program and out of these, 83 completed the induction program and 38 were eligible for randomization. The readers will find all appropriate figures and details in our paper included in these proceedings (see the chapter "Treatment of Small Cell Lung Cancer with Non-Cross-Resistant Induction and Intensive Consolidation Chemotherapy and Autologous Marrow Transplantation"). So far our conclusions from this ongoing study are that after induction therapy, 46% of SCLC patients were eligible for late intensification, and late intensification plus ABMT resulted in longer DFI and survival in limited-disease patients. However, we obtained only a small number of potential cures, and the high relapse rate owed to disease at the primary site indicated the need for adjuvant radiotherapy.

Obviously, I think that we need better induction and intensification regimens in this setting of late intensification. With respect to Dr. Souhami's data, I think that cyclophosphamide used alone is certainly not efficient enough. Now I have a question for Gary. You are thinking about cisplatin as a drug for intensification. I wonder about this choice because cisplatin is not heavily myelotoxic, so maybe it is not a suitable drug for the intensification regimen you may think about. Instead of platinum, wouldn't it be better to use one of the platinum analogs that are myelotoxic?

Dr. Spitzer: I think that nonnephrotoxic platinum derivates without myelosuppression would be desirable in combination chemotherapy. However, restrictions in this country make us committed to use the maximum tolerated dose of cisplatin that does not produce renal toxicity. We feel two courses of 120 mg/m^2 when patients have not had prior platinum therapy should be tolerated. At this moment I would encourage questions to any of the speakers, Dr. Souhami, Dr. Symann, Dr. Harper, and myself.

Dr. Frei: I think the demonstration that you may do very well with the first course of cyclophosphamide but that by the second course you have lost the effectiveness--that is, you have "resistance"--is an extremely important one. There is some evidence that this is also true for cells and cultures, that resistance develops very rapidly. Dr. Skipper has done in vivo studies in L1210 mice that are in accord with that. In view of that, it seems to be a rather bleak approach to use cyclophosphamide for induction therapy and then use it for intensification. Probably you have already selected out a relatively resistant population, which is going to compromise your intensification. We haven't had a discussion about cross-resistance among alkylating agents.

Dr. Spitzer: The reason we chose the drugs that people had been responding to (drugs used for their induction therapy)--although now, of course, we are introducing platinum--is that there are so few active second-line drugs in oat cell carcinoma that we did not feel we could choose another drug that residual tumor cells would have sensitivity to. Residual tumor cells would probably be sensitive to drugs that had already produced a response. But what would you choose?

Dr. Frei: Well, I think that if you wanted to use cyclophosphamide, for example, in the intensification part of it, probably you could develop an induction regimen that would include Adriamycin, vincristine, VP-16-213, or procarbazine, or what have you, and that would get you as good a complete remission rate as you can get with cyclophosphamide. That would have to be worked out, but I think there are enough active drugs for the disease. You could use melphalan, an alkylating agent that is probably as active as cyclophosphamide and is, at least in most experimental systems, not cross-resistant.

Dr. Souhami: Could I ask Dr. Frei whether, from his experience, he really believes in clinical non-cross-resistance? It seems to me that when we look at SCLC, and many other tumors, when you've got a patient who is resistant to combination chemotherapy of one type you hardly ever get worthwhile responses even to seemingly unrelated compounds. Do you think that non-cross-resistance of an exploitable type is a fact in bronchogenic carcinoma and other tumors?

Dr. Frei: I think that when you've got a very aggressive tumor like SCLC or lung cancer you've only got one or two shots of treatment anyhow. By the time you've taken your first shot the patient is further down the line. The patient's compliance is smaller in the sense of organ function and what have you. The tumor is bigger, it has become heterogeneous, and so forth. So there are lots of reasons other than the issue of cross-resistance that puts you down with those agents. But you take Hodgkin's disease, at least in some studies, the doxorubicin, bleomycin, vinblastine, and dacarbazine regimen (ABVD) does pretty well after mechlorethamine, vincristine, procarbazine, and prednisone (MOPP), and MOPP a second time around will do relatively well. So I think we're victims of the clinical evolution. The second time we treat a patient with SCLC, he is a very different patient from the first time.

Dr. Spitzer: Dr. Frei, can you comment a little more for us on what you feel the intensity and the therapy would be if we want to go past just an improved response rate and get long-term disease-free survival? Do you feel that this patient group can tolerate the levels we might have to use to achieve this end result?

Dr. Frei: Well, there are just a couple of principles and I think they are relatively old. Number one, I certainly agree with what has ascended in this conference so far, that the best time to use intensification for eradication and cure is when the patient has minimal disease, when the patient is in complete remission. I feel very strongly that if it is at all possible you ought to get the patient in complete remission with something other than what you use for the intensification. Going way back there is really no effective, definitive way that is not combination

chemotherapy. I think the use of a single agent such as cyclophosphamide with the expectation that you are going to eradicate 10^6, 10^7, or 10^8 cells even for someone with minimal disease is counter dogma. There is no reason based on a long experience in chemotherapy that you can do that, so you've got to go with combinations.

Now your best agents again in terms of resistance production and so forth are the alkylating agents, not the others. So I think we have to look harder at combinations of alkylating agents. I think we need newer, more effective agents to be sure, but right now I think that is about where we are with the tools that we have.

Dr. Spitzer: Dr. Dicke, do you want to ask some questions?

Dr. Karel Dicke: I think that the timing of intensification might be of extreme importance. For instance, I can imagine that when you give it as late intensification, you may already have relatively strong resistant clones that make it very difficult to cure the patient. I really think that an early intensification program where you have minimal disease and where you may have a chance that you have not yet developed highly resistant clones against chemotherapeutic agents is real good timing. This is in accordance with Dr. Frei's comments.

Dr. Spitzer: What would you call early and what would you call late?

Dr. Dicke: Well, early is after three courses of induction chemotherapy.

Dr. Spitzer: So you could take two approaches. You could say that most people would agree that in most diseases standard chemotherapy probably finishes its effectiveness after six courses, and it is a question of whether you would introduce intensification at the end of the effectiveness of the standard therapy or, for the reason of early induced resistance, you would do it at the end of three courses.

Dr. Symann: One practical consideration in SCLC is that usually after three cycles of induction chemotherapy, you don't document any further benefits of chemotherapy. In my study at the time we designed this regimen, we were hoping to derive some benefits from a non-cross-resistant approach. Actually, we did not observe any, and so on practical grounds I think that we should intensify after three runs of a good induction regimen.

Dr. Bob Lowenberg: One more question to Dr. Dicke. Karel, you have just told us that for oat cell carcinoma you prefer to propose a high-dose cytotoxic regimen in the early phase with minimal residual disease. But why don't you want to do that in acute myelogenous leukemia (AML) in first remission?

Dr. Dicke: The difference between oat cell and leukemia is the natural history. In oat cell, a second remission (i.e., a state of minimal disease) can hardly be achieved, which is not the case in acute leukemia.

Dr. Spitzer: Dr. August and Dr. Philip have some data on neuroblastoma.

Dr. Charles August: I would like to present some updated results from our program at the Children's Hospital of Philadelphia. We now have, in addition to the six patients that Dr. Graham-Pole described, a total series of 15 patients who received marrow and were conditioned with our protocol. All of these children had advanced stage III or stage IV disease. They were end-stage patients whose life expectancies were probably on the order of only a few months. I show simply the hematopoietic reconstruction. Of 15 patients, 13 achieved neutrophil counts greater than $1000/mm^3$, and the two that did not died of early toxic reactions. Again, the therapy here was conventional doses of Adriamycin and teniposide and

then the high doses are 210 mg/m² of melphalan followed by 10.0 Gy of fractionated total body irradiation (TBI). With respect to red cell reconstitution and freedom from platelet transfusions, the results are less good. Only about two-thirds of the patients recovered red cell production and adequate platelet production.

The clinical results are as follows: Three patients were not evaluable. Complete remission (CR) was achieved in nine and represents a 75% CR rate. One achieved a partial remission (PR) and two pretty much failed altogether. Three patients died early and there were four relapses, which occurred 3, 3, 5, and 7 months after undergoing ABMT. These patients went on to die 6 months, 3 months, 4 months, and 2 months later, respectively. A word about the three early deaths: The protocol is highly toxic, and two infections took place in the context of severe oral and gastric and enteral mucositis. Two mysterious cardiorespiratory arrests occurred that were not observed by either nurses or physicians so that we don't know quite what happened except that the children died suddenly. Six children of this group of 15 are now surviving. The 43+- and 30+-month survivors represent continuous survivals in complete remission that were reported in our June 1984 *Journal of Clinical Oncology* paper. A 17-month survivor and a 14-month survivor are new patients, and there are two more who are obviously so early out that we can't really make any statements about them. We have two survivors who at the moment have had relapses and who will inevitably die. Overall then, what we are seeing is still on the order of 25-40% salvage in this particular group of patients.

Our allogeneic series is small, but there are two long-term survivors at 62+ and 44+ months out of five transplants that we have done. There again is not a perceivable difference between allogeneic and autologous marrow transplants in these groups of patients. The ablative program is similar to that presented by Dr. Graham-Pole. It is a combination of teniposide and Adriamycin in conventional doses and melphalan at 210 mg/m² followed by fractionated total body X-ray (3.33 Gy x 3) that is given sequentially over 7 days followed by bone marrow rescue.

Dr. Philip: In a group study, the massive regimen used for neuroblastoma is continuous infusion of vincristine, fractionated TBI, and high-dose melphalan followed by ABMT, which is always a purged marrow. This regimen, especially TBI, was chosen because of the very good results from the Philadelphia group that you have just seen. The toxic deaths were two out of 16 cases, and in only one out of 16 was severe morbidity observed. This means that this regimen, including fractionated TBI, is very well tolerated in this group of patients with very bad prognosis.

This is the overall survival of the 10 patients that were grafted in first CR of stage IV neuroblastoma, a condition that you know goes to no survival at 2 years in most series. The mean and median survival time since diagnosis is 18 months. In this group 78% of the patients are alive without evidence of disease compared with 24% in our previous group with no ABMT strategy. These are very early results but they are encouraging.

We go now to the patients who were not treated in CR. Eleven cases are evaluable for response of the protocol. Seven patients were in relapse. We achieved one CR, three PRs, and three no response; the total response rate was 57%. Four of the 11 patients were in partial remission, and two were in complete remission after the massive therapy, and two others had less than partial remissions. This means the response rate in this group was 100%. Seven of the 11 patients are alive, including six with nonprogressive disease. In conclusion, this primary background is able to show first that massive therapy is tolerable with a very small mortality and morbidity.

Dr. John Kemshead will discuss later the purging procedure. The primary clinical results are encouraging, however, with a response rate of 72.7% in our patients. Nonprogressive survival at 18 months of 78% versus 24% in the previous comparable group is very promising, and we are now going on with a new study with ABMT strategy as consolidation of first complete remission in all stage IV patients. Since January 1984, 16 patients entered the study and all marrow has been purged with the Kemshead technique.

IV. Clinical Studies in Solid Tumors: Part Two

Natural History of Stage IV Breast Cancer

A. U. Buzdar

Breast cancer is a major cause of death in U.S. women. In 1983, approximately 38,000 women died of this disease (1). Both a responsive tumor and a significant palliation can be achieved in a higher percentage of patients. Treatment could be divided into two broad categories, hormonal therapies and chemotherapy.

Tumors will respond to hormonal therapies in 40-50% of breast cancer patients. The median duration of response to first-line hormonal therapy is 10-14 months (2-4) and 4-8 months with secondary and tertiary hormonal therapies (5-7). Patients with hormonally responsive tumors survive significantly longer than do patients with hormonally nonresponsive tumors (8). Cytotoxic drugs with significant antitumor activity in this disease include the following: doxorubicin, cyclophosphamide, fluorouracil, methotrexate, *Vinca* alkaloids, and mitomycin. First-line therapy, with various combinations of the above drugs, results in an objective response in 60-80% of the patients; of these patients, 15-25% are in complete remission (CR). The median duration of response has varied from 9-15 months (9-11); the majority of patients who achieve CR develop progressive disease and only 15% remain in remission beyond 3 years (11,12).

Median survival from the initiation of doxorubicin-containing chemotherapy and according to the type of response is shown in Table 1. Patients who achieved CR experienced the longest survival, and patients with progressive disease had the worst prognosis (13). The results of second-line chemotherapy have been disappointing. With secondary chemotherapy, 15-35% of the patients achieve objective remission (14-16). The median duration of remission with second-line chemotherapy is 4-6 months.

Survival from first recurrence has been evaluated in a few studies to determine the impact of various treatment modalities on survival. The results of these studies have been conflicting--a few studies showed no change in survival (17,18). In contrast, a study from The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston illustrated an improved survival for patients treated in the past decade compared with those treated in the two previous decades (19). In this study, groups of more than 200 patients registered consecutively from 1956, 1966, and 1975 were analyzed. A majority of patients in each decade received various endocrine therapies, and 32% of the patients in the 1950's and 50% in the 1960's received single-agent chemotherapy, as either initial or subsequent treatment. Patients (82%) in 1975 were treated with combination chemotherapy, whereas none had combination chemotherapy in the previous two decades. The median survival from first recurrence was 11, 12, and 22 months for patients in the 1950's, 1960's, and 1970's, respectively. Survival of patients in the 1970's was significantly superior to that of patients in the previous two decades ($P<.01$), but survival did not significantly improve from the 1950's to the 1960's ($P=.14$). Combination chemotherapy, which may have resulted in improved survival, did not significantly affect the mortality rate since most patients died of progressive disease.

In a few studies, intensive high-dose chemotherapy has been evaluated as first-line therapy in this disease. Tormey et al used a complex chemotherapy schema, which included doxorubicin, vincristine, methotrexate, dibromodulcitol, bleomycin, and hormonal agents (20). In 23 previously untreated patients, a 78% complete response was observed. The median duration of complete response was 13.5 months and median survival was 25.4 months. Intensive chemotherapy resulted in a higher complete response, but this did not have a significant impact on survival. The survival of this subgroup of patients was comparable to that of patients treated with conventional-dose chemotherapy.

In another study, high-dose chemotherapy with fluorouracil, doxorubicin, and cyclophosphamide was evaluated (Table 2). Patients were randomized to receive either a conventional dose of the same drugs or

Table 1. Survival of Patients from Initiation of Chemotherapy[a]

Type of Response	No. of Patients	Median Survival Time (mo)
Complete	99	32
Partial	298	24
No change	166	17
Progressive disease	56	4

[a]*Adapted from Swenerton KD et al. Cancer Res 1979;39:1552-62.*

Table 2. Conventional-Dose versus High-Dose Combination Chemotherapy[a]

Variable	Conventional Dose	High Dose
Drugs		
Fluorouracil	500 mg/m², days 1 and 8	500 mg/m² x 5 days
Doxorubicin	50 mg/m², day 1	85 mg/m², day 1
Cyclophosphamide	500 mg, day 1	1500 mg/m², day 1
No. of patients	28	32
Response after 3 cycles of chemotherapy (CR+PR)	39%	70%
Overall response rate (CR+PR)	82%	84%
Median survival (mo)	22	19

[a]*Adapted from Malik R et al. Proc ASCO/AACR 1982;(C-303):79.*

Note: *CR, complete response; PR, partial response.*

high-dose chemotherapy for three courses in a protective environment, in addition to prophylactic antibiotics (21). After three courses of chemotherapy, patients treated with high-dose chemotherapy achieved a higher objective response, but overall responses were similar and survival of both subgroups was also similar.

High-dose chemotherapy with autologous bone marrow transplantation support was evaluated in two studies of second-line therapy in metastatic breast cancer patients at M. D. Anderson Hospital (22,23). In the first study, mitomycin was used (Table 3). High-dose mitomycin did not result in a high response, compared with that experienced with the conventional dose of this drug. Autologous bone marrow transplantation did overcome the myelosuppressive toxicity, but other organ toxicities limited the dose

Table 3. High-Dose Chemotherapy with Autologous Bone Marrow Transplant in Second-Line Therapy

Drug	Dose	No. of Patients	No. of CR+PR	(%)
Mitomycin[a]	30-50 mg/m²	20	5	(25)
AMSA[b]	200-250 mg/m²	16	2	(12.5)

Note: *CR, complete remission; PR, partial remission.*

[a]*Adapted from Schell F et al. Autologous bone marrow transplant workshop, Jan. 26-28, 1981.*

[b]*Adapted from Tannir N et al. Cancer Treat Rep 1983;67:599.*

escalation in this study. In the next study, amsacrine (AMSA), an acridine derivative, was evaluated as second-line therapy. The response rate with this drug was disappointingly low (Table 3).

In summary, current treatment approaches result in remission in a significant fraction of patients, but these remissions have not been sufficiently durable to translate into a meaningful improvement in long-term survival. Further cytoreduction in responding patients with alternate drug programs, or the same drugs with autologous bone marrow support, might result in improved disease-free survival.

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The Rationale for High-Dose Chemotherapy with Autologous Bone Marrow Support in Treating Breast Cancer

W. P. Peters

The optimal treatment for metastatic breast cancer continues to be a subject of controversy and conceptual dispute. Despite the clinical efficacy of more than 15 antineoplastic agents, no curative therapeutic approach to metastatic breast cancer has yet been developed. The prognosis for patients with metastatic breast cancer who are treated with currently available chemotherapy (1) is worse than that for patients with acute myelogenous leukemia who achieve a complete remission (2). For many of the effective agents used in treating breast cancer, the dose-response effect of chemotherapy has been clearly demonstrated. However, in the clinical setting, full utilization of the principle of maximal dose administration is limited by myelosuppression. In recent years, this increased understanding of the fundamental importance of administered dose rate, coupled with the recognition of synergism and non-cross-resistance among selected chemotherapeutic agents (3), has renewed interest in treating breast cancer using escalated doses of chemotherapy with amelioration of myelosuppression by autologous bone marrow support. The clinical trials to date, however, have been limited.

RATIONALE FOR DOSE ESCALATION IN BREAST TREATMENT

A steep dose-response curve for treating experimental murine leukemias (4) and solid tumors (5) with alkylating agents has been clearly demonstrated. For example, treatment of L1210 leukemia with a single 150-mg/kg dose of cyclophosphamide will result in killing approximately 3 logs of cells. Doubling the administered dose to 300 mg/kg will result in 7 logs of tumor kill, a 10,000-fold therapeutic advantage (4,6). Similar data have been obtained with experimental solid tumors such as the Lewis lung carcinoma, colon cancer 38, Ridgeway osteogenic sarcoma, and C3H mammary adenocarcinoma (7).

Among the solid tumors of adults, metastatic breast carcinoma is one of the most responsive to the various pharmacologic subtypes of chemotherapeutic agents at conventional doses. Chemotherapy with single agents will consistently produce objective responses in 15-35% of previously untreated patients (8); combination chemotherapy will produce objective responses in approximately 70%, 20% being complete responses (9), although rarely are the responses durable. Of particular relevance is the activity of alkylating agents that produce responses in breast carcinoma. Table 1 summarizes response data derived from phase II studies of various classes of alkylating agents. Objective complete and partial responses for these single agents are generally in the range of 15-25%. In most cases, the dose-limiting toxicity for the alkylating agents is myelosuppression; therefore, they are candidates for inclusion in intensive autologous marrow therapy.

Clinical data supporting a steep dose-response effect for chemotherapy has been demonstrated in the treatment of advanced breast cancer and in the adjuvant treatment of primary disease. Retrospective analyses have demonstrated that for patients with advanced breast cancer, there is a clear therapeutic advantage in full doses of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF). Compromise of the administered dose to less than 65% of full dose substantially reduces therapeutic efficacy. The analysis demonstrating the dose-response relationship in the adjuvant treatment of breast cancer, although consistent with the above data, is more controversial and subject to potential errors of statistical methods (10).

Basing his estimations on pharmacokinetic considerations, Skipper (11) has calculated the in vivo exposure of breast cancer cells to each of

Table 1. Alkylating Agents Given at Conventional Doses and Tumor Response of Breast Cancer (Derived from Various Phase II Trials)

Alkylating Agents[a] by Class	N	CR + PR[b]	Response Rate(%)
2-Chloroethylamines			
Nitrogen mustard	95	21	22
1-Phenylalanine mustard	35	10	29
	177	34	19
Cyclophosphamide	414	112	27
Piperazinedione	58	3	5
Chlorambucil	60	11	18
Aziridines			
Triethylmelamine	20	2	20
Thio-TEPA	105	18	17
Epoxides			
Dianhydrogalactitol	26	1	4
Dibromodulcitol	154	44	29
	82	24	29
Alkyl alkanesulfonates			
Dimethylmyleran	1	0	0
N-Alkyl-*N*-nitrosoureas			
BCNU	76	16	21
	143	17	16
CCNU	170	20	12
	143	17	12
meCCNU	100	4	4
	54	2	4
Streptozotocin	25	0	0
Triazines			
DTIC	29	2	7
Others			
Cisplatin	14	2	14
	35	19	54
Procarbazine	21	2	5
Hexamethamelamine	91	2	3
Mitomycin C	110	26	24

[a] *BCNU, 1,3-bis-(2 chloroethyl)-1-nitrosourea; CCNU, 1-(2 chloroethyl)-3-cyclohexyl-1-nitrosourea; meCCNU, 1-(2-chloroethyl)-3(4-methylcyclohexyl)-1-nitrosourea; DTIC, dimethyl triazeno imidazole carboxamide.*

[b] *CR + PR, complete and partial responses.*

the component drugs of the CMF regimen at full dose and at fractionated dose reductions. The exposure relationship for methotrexate and fluorouracil decreases linearly for any given dose reduction, but the decrease is nearly logarithmic for the alkylating agent cyclophosphamide. Thus, a change in the administered dose of cyclophosphamide would be expected to have a considerable effect on therapeutic efficacy. Further, preliminary analysis of data (see Fig 1) derived from our studies of high-dose combination alkylating agents with autologous marrow support in breast cancer suggests that dose escalation is associated with an enhanced rate of tumor volume regression (12), which appears to be nonlinear with increasing dose.

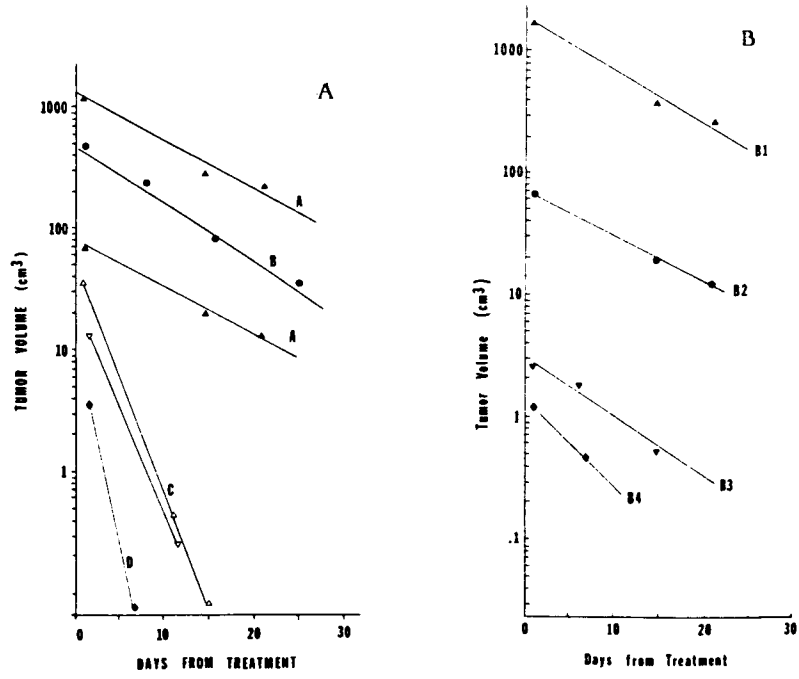

Figure 1. A, *Tumor volume measurements for individual lesions charted against days from the initiation of chemotherapy with high-dose combination alkylating agents. Each line is from an individual patient lesion. Patients were treated at* (a) *1.5 g/m² CPA, 75 mg/m² cDDP, 150 mg/m² BCNU;* (b) *3.0 g/m² CPA, 150 mg/m² cDDP, 300 mg/m² BCNU;* (c) *5.6 g/m² CPA, 165 mg/m² cDDP, 750 mg/m² BCNU; and* (d) *7.5 g/m² CPA, 180 mg/m² cDDP, and 750 mg/m² BCNU.* B, *Tumor volume measurements for individual lesions (B1, B2, B3, B4) of a single patient treated with 1.5 g/m² CPA, 75 mg/m² cDDP, 150 mg/m² BCNU plotted against days from initiation of treatment. The rate of tumor volume regression appears relatively independent of initial tumor size.*

The response data presented in Table 1 demonstrate activity for several alkylating agent classes in excess of 20%. Recent experience in melanoma suggests that even for this chemotherapy-insensitive tumor, substantial improvement in response rate can be achieved with dose escalation of selected alkylating agents (13). Coupled with in vitro and experimental in vivo (14,15) evidence for non-cross-resistance among the alkylating agents, their established steep dose-response curve, and for selected agents, nonoverlapping nonmyelosuppressive toxicity, these represent attractive drugs for use in combination therapeutic regimens for breast cancer employing autologous bone marrow support (12).

Clinical studies of high-dose therapy with autologous bone marrow transplantation (ABMT) in breast cancer have been limited. Table 2 summarizes the published data. The series are small and the treatment programs heterogeneous. The definitions of high-dose therapy are not uniform. Almost all the patients received extensive prior therapy before enrollment in the high-dose protocols. Although the data are severely limited, two observations appear warranted. First, extramedullary toxicity, although generally frequent, was fatal in five of the 58 reported breast cancer patients (8%). In similarly collected series, fatal toxicity related to therapy occurred in eight of 32 patients (25%) with diffuse histiocytic lymphoma and 12 of 32 patients (37%) with germ cell tumors treated with a variety of intensive regimens and ABMT (16). This suggests that the intensity of the therapeutic regimens used for breast cancer may have been less than that applied to these other refractory malignancies or that despite the extensive previous therapy, the

Table 2. Reported Series of High-Dose Therapy with Autologous Bone Marrow Support in Breast Cancer

Reference	No. of Patients		High-Dose Treatment[b]	Response		Response (%)	Toxic Deaths
	Total	Breast[a]		Complete	Partial		
Peters (12)	19	6	CPA, cDDP, BCNU, PAM	3	3	100	2
Douer (20)	14	1	CPA, VBL, TBI	1		100	1
Tobias (21)	9	1	CPA, ADR		1	100	0
Stewart (22)	5	5	CPA, TBI	2[c]		50	1
Robinson (23)	15	1	MTX-LV, HN2, ACT-D		1	100	-
Gale (24), Gale et al (25)	-	4	MMC, ADR, CPA, VBL, MTX	1	3	100	0
Schnell (26)	15	15	MMC	4	3	47	1
Lazarus (27)	33	3	PAM		2	67	0
Corringham (28)	17	3	PAM			0	0
Subtotal	127	39		11	13	62	5
Tanir[d]	17	16	*m*-AMSA		2	12	0
Wolff (2)	26	3	VP-16-213			0	0
Total	170	58		11	15	45	5

[a] *Breast cancer patients evaluable for response.*

[b] *CPA, cyclophosphamide; cDDP, cisplatin; BCNU, 1,3,bis-(2 chloroethyl)-1-nitrosourea; PAM, melphalan; VBL, vinblastine; TBI, total body irradiation; ADR, Adriamycin (doxorubicin); MTX-LV, methotrexate-leucovorin; HN2, nitrogen mustard; ACT-D, dactinomycin; MMC, mitomycin C; m-AMSA, amsacrine.*

[c] *One additional patient died without evidence of disease or toxicity.*

[d] *Unpublished results.*

patient's condition at the initiation of therapy was better. Second, excluding patients treated with VP-16-213 or amsacrine, both of which appear inactive, the overall response rate was 67%, and the complete response was 35%. Despite the heterogeneity of the therapeutic approaches, these results are impressive for this group of heavily pretreated patients. Optimization of the regimen and treatment of earlier disease, perhaps even in the adjuvant setting, may allow this modality to provide a significant therapeutic advantage. Single-agent studies at intense doses are essential in establishing enhanced activity and defining toxicity. However, quantitative considerations of drug resistance and tumor heterogeneity suggest that appropriately designed combination therapy might enhance therapeutic results. The superiority of multiple agents over single drugs has been demonstrated in studies of conventional doses. In a phase I trial, we have demonstrated that selected alkylating agents may be combined at nearly full individual agent transplant doses before extramedullary toxicity becomes prohibitive (12). Careful, controlled clinical trials will be needed to prove efficacy. The optimal regimen and timing for therapy remain to be established.

PROBLEMS ACCOMPANYING INTENSIVE CHEMOTHERAPY AND ABMT

Breast cancer is a heterogeneous disease in its natural history and in its response to therapy. The overall probability of cancer-related death within 18 months of diagnosis of metastatic breast cancer is 50% despite response to conventional therapy. However, there is also a small fraction of patients alive with metastatic breast cancer many years after diagnosis (17). These considerations emphasize the need to carefully devise therapeutic trials of intensive therapy to assure a favorable risk-benefit ratio. However, the failure of currently available therapy to produce cures emphasizes the need for adequate clinical evaluation of new approaches.

Contamination of bone marrow with metastatic cancer is a major potential problem in the development of therapeutic approaches involving ABMT. Recent studies have demonstrated that 28% of women without clinical evidence of metastatic disease at the time of presentation with primary disease had tumor cells detected in the bone marrow by an immunological method (18). Although occult tumor cell involvement of bone marrow may prove to be present with some frequency in breast cancer, its relevance to high-dose therapeutic approaches remains to be established. For example, long-term complete remissions have been obtained in patients presenting with bone marrow involvement with Burkitt's lymphoma (19). In these patients, from whom bone marrow was harvested after complete response had been achieved, high-dose therapy appears to have produced "cures" despite the high probability of marrow contamination with cancer cells. Patterns of relapse may prove useful in assessing the importance of bone marrow contamination. It is likely that occult tumor contamination of bone marrow will prove in some patients to be a factor in the failure of therapeutic approaches involving ABMT and that investigations of marrow "clean-up" will be needed. However, at the current time, development of more effective cytoreductive regimens are essential to realize this potential benefit.

SUMMARY

Metastatic breast cancer has a poor prognosis and has proved refractory to cure by conventional chemotherapeutic approaches despite the availability of drugs producing objective response. Clinical and experimental data support a steep dose-response effect in the treatment of breast cancer, and limited data from programs involving intensive chemotherapy with marrow support suggest an enhanced response in refractory disease. The use of intensive chemotherapy programs with ABMT offers the hope of improving therapeutic results in metastatic and perhaps primary breast cancer. However, the treatment is costly, time-consuming, and toxic. It should be used in trials designed with curative intent, care-

fully conducted with adequate numbers so that therapeutic benefit, if present, can be established and toxicity can be understood and minimized.

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Intensive Chemotherapy and Autologous Bone Marrow Transplantation for the Treatment of Refractory Malignancies

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INTRODUCTION

Myelosuppression is the dose-limiting toxicity for most antineoplastic agents, yet administering larger quantities of drugs results in greater antitumor effects (1). Bone marrow transplantation can ameliorate the dose-limiting myelosuppression produced by intensive chemotherapy and permit increased dosages, thereby possibly improving antitumor responses (2). Our phase I studies with autologous bone marrow transplantation (ABMT) using high-dose 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU), melphalan, and the combination of melphalan and BCNU have established the maximally tolerated doses of these agents (3,4). This report summarizes the antitumor responses obtained with these intensive chemotherapy regimens in 228 evaluable patients.

STUDY DESIGN

BCNU

The total BCNU dose was administered intravenously over 2 h in divided doses (each 600-1200 mg/m^2) on each of 3 consecutive days, as previously described. Three to 7 days after the last dose of BCNU, cryopreserved marrow (approximately 1 x 10^8 nucleated bone marrow cells/kg) was rapidly thawed and infused intravenously.

Melphalan

The total dose of melphalan was administered by intravenous bolus injection in divided doses (each 120-225 mg/m^2) on each of 3 consecutive days, as previously described (4). Seventy-two hours after the last dose of melphalan, cryopreserved marrow (approximately 1 x 10^8 nucleated bone marrow cells/kg) was rapidly thawed and infused intravenously.

Melphalan-BCNU Combination

The total melphalan dose (90-180 mg/m^2) was administered by intravenous bolus injection in divided doses over 3 days. In the following 3 days, the divided doses of BCNU (total dose of 600-1200 mg/m^2) were given over 2 h. Three days after the last dose of BCNU, cryopreserved marrow (approximately 1 x 10^8 nucleated bone marrow cells/kg) was rapidly thawed and infused intravenously.

EVALUATION

Only patients with measurable disease and survival to at least day 28 after autografting were evaluable for response, unless progression was noted earlier. Complete responses (CRs) were defined as the disappearance of all symptoms and signs referable to tumor. Partial responses (PRs) were defined as a greater than 50% reduction in the sum of the perpendicular diameters of all tumors and no appearance of new lesions. Less than 50% improvement, stability, or progression (at any time) were all

Table 1. Response of Refractory Malignancy to BCNU and Autografting

Diagnosis	No. Evaluable	Response[a]			Duration of Response (mo)[b]
		CR	PR	Rate (%)	
Melanoma	31	5	9	45	5 (2-43+)
Lung cancer	13	0	6	46	1 (1-2)
Glioblastoma	27	2	10	44	4 (1-71+)
Gastrointestinal cancer	9	1	1	22	5, 24
Hematologic malignancy	8	2	1	38	1, 5, 28
Sarcoma	4	0	3	75	1, 2, 9
Miscellaneous	7	1	1	29	3, 3
Total	99	11	31	42	

[a] *CR, complete response; PR, partial response.*

[b] *Unmaintained response. When range is reported in parentheses, accompanying figure represents median.*

denoted as no response (NR). Time to progression was calculated from day 0, the day of marrow infusion.

RESULTS

BCNU

Ninety-nine patients with refractory malignancies were evaluable for antitumor response (Table 1). Eleven of the 99 patients (11%) had CRs, including patients with melanoma (5), recurrent glioblastoma multiforme (2), colon cancer (1), leukemic meningitis (1), Hodgkin's disease (1), and adenocarcinoma of unknown primary with brain metastases (1). In addition, 31 patients (31%) achieved PRs, for an overall response rate of 42%.

Metastatic malignant melanoma was the most common tumor treated in our study: 31 patients were evaluable. All patients received 1200 mg/m^2 BCNU. Five patients had disease confined to skin, lymph nodes, or both; 26 patients had visceral metastases. Nineteen of the patients had not received prior chemotherapy. Five of 31 patients (16%) had CRs, and an additional nine patients had PRs (29%). Four of the five CRs and seven of the nine PRs occurred in the 19 previously untreated patients. The difference in response rate between the previously treated patients (3/12, 25%) and the untreated group (11/19, 58%), approached statistical significance (P=.06). The median time to progression (unmaintained response) in the responding patients was 5 months (range, 2-43+ months), with 13% (4/31) of the patients having a response that extended 1 year or more. These four patients had not received prior chemotherapy.

Twenty-seven patients with recurrent glioblastoma multiforme were evaluable. Twelve (44%) responded. An additional nine patients were treated with high-dose BCNU and autografting as adjuvant therapy. Three patients had progressive disease 1, 12, and 16 months after therapy, and

one patient died of diffuse interstitial pneumonitis at 3 months with residual disease noted at necropsy. Another patient died of liver failure owed to small cell lung cancer, without evidence of glioblastoma multiforme, 5 months after marrow transplant. Four patients are alive without progression 12, 13, 32, and 54 months after treatment.

Nine patients with gastrointestinal cancer were evaluable. Responses were observed in two of four patients with metastatic colon cancer, including one patient whose hepatic metastases was completely resolved for 24 months and one patient whose PR lasted for 5 months. No responses were seen in patients with gastric (two), esophageal (one), pancreatic (one), or hepatic (one) cancer.

Two of eight patients with hematologic malignancies had CRs. One patient with acute undifferentiated leukemia in marrow remission but with refractory leukemic meningitis achieved a CR and continued in unmaintained marrow and meningeal CR for 28 months, but he died at home with a febrile illness. A patient with Hodgkin's disease had a CR but died of bacterial pneumonia at 6 weeks posttransplant; no tumor was found at necropsy. The third patient who responded was a patient with diffuse histiocytic lymphoma who had a PR lasting 5 months.

The remaining patients who responded to the high-dose BCNU and autografting regimen (three with soft tissue sarcoma, one with recurrent brain metastases from an adenocarcinoma of unknown primary, and one with metastatic epidermoid carcinoma of the tonsil) generally had brief responses.

Melphalan

Eighty-one patients with refractory malignancies were evaluable for antitumor response (Table 2). Seventeen of the 81 patients (21%) had CRs, including patients with melanoma (six), neuroblastoma (five), Ewing's sarcoma (two), soft tissue sarcoma (one), germ cell tumors (one), and cancer of the ovary (one), and colon (one). In addition, 33 patients (42%) achieved PRs, for an overall response rate of 62%.

Nineteen patients with malignant melanoma were evaluable after treatment with melphalan (180-225 mg/m^2) and autologous marrow transplant. Six patients (32%) achieved CRs and eight patients (42%) had PRs. The response rate with high-dose melphalan and autografting (74%) was significantly better than the response rate observed with high-dose BCNU (45%; P =.03). The median duration of unmaintained response was similar, but only one patient maintained a response longer than 1 year. Unlike the results obtained with high-dose BCNU, there was no adverse effect of having received previous chemotherapy: ten of thirteen previously treated patients responded (77%; three with CRs, seven with PRs) compared with four of five patients (80%; three with CRs, one with a PR) who had not had prior chemotherapy.

Seven of ten patients with refractory neuroblastoma responded to high-dose melphalan and autografting. Although there were five CRs, the median time to progression was 6 months, and the longest response was only 10 months. The protocol has now been modified to include 12 Gy of total body irradiation, with the hope of improving the duration of response.

Seventeen patients with sarcomas were evaluable for antitumor response. Eight of 10 patients (80%) with Ewing's sarcoma responded, but the responses were of brief duration. Only one patient of seven with soft tissue sarcomas had a CR, and this patient was lost to follow-up 4 months after transplantation.

Four of six patients with germ cell tumors responded, (although one patient was lost to follow-up after 4 months), and one patient still showed a CR 21+ months after treatment. The other two responding patients had brief (2-month) PRs. Of seven patients with refractory ovarian cancer, four responded, two patients having continued responses 5+ and 18+ months after autografting. Although no CRs were seen in six women with breast cancer, four PRs were achieved, one of which lasted more than 1 year. Two of three patients with refractory Hodgkin's disease had brief PRs. Finally, a patient with a giant cell tumor of bone and a patient with small cell lung cancer each had 3-month PRs. Two patients with esophageal cancer, one with a mesothelioma and another with cervical

Table 2. Response of Refractory Malignancy to Melphalan and Autografting

Diagnosis	No. Evaluable	Response[a] CR	PR	Rate (%)	Duration of Response (mo)[b]
Melanoma	19	6	8	74	5 (2-14)
Neuroblastoma	10	5	2	70	6 (2-10)
Ewing's sarcoma	10	2	6	80	3 (3-7)
Other sarcoma	7	1	0	14	> 4+
Germ cell tumors	6	1	3	67	2, 2, >4+, 21+
Ovarian cancer	7	1	3	57	3, 5, 5+, 18+
Breast cancer	6	0	4	67	2, 3, 3, 14
Colon cancer	7	1	3	57	2, 2, 3, 3
Hodgkin's disease	3	0	2	67	2, 6
Miscellaneous	6	0	2	33	3, 3
Total	81	17	33	62	

[a]*CR, complete response; PR, partial response.*

[b]*Unmaintained response. When range is reported in parentheses, accompanying figure represents median.*

cancer, had diseases that failed to respond to high-dose melphalan and autografting therapy.

Melphalan-BCNU Combination

Since the results with autografting using high-dose melphalan and BCNU as single agents in the treatment of metastatic melanoma were quite promising, with a high response rate with melphalan but a somewhat improved duration of response with BCNU, we combined the two drugs in a phase I-II trial for the treatment of these patients. The initial dose selected for melphalan and BCNU was 50% of the maximally tolerated dose of each drug found in the previous phase I trials (i.e., 90 mg/m^2 for melphalan and 600 mg/m^2 for BCNU). The drug doses were individually escalated in subsequent patients until the maximal dose of each was achieved (melphalan/BCNU [mg/m^2]: 135/600, 135/900, 180/900, 180/1200). Forty-eight patients were evaluable for antimelanoma activity (Table 3). There were seven CRs (15%) and 23 PRs (48%) for an overall response rate of 63%. The median duration of unmaintained response was 5 months (range, 2-29+ months), but six responses (13%) lasted 1 year or more. Thus, the response rate appears to mimic the results with high-dose melphalan and is also statistically superior to high-dose BCNU. There was not a statistically significant different response rate between patients who had and who had not been previously treated (9/12 compared with 21/36, P=.17). Most of the patients had visceral organ involvement (40 patients), and eight patients had recurrent disease limited to the skin, lymph nodes, or both. There was no statistically significant difference in the response rate between the groups (P=.31). The results obtained with each dose

Table 3. Response of Melanoma to Melphalan-BCNU-Autografting Combination Therapy

Category	No. Evaluable	Response[a] CR	PR	Rate (%)
Extent of Disease				
Skin, lymph nodes, or both	8	0	5	63
Visceral organs	40	7	18	63
Prior Therapy				
Untreated	36	5	16	58
Prior Treatment	12	2	7	75
Total	48	7	23	63

[a]*CR, complete response; PR, partial response.*

Table 4. Effect of Dose of Melphalan and BCNU in Autografting Therapy for Melanoma

Dose of Melphalan/BCNU (mg/m²)	No. Evaluable	Response[a] CR	PR	Rate (%)	Duration of Response (mo)[b]
90 / 600	9	1	3	44	2, 4, 6, 29+
135 / 600	7	2	2	57	>3+, 9, 9, 13
135 / 900	4	1	2	75	4, 4, 12
180 / 900	19	2	12	74	5 (2-16)
180 / 1200	9	1	4	56	4 (2-7)
Total	48	7	23	63	5 (2-29+)

[a]*CR, complete response; PR, partial response.*

[b]*Unmaintained response. When range is reported in parentheses, accompanying figure represents median.*

level are shown in Table 4. The maximally tolerated dose was found to be at the 180/900 mg/m² level. Differences between the response at the 180/900 mg/m² dose level and responses at other dose levels were not statistically significant, but the difference between it and the response at the lowest dose level (90/600 mg/m²) approached significance (P=.11).

SUMMARY

Our experience with treating patients with metastatic melanoma particularly demonstrates the potential of intensive chemotherapy with ABMT. With conventional chemotherapy the response rate is usually reported to be in the 20-25% range, and even lower response rates are reported for patients with multiple visceral metastases. Using the high-dose intensive regimens with autografting, statistically significant superior response rates can be achieved. Since improved responses can be obtained, attention must now be directed at improving the long-term duration of response.

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Massive Chemotherapy and Autologous Bone Marrow Transplantation in Progressive Disease of Nonseminomatous Testicular Cancer: A Phase II Study on 15 Patients

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INTRODUCTION

Recent progress in the chemotherapy regimens available for nonseminomatous testicular cancer produced substantial improvement in the therapeutic strategy for this disease. Since the introduction of cisplatin, regimens like VAB-6 (13) or PVB (6) have induced objective responses followed by long-term remission in 50% of the patients in stage II of the disease and 45-70% of the patients in stage III. However, conventional dose chemotherapy regimens, even those including VP-16-213, did not change the outcome of the patients who either did not achieve complete remission (CR) or relapsed during therapy (1,4,8,12,15).

The dose-response relationship in chemosensitive tumors is well demonstrated both in vitro and in vivo (10,11). The efficacy of autologous bone marrow transplantation (ABMT) to reduce the duration of aplasia after massive chemotherapy is evident (3,10,11). Using single-dose alkylating agents, at least three autologous bone marrow teams have reported a dose-response relationship in nonseminomatous testicular cancer (3,5).

Since 1982 several French autologous bone marrow teams have studied the efficacy and tolerance of single-dose melphalan, in addition to the combination of high-dose melphalan (HDM) and VP-16-213 at increasing dose levels, in patients with nonseminomatous testicular cancer who had either relapsed or had progressive disease. The results of phase II of this study, in which 15 patients (as of this writing) have been entered, are reported here.

PATIENTS AND METHODS

Fifteen patients from five institutions in France entered the study. The patient distribution was as follows: six patients were from the Centre Leon-Berard, Lyon; three from the Institut Paoli-Calmette, Marseilles; three from the Institut Gustave-Roussy, Villejuif; two from the Centre Rene Gauducheau, Nantes; and one from the Hopital St. Jacques, Besancon. Details on these patients are summarized in Table 1. All except patient 11 had nonseminomatous testicular cancer of the dysembryoma, choriocarcinoma, or a mixed type. Patient 11 had a Sertoli tumor. The median age of the patients was 29 years (range, 4-50 years). All patients received initial cisplatin that contained regimens of VAB-6 (14 patients) and PBV (1 patient). Nine patients also received salvage therapy, which included cisplatin or VP-16-213 or both, or ifosfamide or Adriamycin or both. All had relapsed or did not respond to these regimens at the time of ABMT. Fourteen patients had progressive disease and one had a partial response upon entering phase II of the ABMT study.

HDM, in combination with VP-16-213, was the therapy selected for the following reasons. HDM alone produced a 45% CR rate in an advanced phase of nonseminomatous testicular cancer (12). Several teams involved in this study also participated in a phase II study of HDM in advanced solid tumors of types other than testicular cancer (D Maraninchi, unpublished data). Rapid excretion (i.e., < 8 h) of HDM and its metabolites allowed us to use nonfrozen autologous bone marrow (2). As a single agent, VP-16-213 produced a 40% CR rate in resistant nonseminomatous testicular cancer (1,8,12,15). A summary of the massive therapy regimen used in this study follows (also see Table 2).

Table 1. Patient Characteristics

Patient/Age/ Diagnosis	Previous Therapy	Months from Diagnosis to ABMT	Clinical Status Prior to ABMT	Protocol (by mg/m^2)	Response	Details of Response	Duration of Response (days)	Additional Therapy	Status 1/8/84
1/36/NSTC	PVB	8	PD Liver and chest CNS AFP & HCG[a]	HDM 200 VP-16-213 600	PR	Persistence of chest metastasis HCG & AFP	(35) NE	No	Died day 35
2/26/NSTC	VAB-CDDP	7	PD Chest HCG	HDM 200 VP-16-213 400	NR	Stable; chest metastasis; HCG	--	CDDP	Died day 126 CNS metastasis
3/23/NSTC	VAB-CDDP	8	PD Liver and chest AFP-HCG	HDM 200 VP-16-213 1000	PR	HCG-AFP	(25) NE	No	Died day 25
4/4/Yolk sac tumor (retroperitoneum)	VAB-CDDP VP-16-213 HD-CDDP	27	Retroperitoneum localization PD-AFP	HDM 200 VP-16-213 1000	CR	Normalization AFP	(28)	No	Died day 120 PD
5/50/NSTC	VAB-CDDP VP-16-213 HD-CDDP	14	Subclavicular lymph nodes chest PD-AFP	HDM 200 VP-16-213 1000	PR	Stable lymph nodes chest 50% AFP	(70)	Surgery X-ray VCR-CDDP-VP-16-213 x 2 + ABMT	Died day 168 (after 2 ABMTs)
6/30/NSTC	VAB-CDDP	8	PD chest AFP	HDM 200 VP-16-213 1000	PR	Normalization AFP	(37) NE	No	Died day 37
7/34/NSTC	VAB-CDDP VP-16-213 HD-CDDP	26	PD chest AFP & HCG	HDM 140 VP-16-213 1000	PR	Chest lesions PR > 50% AFP & HCG	(20) NE	No	Died day 20
8/27/NSTC	VAB-CDDP VP-16-213 CDDP	15	PD chest HCG	HDM 140 VP-16-213 1000	PR	>50% of chest lesions HCG	(35)	CDDP VP-16-213	Died day 119 PD
9/30/NSTC	VAB-CDDP	7	PR chest	HDM 140 VP-16-213 1000	NR	Chest-scan stable disease	--	Surgery	Died day 120

10/32/NSTC	VAB-CDDP Irradiation	32	PD chest + lymph nodes; AFP	HDM 140 x 2		CR in chest & some lymph nodes AFP PR mediastinum	(129)	No	Died day 207 PD
11/30/NSTC; Sertoli cell tumor	VAB-CDDP VCR-ADM Act. D	10	PD Cutaneous tumor lymph nodes chest	HDM 140 x 2	CR	All tumor targets disappeared	(196)	No	Died day 196 Leukemic AML
12/30/NSTC	VAB-CDDP VCR-ADM Irradiation	12	PD chest lymph nodes testicular tumor AFP & HCG	HDM 140	PD	Unchanged chest X-ray HCG	--	CDDP	Died day 105 PD
13/22/NSTC	VAB-CDDP VP-16-213	13	PD lymph nodes chest HCG	HDM 140	PD	Unchanged chest X-ray HCG	--	No	Died day 60 PD
14/27/NSTC	VAB-CDDP VP-16-213	8	PD lymph nodes chest AFP & HCG	HDM 140	PD	Chest progression liver HCG	--	No	Died day 77 PD
15/27/NSTC	VAB-CDDP VP-16-213	11	PD HCG mediastinal & aortic lymph nodes chest metastasis	HDM 140	PD	Liver metastasis chest metastasis HCG	--	No	Died day 145 PD

Note: *ABMT, autologous bone marrow transplantation; NSTC, nonseminomatous testicular cancer; PD, progressive disease; CNS, central nervous system; HDM, high-dose melphalan; PR, partial response; NE, nonevaluable; NR, no response; HD-CDDP, high-dose cisplatin; CR, complete response; Act. D, actinomycin D; AML, acute myelogenous leukemia.*

[a]*The fetal tumor antigens AFP and HCG have increased.*

Table 2. Summary of the Single-Dose Regimen Used in this Study

High-dose Therapy[a]	No. of Patients	Patients[b]
HDM 140 mg/m²	6	10-15
HDM 140 mg/m² VP-16-213 1 g/m²	3	7-9
HDM 200 mg/m² VP-16-213 400 mg/m²	1	2
HDM 200 mg/m² VP-16-213 600 mg/m²	1	1
HDM 200 mg/m² VP-16-213 1 g/m²	4	3-6

[a] *HDM, high-dose melphalan; VP-16-213, etoposide.*

[b] *These are the patients listed by number in Table 1.*

As a single agent, HDM (Alkeran, Burroughs Wellcome, Research Triangle Park, NC) was administered to six patients (patients 10-15, Table 1) at a dose of 140 mg/m². The drug was given intravenously by a rapid infusion. All patients received a hyperhydration regimen consisting of 3 l/m²/day of 5% dextrose. This regimen was started 24 h before and discontinued 24 h after the bolus injection. Furosemide was administered when necessary to obtain a urine flow rate of about 2 ml/m²/min. Pharmacokinetic studies have been reported elsewhere (2) (C Ardiet et al., unpublished observations).

HDM has been combined with VP-16-213 at doses of 140 mg/m² in three patients (patients 7-9, Table 1) and 200 mg/m² in six patients (patients 1-6, Table 1). An increased dose of VP-16-213 was used in combination with HDM: 400 mg/m² in patient 2, 600 mg/m² in patient 1, and 1 g/m² in all other patients (patients 3-9).

Vepeside (Sandoz Pharmaceuticals, East Hanover, NJ) was administered intravenously by a slow 60-min infusion in 250 ml of saline. Part of the dose was infused every 12 h for 2 days (400 mg/m²) or for 4 days (1 g/m²). The dose administered to each individual patient has been summarized in Table 1.

Patients 10 and 11 received two courses of HDM (140 mg/m²). Patient 5 received HDM (200 mg/m²) and VP-16-213 (1 g/m²) followed by both cisplatin (200 mg/m²) and VP-16-213 (500 mg/m²), as reported by Ozols (8), and cisplatin (200 mg/m²) and VP-16-213 (1 g/m²) and ABMT (see Table 1).

Bone marrow harvest was performed when massive therapy was indicated. During the procedure, four aspirations and four biopsies were performed in each case. Results of both the aspirations and the biopsies were always normal. Bone marrow cells were not frozen before transplantation, which was when HDM alone was administered (10). In the other cases, the stem cells were frozen, stored, and thawed, as described previously (10).

In the hematopoietic recovery phase after transplantation, patients were isolated with or without laminar air flow, and oral decontamination was always used. Sterile food and parenteral nutrition were also used, as was previously reported by the five teams.

Response to massive therapy was evaluated by both measurable tumor targets and biologic markers, which was in accordance with the recommendation of the World Health Organization (7). Informed consent to enter phase II of the study was obtained.

Table 3. Summary of Responses of 15 Patients to Massive Therapy in the Phase II Study

Massive Therapy	No. of Patients	Response				
		CR	+ PR	NR	PM
HDM 140 mg/m²	6	1	1	-	4
HDM 140 mg/m² VP-16-213 1 g/m²	3	-	2	1	-
HDM 200 mg/m² VP-16-213 400 mg/m²	1	-	-	1	-
HDM 200 mg/m² VP-16-213 600 mg/m²	1	-	1	-	-
HDM 200 mg/m² VP-16-213 1 g/m²	4	1	3	-	-
Total	15	2	7	2	4

Note: *HDM, high-dose melphalan; CR, complete response; PR, partial response; NR, no response.*

RESULTS

Tumor Response Data

An objective response was observed in 9 (60%) of the 15 patients, which included 2 CRs and 7 partial responses (PRs) (Tables 1 and 3). In patient 11, cutaneous, pleural, testicular, and lumboaortic lesions disappeared for 196 days. The patient died of acute myelogenous leukemia (AML) while in CR. Biologic markers decreased dramatically in patients 1, 3, 5-8, and 10. In patient 1, chest lesions were persistent. In patients 7 and 8, chest lesions decreased by 50%, whereas in patients 5 and 10, chest lesions decreased as well--but with persistence of other lesions (Table 1). In two patients (patients 2 and 4, Table 1), biologic markers increased immediately after massive therapy, then quickly decreased; in patient 2, chest lesions remained stable; and in patient 4, normal values of markers did not last for more than 28 days.

It is important to note that only two of six patients from the HDM-treated group had a response (patients 10 and 11, Tables 1 and 3), but seven of nine patients responded to the combination of HDM and VP-16-213. Of the seven patients who received 1 g/m² of VP-16-213, we observed five PRs and one CR.

The duration of response, however, is extremely short in this group of patients (i.e., 70 days for evaluable patients; 120 days, median survival post-ABMT for the nine responders). Of the nine responders, only two who were in CR were observed.

Toxicity Data

Marrow toxicity is clearly more profound when VP-16-213 is combined with HDM (median time of <200 granulocytes was 13 days vs. 6 days). For the entire group we observed a median of 16 days with a WBC lower than 1000/mm³, 16 days with fewer than 500 granulocytes/mm³, 13 days with fewer

Table 4. Marrow Toxicity in 15 Patients

Patient	Drugs	Bone Marrow Recovery (days) <1000 WBC	<500 PMN	<200 PMN	<50,000 Platelets	Mucositis	Infectious Complications	Miscellaneous Complications
1	HDM-VP-16-213	14	16	13	35	?	--	Fatal pulmonary fibrosis
2	HDM-VP-16-213	14	17	13	21	++	--	--
3	HDM-VP-16-213	25	25	25	25	++++	Interstitial pneumonitis viral + aspergillosis	--
4	HDM-VP-16-213	27	29	24	51	++	Nondocumented pneumonitis	--
5	HDM-VP-16-213	23	22	13	23	+++	Nondocumented hyperthermia	Regressive VOD
6	HDM-VP-16-213	16	16	14	37	++++	*Candida* pneumonitis	Hepatic cytolytic syndrome
7	HDM-VP-16-213	>20	>20	>20	>20	+++	*Streptococcus* sepsis and pneumonitis	--
8	HDM-VP-16-213	10	7	4	20	++	Septic shock (pseudo-A)	--
9	HDM-VP-16-213	11	7	6	8	++++	--	--
10	HDM x 2	22	16	5	16	++	*Staphylococcus* sepsis 2 ABMT *Candida* sepsis	--
11	HDM x 2	31	28	5	25	++	*Staphylococcus* sepsis + oral candidiasis	AML
12	HDM	10	12	7	45	0	--	--
13	HDM	7	10	9	9	?	AML	--
14	HDM	?	23	?	27	+++	Head & neck infection	--
15	HDM	9	11	6	9	?	Nondocumented sepsis	--

Note: *HDM, high-dose melphalan; VOD, veno-occlusive disease; ABMT, autologous bone marrow transplantation; AML, acute myelogenous leukemia.*

than 200 granulocytes/mm^3, and 22 days with fewer than 50,000 platelets/mm^3.

Severe morbidity was observed in this group of patients with end-stage disease during marrow aplasia (Table 4). Additional observations included the following: septicemia occurred in 10 patients (patients 3-7, 10, 11, and 13-15); fevers of unknown origin in four patients; fungal infections in four patients (patients 3, 6, 10, and 11); and pneumonitis in four patients (patients 3, 4, 6, and 7) (Table 4).

Several miscellaneous complications were also observed, such as fatal pulmonary fibrosis in patient 1, progressive veno-occlusive disease of the liver in patient 5, hepatic cytolysis of unknown origin in patient 6, and AML (related to the two courses of HDM) in patient 11. Mucositis was observed in all but one of the evaluable patients and was obviously more severe when VP-16-213 was combined with HDM (Table 4).

Of the 15 patients, 11 had at least one complication, which was responsible for the deaths in one of six patients who received HDM alone and in five of nine patients who received VP-16-213 and HDM.

DISCUSSION

With an overall response rate of 60%, this phase II study confirmed the dose-response relationship in testicular cancer (3,5,9) and in solid tumors (10). This response rate is superior to all other comparable data with conventional dose therapy, which is 45% for melphalan (12) and 40% for VP-16-213 (1,8,12,15). Our data clearly indicate that VP-16-213 is a valuable drug when ît is combined with melphalan, as was previously suggested by Postmus et al (13). Although relatively severe morbidity was observed in this group of patients with end-stage disease, it is our experience that it is much less severe in patients who are in an earlier stage of the disease (10). Postmus et al suggested in their phase I study that VP-16-213 data indicated a 1.5 g/m^2 dosage for VP-16-213 when ABMT rescue is available. In our study, increased toxicity is observed when VP-16-213 and HDM are given in combination. The toxicity of a regimen of VP-16-213 (1 g/m^2) and HDM (140 mg/m^2) is acceptable. This schedule will be used in our future studies.

The major difference between the data on testicular cancer and our previous experience with lymphomas is the predominance of CR in the responders of the non-Hodgkin's malignant lymphoma group, whereas in testicular cancer, the majority of responses are only partial. However, the use of massive therapy produced a dose-response in nonseminomatous testicular cancer and should be used as part of a first-line salvage therapy.

According to the data of Blijham et al and Buckner et al, high-dose alkylating agents as single agents are not sufficient to salvage non-seminomatous testicular cancer (6,7). In this group of patients, the best salvage therapy data published are undoubtedly that of Ozols et al, who use two sequential courses of VP-16-213 and cisplatin in association with other drugs (8). Massive therapy and ABMT were used by Ozols et al in incomplete responders only. Ozols and coworkers clearly demonstrated that one course of massive therapy is not sufficient to salvage these patients. Therefore, we will use two courses of their regimen as first-line rescue therapy for this group of patients. The complete responders to first-line therapy will receive VP-16-213 (1 g/m^2) and HDM (140 mg/m^2) as late intensification. If the model we have proposed for relapse of NHML is valid for treatment of other solid tumors, long-term survivors and even a cure can be expected in a fraction of this patient population, in which a mortality rate of 100% is still observed.

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Autologous Bone Marrow Transplantation in Patients with Advanced Teratocarcinoma

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INTRODUCTION

The prognosis of patients with metastatic teratocarcinoma of the testis has been drastically improved with treatment combining vinblastine, bleomycin, and cisplatin (VBP) (1). More than half of the patients may be cured. The prognosis is less favorable for patients with bulky disease at diagnosis and worse for patients who relapse after the VBP treatment. Autologous bone marrow transplantation (ABMT) facilitates high-dose treatment with myelotoxic drugs without the danger of irreversible marrow damage (2). We have studied ABMT for three patients with progressive teratocarcinoma.

PATIENTS AND TREATMENT

The clinical data are summarized in Table 1. Patient A. G. had bulky disease at diagnosis; he responded to the VBP treatment. Secondary retroperitoneal lymphadenectomy revealed no residual tumor. However, he developed cerebral disease for which he was operated on and irradiated. In the following weeks, pulmonary metastases regrew rapidly. Chemotherapy was continued with vinblastine, ifosfamide, and cisplatin (VIP). Marrow was obtained after the third course of VIP. The response was transient and B-human chorionic gonadotropin levels rose again. He received a course of cyclophosphamide and VP-16-213 at lower doses with a transient response again. High-dose treatment was given as described by Spitzer et al (2). Hematopoietic restitution was prompt and uneventful. The tumor response was very transient, and the patient died 3 months later of his tumor.

The course of disease was very similar in the second patient, who had primary disease in the mediastinum, and in the third patient, who failed to respond to the VBP combination. The third patient received cisplatin in addition to the cyclophosphamide and VP-16-213 before ABMT. The third patient is still alive with progressive pulmonary metastasis and rising levels of human chorionic gonadotropin.

DISCUSSION

The combination of cyclophosphamide and VP-16-213 is severely myelotoxic, but does not preclude marrow recovery from residual stem cells to the extent that total body irradiation does. The effect of marrow infusion on hematopoietic restitution was studied by comparing recovery of blood counts after ABMT to that after a previous course of chemotherapy with lower doses. Figure 1 shows such a comparison. After high-dose treatment a lower and earlier nadir and a prolonged cytopenia may be expected. Indeed, the nadir occurred earlier and was lower than that after lower dose treatment, but the recovery of leukocyte counts was as fast after high-dose treatment and marrow infusion as after low-dose treatment without marrow infusion. Therefore, it is likely that marrow infusion had an effect on hematopoietic recovery following this treatment.

CONCLUSION

The value of ABMT for the treatment of advanced teratocarcinoma remains to be determined. One possibility is an earlier attempt at eradicating the tumor with high-dose treatment. Patients with bulky

Table 1. Summary of Autologous Marrow Grafts in Progressive Teratocarcinoma

PATIENT	STAGE AT DIAGNOSIS	PRIOR CHEMOTHERAPY	STAGE AT AUTOL.TRPL.	CONDITIONING TREATMENT	RESULT
A.G.21,M.	IV, BULKY DISEASE	VBL,BLM,PLT X 4 VBL,IFO,PLT X 4 CY, VP-16, BLM	PROGR.DIS.	CY 1.5G/M^2 X 4 VP-16 200MG/M^2 X 4	<PR
R.M.27,M,	IV,EXTRAGON.	VBL, BLM, PLT X 4 VP-16,IFO,PLT X 3	PROGR. DIS.	CY 1.5G/M^2 X 4 VP-16 200MG/M^2 X 4	<PR
H.S.21,M.	IV	VBL, BLM, PLT X 2 VP-16,CY,PLT,BLM X 4	PROGR. DIS.	CY 1.5G/M^2 X 4 VP-16 200MG/M^2 X 4 PLT 50 MG X 4	<PR

Note: *VBL, vinblastine; BLM, bleomycin; PLT, cisplatin; IFO, ifosfamide; VP-16, etoposide; CY, cyclophosphamide; PR, partial remission.*

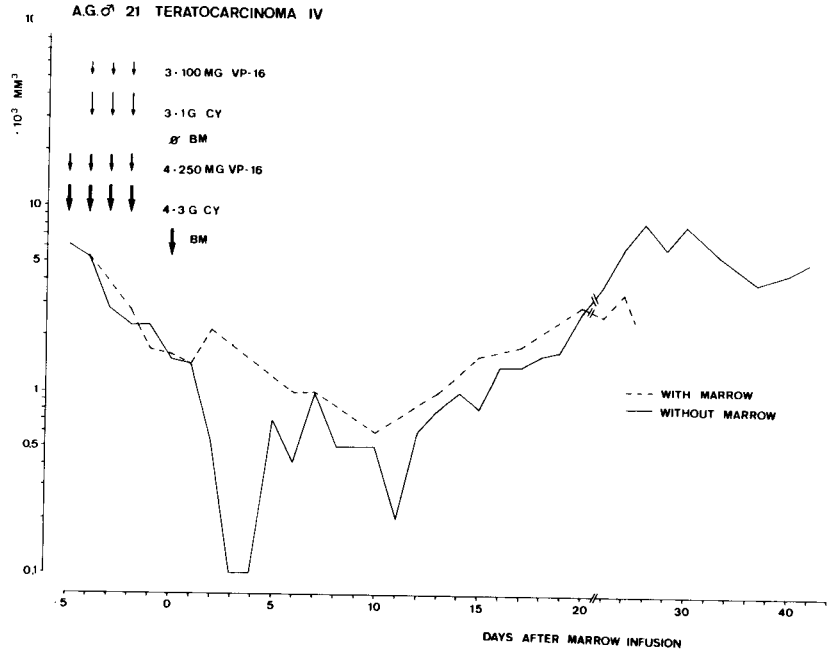

Figure 1. Recovery of white blood cells with and without autologous marrow infusion. Log_{10} plot of 10^3 cells/mm^3. Abbreviations: Cy, cyclophosphamide; BM, autologous bone marrow.

disease who have microscopic residual tumor at second-look operations may be the best candidates for high-dose combination chemotherapy and ABMT.

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Panel Discussion: Session IV

G. Herzig and I. H. Krakoff, Moderators

Dr. Geoffrey Herzig: The crucial question to answer is whether we have reached a point where there is evidence that high-dose therapy with autologous bone marrow transplantation (ABMT) has any proven effectiveness in the treatment of solid tumors. My opinion is that from our data we are convinced that the response of melanoma both to melphalan and to 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU) as single agents is better than the response of melanoma to those two agents in conventional doses or to any other chemotherapy. I am not convinced yet from the data that we have better responses in any other tumors. The one exception might be colon cancer, not just from our data but from a larger series of patients treated by Bill Knight in San Antonio showing a remarkable response of colon cancer to high-dose melphalan. I think these two tumors (melanoma and colon) are good examples of a situation where a standard phase II trial of high-dose therapy with ABMT can produce a positive result with a small group of patients, because with tumors that are refractory to virtually all therapies that have been tried even obtaining a response rate on the order of 20-40% can be meaningful.

Another situation in which the effectiveness of ABMT can be evaluated is exemplified by tumors such as small cell lung cancer (SCLC) and ovarian cancer, which are clearly responsive to current conventional therapy but in which only a very small proportion of patients are being cured. Here the options are greater but perhaps the best studies would involve intensification therapy of patients in first remission. The question then is how can we best evaluate intensification therapy? Should these be single-agent studies? Should they be intensifications of current regimens with drugs whose dose maxima we know, but used in combination; for example, intensifying the cyclophosphamide in a combination or nitrosourea in a combination?

Finally, we deal with the other large category of tumors, those tumors that are responsive to chemotherapy but where the responses are of relatively short duration and not of much survival benefit. I think the real difficulty in this setting, as was brought up earlier, is to get these patients referred for ABMT studies early in their course. It appears that many oncologists find it difficult to accept the idea of a course of therapy that is going to lead to a 3- to 4-week hospitalization and carry a 10-20% risk of a fatal outcome. To accept that as a mode of therapy early in the course of disease even when it is clear from all the available data that that disease is going to be fatal in 100% of the patients in let's say a year or 2 is difficult. So, I would appreciate any comments people have on ways around these problems too, and I would just like to open these points to discussion and anything else that anybody else has.

Dr. Karel Dicke: Basically there are two principles here. That is the tumor that is responsive to chemotherapy and where we try to give additional chemotherapy to change the natural history of the disease or in case of colon and melanoma where we know that at this moment there are no extremely active drugs. In these resistant tumors we try to change the natural history by high-dose chemotherapy in the initial phase of the disease. Now I feel that we may hit a homerun in those diseases that respond to normal-dose chemotherapy, rather than in melanoma and in colon where we know that we don't have, in my opinion, the right drugs at this moment.

Dr. Herzig: Well no, I mean I only feel that way because those tumors have responded to high-dose therapy. Colon cancer for example certainly needs to be treated and since there are no other obvious, good approaches I think the question of whether intensive therapy with agents that we have available will change the response of colon cancer should be answered. I was surprised that melphalan had any activity; I certainly

did not expect it. I think all of us get discouraged in trying to do phase II studies in refractory tumors until responses occur, and then everybody gets very enthusiastic about it again; the fact that it happened in two different very refractory tumors, melanoma and colon cancer, I think is an indication that we ought to continue to pursue this approach. Clearly we are not going to cure many or perhaps any patients with single-agent high-dose therapy, but these are the kinds of studies that lead to the development of combinations and allow you to treat patients earlier in the course of their disease. In melanoma, for example, I think there is good reason now to treat patients who have minimal disease but are at very high risk, for example the group of patients with more than three positive lymph nodes at diagnosis. The same thing may be said soon about colon cancer; that is, using high-dose melphalan in an adjuvant setting in a group of patients with colon cancer who are very high risk at the time of diagnosis.

At the same time the idea of intensifying therapy in sensitive tumors has come to most people's minds recently. For example, in ovarian cancer we know that melphalan is an active agent; we know that we can give melphalan in high doses now, maybe 10 times the dose that was evaluated in the older studies, with very little in the way of nonmarrow toxicity and with good potential of producing cures in a larger proportion of patients. I think that these studies need to be pursued too. It is encouraging to me that we have gotten to the point where at least in our institution gynecologists have recognized that the current results with ovarian cancer are not satisfactory and that the patients in the poor prognosis group are all ultimately relapsing and dying, and they are anxious to try something else as well.

Dr. William Peters: I would like to comment on Dr. Herzig's original question. This type of therapy is toxic, expensive, and time consuming. And it seems to me that if one is going to approach these diseases we ought to really separate very clearly in our minds those that are refractory and those that are responsive and what our approach to them ought to be. In those that are refractory, we will have to look for agents that will be substantially better than anything that we have now in order to get any real mileage. We are going to have to increase the CR rates at least double what we see now in order to make pursuing those agents or those combinations more meaningful. In those diseases where the response is substantial and where we can achieve some good responses with high-dose therapy, it seems to me a most reasonable approach to move the therapy earlier in the course of the disease--to the setting of minimal disease--in an effort to achieve cure. I think that the treatments in refractory disease really require very few patients to demonstrate whether or not the therapy is actually going to be effective, because the differences that one is going to look for have to be very big. When one is looking at the more-sensitive diseases, you have to get into the numbers game and that becomes much more complicated.

Dr. Herzig: Well I agree with that. That is what I was trying to say. In terms of refractory tumors like colon cancer and melanoma, it does not require many patients. What you are looking for is a major response; it is not a question of prolonging the measurable disease response for another 2 months. In the other studies with the more sensitive tumors I think you are also right. In my mind the endpoint of those studies again is not prolonging remission duration by 6 months or 1 year, it is increasing the proportion of patients who achieve remission. It does require a long follow-up, and it makes those studies difficult. It is hard to wait for the answers.

Dr. Gary Spitzer: What response rates and what kind of range of complete response rates are you looking for when you start these studies in these resistant tumors? You would always get an occasional long-term disease-free survivor with standard therapy.

Dr. Herzig: In tumors that have no obvious response, we are looking for a 20% response rate as the minimum for a phase II evaluation. To

carry anything further--what we would want is an agent that has a 20% complete remission (CR) rate before putting it into combinations in high-dose therapy. We have been able to cure tumors with combinations of three or four agents that individually produce responses on the order of 20% as single agents; if we can do that with individual agents at high dose then I think that is the kind of progress we could expect.

Dr. Spitzer: So what complete response rate would you be looking for in a melphalan and BCNU program?

Dr. Herzig: In the case of the two drugs, we would want to see something better than either of the drugs alone--at least additive. The data are not yet complete because the duration of the response I think is important. But my impression is that the combination is not as good as we would have wanted to see to continue it; I don't think it is additive. We are now considering the question of giving more than one course of therapy. The results to date have been achieved with a single course of high-dose therapy, but we almost always have enough bone marrow from one storage to give two courses of therapy. We know that for some agents like melphalan that you can give two courses without any apparent cumulative toxicity. So one option is to give more than one course of melphalan alone; another would be to use the melphalan and BCNU in sequential courses at their full single-agent doses, rather than the reduced dose of BCNU required in the combination. It would also be possible to use the melphalan-BCNU combination followed by a second course of melphalan alone, because we know that we could not give more BCNU (due to cumulative pulmonary toxicity).

Dr. Peters: There is another endpoint which should not be ignored. We should derive as much data as we can from the responses in phase I and phase II trials as is possible. We should very carefully look at the first derivative of tumor volume. I think Dr. Souhami has begun to show the value of that approach, and some of our data point in that direction as well. While it is not the sole criterion of response, it will I think add substantially to the decision as to which agents actually produce more effective early regression and help us in the end in designing combinations that will allow us to achieve what we finally want. We ought not to ignore the possibility of getting as much data as we can.

Dr. Spitzer: I just wanted to ask you another point of design. I am curious why you would want to give second courses to patients at these high doses of drugs if we have only had a partial remission (PR) on the first course. Here we have given five times the usual dose equivalent to 4 or 5 months' therapy when you only had a 1.5-log reduction. Yet we hope it would be more effective than 4 or 5 months of standard therapy because it is being given in one bolus; we hope it is equivalent to 8 or 9 months or whatever. What is the purpose of a second course in a PR patient?

Dr. Herzig: Well, in my mind it was the same as the purpose of second courses in any other chemotherapy. I mean it is unusual to have a CR after the first course of therapy even when you are dealing with a combination of agents that you know is effective and will produce a cure after, say, three courses of therapy. I see no conceptual difference between that and the anticipated response to high-dose therapy; why if you are able to produce a PR with one course won't two courses convert some of those to CRs, and if you have had a CR why won't a second course of therapy increase the duration of those CRs?

I think there is another problem in solid tumors in that it is very difficult sometimes to know whether you are dealing with CR or PR because you don't have a direct way of measuring tumor response. You use indirect measures--you see tumor shrinkage, there is a residual abnormality--but you have no way of knowing really whether that is fibrosis or necrotic tissue or whether there really is residual tumor, and you are not going to subject all patients to surgical exploration to find out. So all those patients are counted as PRs, but in our melanoma experience for example, some of the PRs have been just as long lasting as the CRs and I think they

probably were CRs; if we would have resected those residual abnormalities we would not have found viable tumor tissue there.

Dr. Peters: There is experimental data that Howard Skipper has published that show the tumor stem cell component actually is far more substantially reduced in solid tumors than indicated by tumor volume. In fact if you achieve a PR, you may achieve 4-5 logs of stem cell clonogenic tumor reduction. So, measurement of tumor volume may vastly underestimate the tumor response that you have achieved, and if you sequence two high doses close together, not allowing a lengthy period of 8-10 weeks between, you may achieve substantial improvement in the regimen.

Dr. Irwin Krakoff: I would like to make a comment related to the data Dr. Biron showed. It will be, I assume, an unpopular comment in this particular crowd. I and others have written papers and editorials that say in effect that more is better. I have believed that all my life, and I guess I still do. With a model such as testicular cancer where we can now with relative ease induce complete, lasting, curative remission in perhaps 80% of patients, but fail abjectly in the other 20%, we know that the only thing that really influences survival is CR. It is beginning to appear to me that the attempt to seek out more and more CRs with intensive therapy, as demonstrated by Dr. Biron, can in fact produce a few additional CRs, but those that are achieved with great difficulty, it seems to me, do not result in cure. I wonder whether those additional CRs might be only a quantitative difference that may not be important. The reason we cure testicular cancer 80% of the time is because we are able to achieve a qualitative result that we could not do with previous therapy and with single agents. By quantitatively extending that I am not sure that we are really aiming in the direction of cure. Now that is not what the theme of this meeting is and I don't expect that to meet with general agreement, but I am concerned that kind of pursuit may not be in the direction of getting more cures.

Dr. Spitzer: I would agree with Dr. Krakoff that the CR sought in a PR patient is probably not going to be durable. However, we should follow a sequence in each of these tumor systems. First, we have to document the actual potential cytoreduction of high-dose therapy in a tumor type by being able to evaluate the response that is in PR patients; then we can move on into a group of patients who do have CRs with an extra degree of sensitivity but a low cure rate. I think that what Taylor and I are proposing is initially studying the portion of partially responding patients to determine really whether this program is even active when we can still measure it. We are not really optimistic that we will have a high cure fraction unless we take that group of patients in CR that Dr. Wharton showed still were not cured.

Dr. Robert Bast: While you are on the subject of ovarian carcinoma and a steep dose-response curve, what are the chances that you will miss activity altogether in patients that have been heavily pretreated through six cycles or more of therapy before you go to your high-dose treatment course?

Dr. Spitzer: I do not know what the answer is. I can only speak to the data we have heard today where obviously we are still detecting activity on the partially responding oat cell patients after three courses and where Dr. Symann documented very clearly activity with a 100% CR rate with intensification after five or six courses. So in the one model system that it has been tested there is still documented activity after five or six chemotherapy courses. I think we could move ovarian cancer up a little step higher in sensitivity than oat cell because there are single-drug CRs with ovarian cancer. Therefore, there might be just that little increment of increased sensitivity over oat cell, and we may still detect it.

Dr. Souhami: We treated 18 patients with ovarian cancer with minimal residual disease with very high dose cyclophosphamide as the initial

treatment. But it becomes very difficult to know what you have done unless you are going to undertake rigorous restaging procedures, which are another burden for the patient. It becomes very difficult to quantify response rates when you bring the treatment very early even when it might have the best possible chance of success. So I find it very difficult to know how to design ovarian cancer studies when the high-dose chemotherapy is introduced rather early in the course of the disease.

Dr. Bast: We finally have a marker that is adequate to follow disease in the short run for the first two or three courses of chemotherapy. With CA125 in fact you could follow the rate of response in ovarian cancer just as Bill Peters has done looking at readily measurable lesions in melanoma and breast cancer. Now CA125 has its limitations, and it does not replace second-look operations, but if you want to ask that question, in the short run you probably could follow response in about 80% of the patients.

Dr. J. Taylor Wharton: That is certainly a very valid point. However, we have chosen to do our second-look laporotomy at the conclusion of six courses of chemotherapy. It may be that we will do that after two or three courses of chemotherapy. The moment that the patient has a clinical CR we will operate. After two courses of intensification chemotherapy supported by ABMT we consider the issue important enough to operate again and perform 24-30 biopsies so that we will know what has happened to the cancer.

V. Phase I Studies: Part One

High-Dose Aziridinylbenzoquinone with Autologous Bone Marrow Support as Treatment for Solid Tumors

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INTRODUCTION

Aziridinylbenzoquinone (AZQ) is a synthetic quinone compound that was designed to optimize central nervous system penetration by virtue of its lipid solubility and low ionization potential (1). Its mechanism of action is unknown. It appears to cross-link DNA and to produce free radicals. Exposure of cultured cells to a concentration of the drug lethal to 50% of the cells produced inhibition of DNA synthesis (2). The drug has shown a broad spectrum of activity against animal tumors, including intracranially implanted ependymoblastoma, B16 melanoma, P388 leukemia, L1210 leukemia, and colon 26 (3).

Preclinical toxicology studies of AZQ have been performed in beagle dogs, rhesus monkeys, and BALB/c x DBA/2F$_1$ mice. The major toxicity was reversible bone marrow hypoplasia (4). Studies in humans have induced only mild nausea. Leukopenia, thrombocytopenia, and anemia have been the dose-limiting toxicities. Repeated administrations have caused no cumulative extramedullary toxicities (5). AZQ has demonstrated antitumor activity against several human tumors, including brain tumors (6), lymphoma (7), and acute leukemias (8).

High-dose AZQ was chosen for use with bone marrow transplantation because the dose-limiting toxicity of the drug has been myelosuppression. The drug has produced no cumulative nonhematologic toxicity, and clinical studies so far suggest that increased doses of AZQ in certain tumors lead to increased remission rates. For these reasons we believed that patients could tolerate relatively high doses of AZQ with limited toxic side effects and that full recovery of the bone marrow could be expected after autologous bone marrow transplantation (ABMT).

MATERIALS AND METHODS

Nineteen patients have been entered in this study (11 men and 8 women), ranging in age from 26 to 66 years (median, 41 years). All patients had advanced cancer no longer responsive to conventional chemotherapy or radiotherapy. Primary tumors in six were brain tumors; in four, melanoma; in five, lung cancers; in two, lymphoma; and in one each, breast cancer and sarcoma. Hematopoietic, hepatic, and renal functions were within normal limits. Seventeen of the 19 patients entered in this study had a performance status (PS) of two or less on the Zubrod scale; two had a PS of three. Eighteen of the 19 patients had received prior chemotherapy, 16 had received prior radiation therapy, and 13 had received prior surgery. The median number of prior chemotherapy regimens was 2.5 (range, 0-6), and the median duration of chemotherapy was 6 months (range, 0-36 months). Routine laboratory and imaging studies were performed to document the extent of disease. Follow-up studies were obtained at specified intervals to document response. Standard response criteria were followed.

AZQ is supplied in 10-mg vials. The content of the vial is dissolved with 0.5 ml sterile *N*,*N*-dimethylacetamide and further diluted with 9.5 ml sterile 0.01 M phosphate buffer (pH 6.5). The drug is then diluted in normal saline and given intravenously over 1 h.

RESULTS

Phase I studies at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston were completed before the present study

began, and other phase II studies were already in progress. In these studies AZQ was administered intravenously in normal saline over 1 h on a 5-day schedule. The maximum dose given was 8 mg/m^2/day x 5; hematologic toxicity was moderate. Other phase I studies had reached a maximum dose of 10 mg/m^2/day x 5. The starting dose in this study was 12 mg/m^2/day x 5. Subsequent dose escalations and the number of patients at each level are documented in Table 1. Also included is the number of courses given at a particular level, since some patients had two courses.

Four deaths occurred during this study, each one less than 3 weeks after a patient began a chemotherapy course. One occurred during the second course of chemotherapy, when a patient with astrocytoma died on day 18 with a pontine hemorrhage plus hemorrhage in the lungs and gastrointestinal tract. The other patients died during or after the first course. One died on day 19 with a bleeding duodenal ulceration secondary to malignant melanoma in the gastrointestinal tract; one with lymphoma died on day 13 in septic shock secondary to a small bowel obstruction; and the fourth patient died on day 11 with progressive pulmonary carcinomatosis. Thus, 16 of these 19 patients are evaluable for response, and 18 are evaluable for toxicity.

The hematologic toxicities are shown in Tables 2 and 3. As can be seen from these tables, protracted hematopoietic toxicity invariably occurred at doses of 25 mg/m^2 x 5 days and above. The absolute granulocyte count remained less than 500/mm^3 for a median of 14 days at 25 mg/m^2 (8-16 days). Two patients who received 30 mg/m^2 had an absolute granulocyte count of less than 500/mm^3 for 24 days.

The platelet count was reduced at doses lower than were needed to reduce the granulocyte count. At 18 mg/m^2 the median lowest platelet count was 11,000/mm^3 (range, 4000-26,000). However, at this dose the platelet count remained less than 20,000/mm^3 for a median of only 1 day (range, 0-8 days). At the higher dose of 25 mg/m^2, the platelet count was less than 20,000/mm^3 for a median of 11 days (range, 1-20 days). The median time for recovery of platelet counts to greater than 50,000/mm^3 was 26 days at 18 mg/m^2 (24-28 days) and 28 days at 25 mg/m^2 (20-34 days).

Seventeen infectious complications occurred during 24 courses of therapy. These complications included seven episodes of sepsis, five urinary tract infections, three herpes infections, and one episode each of oral thrush and pneumonia. The majority (6/7) of the septic episodes were with gram-negative rods. One septic episode combined *Staphylococcus* and *Candida*, and another episode combined *Klebsiella* and *Candida*. The infectious complications responded to appropriate therapy.

The renal and hepatic toxicities were graded as shown in Table 4. Nine of 11 episodes of renal toxicity were grade I, one episode was grade II, and one was grade III. All of these decreases in renal function corrected themselves with adequate hydration except for the grade III toxicity. This patient developed acute renal failure following resuscitation from a cardiac arrest and subsequently died in renal failure.

Thirteen episodes of hepatic toxicity occurred. Five of these episodes were not related to infection. Three of these were grade II and two of them were grade III. Other toxicities included four episodes of gastrointestinal bleeding, one episode of inappropriate antidiuretic hormone secretion, and one episode of a transient encephalopathy that may have been related to other drugs.

Table 1. Dose Escalation

Level	Intravenous Dose	No. of Patients	No. of Courses
1	12 mg/m^2 x 5 days	4	5
2	15 mg/m^2 x 5 days	3	5
3	18 mg/m^2 x 5 days	4	5
4	25 mg/m^2 x 5 days	5	6
5	30 mg/m^2 x 5 days	3	3

Table 2. Hematologic Toxicity: White Blood Cells and Granulocytes

Dose (Courses)	Days WBC <1000/mm³		Days Granulocytes <500/mm³		Lowest WBC Measured (x 1000/mm³)		Lowest Granulocytes/mm³	
	Median	Range	Median	Range	Median	Range	Median	Range
12 mg/m² (5)	1	0-10	1	0-10	0.9	0.2-1.4	288	12-544
15 mg/m² (5)	0	0-7	2	0-8	1.2	0.3-1.6	143	6-1008
18 mg/m² (4)	5	3-10	8	4-9	0.3	0.1-0.4	155	0-3000
25 mg/m² (6)	15	9-21	14	8-16	0.1	0.1-0.2	0	
30 mg/m² (2)	25+	24-25+	24		0.1		0	

Table 3. Hematologic Toxicity: Hemoglobin and Platelets

Dose (Courses)	Days Hg <10 g/dl		Days Platelets <20,000/mm³		Lowest Platelet Count (x 1000/mm³)		Day of Platelet Recovery >50,000/mm³	
	Median	Range	Median	Range	Median	Range	Median	Range
12 mg/m² (5)	10	6-48+	6	0-8	9	8-43	21	21-41
15 mg/m² (5)	12	0-37+	0	0-2	25	13-68	20	18-22
18 mg/m² (4)	14	7-16+	1	0-8	11	4-26	26	24-28
25 mg/m² (6)	25	16-30+	11	1-20	9	6-13	28	20-34
30 mg/m² (2)	27	21+-32	19		17	10-23	40	35-45+

Table 4. Nonhematopoietic Toxicity Grading

Component	Toxicity Grade I	II	III	IV
Renal				
BUN	21-40	40-60	>60	Symptomatic
Creatinine	1.3-2	2.1-4	>4	Symptomatic
Hepatic				
SGOT	1.5-2 x Normal	2.1-5 x Normal	>5 x Normal	---
Alkaline phosphatase	1.5-2 x Normal	2.1-5 x Normal	>5 x Normal	---
Bilirubin	1.5-2 x Normal	2.1-5 x Normal	>5 x Normal	---
Clinical indication	---	Flap	Precoma	Hepatic coma

Note: *BUN, blood urea nitrogen; SGOT, serum glutamic oxaloacetic transaminase.*

Of the 19 patients entered into this study, 16 were evaluable for response. Four have shown minor responses, all of whom were treated at the higher dose levels of 25 mg/m^2 or 30 mg/m^2. Two of these patients had never been in remission, and two were progressing on second-line chemotherapy.

DISCUSSION

The results of this phase I-II study warrant further investigation. Minor antitumor activity in a group of such chemotherapy-resistant patients is encouraging. Interestingly, although the responses occurred at high doses, either 25 mg/m^2 or 30 mg/m^2, no limiting extramedullary toxicity has yet been encountered at these doses. The early deaths and life-threatening complications were related to myelotoxicity.

The above doses are approximately four times the maximum dose of AZQ given to patients with solid tumors. Only a few agents can be escalated five-fold or more above their usual maximal dose without producing intolerable extramedullary toxicity. Cyclophosphamide and VP-16-213 are two such agents, but even at sixfold escalation they are limited by extramedullary toxicity plus rapidly reversible myelotoxicity. Carmustine and mitomycin, at escalations of only fourfold and twofold to threefold, respectively, produce multiple extramedullary toxicities (9,10). Amsacrine (AMSA) at fourfold escalation produces severe gastrointestinal toxicity (11). The one drug so far investigated that can be escalated sixfold (from 30 mg/m^2 to 180 mg/m^2) with acceptable extramedullary toxicity and simultaneously produce prolonged myelosuppression is melphalan. Possibly AZQ is a drug with the same potential, since no limiting extramedullary toxicity has yet been seen.

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High-Dose Aziridinylbenzoquinone for Patients with Refractory Central Nervous System Neoplasms: A Preliminary Analysis

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INTRODUCTION

In 1983 we initiated a phase I-II study of high-dose aziridinylbenzoquinone (AZQ) supported by autologous hematopoietic reconstitution for patients with primary central nervous system (CNS) tumors refractory to surgery and radiation therapy. AZQ is significant with respect to CNS neoplasms because of its relatively high lipid solubility and its documented antineoplastic activity against a number of tumors in mice--even when such tumors have been intracranially inoculated (1). We were particularly interested in AZQ for a study of therapeutic intensification supported by bone marrow reconstitution and because of its substantial acute and cumulative dose-limiting hematologic toxicity (2,3) in association with its relative lack of other major organ system toxicities. Experience to date suggests that this high-risk patient population can be safely guided through therapy of this type--provided special attention is given to infection control and prevention of thrombocytopenic bleeding.

METHODS

This study was approved by the relevant Human Studies Institutional Review Boards. Following appropriate informed consent, 12 patients (four pediatric and eight adult) underwent bone marrow storage and insertion of Hickman or Broviac lines--often during the same anesthesia. Bone marrow harvesting was accomplished as described previously (4). Hickman line insertion was directed by members of the surgical staff.

All patients had progressive CNS neoplasms refractory to regional therapeutic modalities (radiation $\pm$ surgery) and six had received prior systemic chemotherapy. No patient had received any therapy within 9 weeks of entry into this study. Patient 5 had a partial response (PR) to nonablative doses of AZQ by computed tomography (CT) and neurologic parameters, but was unable to tolerate further AZQ because of cumulative myelosuppression. All other patients had clinical and CT-documented evidence of progressive tumor. All patients were receiving systemic corticosteroids--usually dexamethasone, in doses in excess of 10-12 mg/day. Details of age, gender, histology, and prior treatment are summarized in Table 1. Details of AZQ dose, time to hematopoietic recovery following bone marrow infusion, and treatment response are summarized in Table 2.

Bone marrow cryopreservation was accomplished using controlled rate techniques and 10% dimethyl sulfoxide, as described previously (5).

Patients were treated in standard private rooms and placed on diets of cooked food only. Real flowers and plants were not permitted in the rooms at times of myelosuppression. Nonabsorbable oral antibiotics were not utilized. Initially, parenteral antibiotics were withheld from adult patients until first onset of fever. However, after this approach resulted in bacteriologically documented sepsis in three of our first four adult patients, parenteral broad spectrum antibiotics were subsequently initiated as granulocytes that fell toward 500/μl. Platelet counts were done twice daily after platelets fell below 100,000/μl and maintained above 50,000/μl by transfusion as needed.

AZQ was administered intravenously. The first three patients in this study received 25 mg/m^2/day for 5 consecutive days. The remaining patients each received 30 mg/m^2/day for 5 days. In all cases, a daily dose was administered as a 4-h infusion with one-third of the dose given over 20 min and the remaining two-thirds given over 3 h and 40 min.

Table 1. Patient Characteristics

Patient	Age	Gender	Tumor Type	Prior Treatment
1	45	Male	Astrocytoma-Grade III	Biopsy, Radiation
2	10	Female	Astrocytoma-Grade II	Resection, Radiation
3	47	Male	Astrocytoma-Grade II	Resection, Radiation, Repeat Resection
4	47	Female	Glio. Multiforme	Resection, Radiation, BCNU
5	43	Female	Astrocytoma-Grade II	Resection, Radiation, AZQ, Repeat Resection
6	6	Female	Brain Stem Glioma	Radiation
7	24	Female	Brain Stem Glioma	Radiation
8	12	Female	Pineal Teratoma	Radiat. Velban/Bleo/Cis-Plt
9	9	Female	Brain Stem Glioma	Biopsy, Radiation
10	46	Female	Glio. Multiforme	Resection, Radiation, BCNU, Repeat Resection
11	30	Male	Glio. Multiforme	Resection, Radiation, CCNU, + VCR + PCBZ (1 cycle)
12	59	Male	Glio. Multiforme	Resection, Radiation, BCNU

Note: *Glio, glioma; BCNU, 1,3-bis-(2 chloroethyl)-1-nitrosourea; AZQ, aziridinylbenzoquinone; Radiat, radiation; Bleo, bleomycin; Cis-Plt, cisplatin; VCR, vincristine; CCNU, 1-(2 chloroethyl)-3-cyclohexyl-1-nitrosourea; PCBZ, procarbazine.*

Cryopreserved marrow was brought to the bedside in the form of liquid nitrogen, thawed at 40°C, and infused using aseptic precautions. Bone marrow infusion was done 72 h after the final AZQ dose.

RESULTS

In this limited series of extensively pretreated patients we have observed two PRs, as judged by CT improvement and clinical examination, and two disease stabilizations (Table 2). One patient died with autopsy-documented tumor progression and uncal herniation on day 9 following bone marrow infusion.

Mean ($\pm$ SEM) times to granulocytes and platelet recovery are 17 $\pm$ 2 days and 20 $\pm$ 6 days, respectively. Patients who had received two or more cycles of systemic chemotherapy prior to bone marrow harvest showed a trend toward delayed hematologic recovery (Table 3).

At the onset of this study, we planned to keep platelet counts greater than 25,000/µl. However, patient 3 developed a subarachnoid hemorrhage with a platelet count of 20,000/µl. We then measured patients' platelet levels twice daily and transfusions were given to maintain platelet counts in excess of 50,000/µl--whereafter intracranial bleeding did not occur.

We observed documented bacterial sepsis in three of our first four adult patients. Thereafter, parenteral antibiotics were begun empirically as granulocytes fell toward 500/µl. Nausea and vomiting from AZQ were minimal. Three patients developed moderate elevations of liver enzymes

Table 2. Treatment and Response

Patient #	AZQ dose per day	Days to Hematologic Recovery: PMN ≥ 500/μl	Days to Hematologic Recovery: Platelets ≥ 50,000	Treatment Outcome
1	25 mg/M^2	14	18	No Response
2	25 mg/M^2	22	18	Stable x6 months.
3	25 mg/M^2	11	13	No Response
4	30 mg/M^2	19	36	No Response
5	30 mg/M^2	28	25	Stable x8 months
6	30 mg/M^2	12	16	No Response
7	30 mg/M^2	16	16	Clinically improved x5+ months
8	30 mg/M^2	21	21	CT improved x4+ months
9	30 mg/M^2	14	14	No Response
10	30 mg/M^2	18	26	No Response
11	30 mg/M^2	14	18	No Response
12	30 mg/M^2	Died day 9 of tumor progression.		

Note: *AZQ, aziridinylbenzoquinone; CT, computed tomography.*

Table 3. Impact of Chemotherapy Prior to Bone Marrow Harvest on Hematologic Recovery

Number Cycles of Chemotherapy	n	Days To: WBC≥1000/μl	Days To: PMN≥500/μl	Days To: Platelets≥50,000/μl
≥ 2	4	19 ± 3	22 ± 2	27 ± 3
< 2	7	14 ± 1	15 ± 1	16 ± 1
P	-	NS	< .05	< .01

Note: P *determined by Wilcoxon Rank Sum Test; NS, not significant.*

and bilirubin 2-4 weeks from the start of AZQ. However, infection or sepsis, as well as possible AZQ toxicity, was implicated in these three patients.

DISCUSSION

We studied 12 patients with primary CNS neoplasms refractory to regional therapeutic modalities and five patients had neoplasms that were also refractory to nonablative systemic chemotherapy. Autologous hemato-

poietic reconstitution was effective in this patient population, but required special attention to platelet support and antibiotic use. The administration of chemotherapy [primarily BCNU (1,3-bis-(2 chloroethyl)-1-nitrosourea)] prior to bone marrow storage was subsequently associated with delayed hematopoietic recovery after high-dose AZQ.

Even though our total AZQ dose per cycle was two- to threefold higher than that of a nonablative cycle, our overall rate of improvement (PR plus stabilization) of 33% (4/12) is not, thus far, superior to prior studies of AZQ used at nonablative levels (2,3). It is conceivable that this may change as our experience matures. Alternatively, lack of increased rate of response may be related to the extensive pretreatment prior to AZQ observed in our patients, or perhaps there may be a relatively flat tumor-dose response curve for AZQ when used against primary CNS neoplasms. In any event, since convincing nonhematologic toxicity has yet to be encountered, further dose escalations of AZQ need to be considered.

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Novel Toxicities Associated with High-Dose Combination Alkylating Agents and Autologous Bone Marrow Support

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INTRODUCTION

The dose-response curve for almost all known nonhormonal antitumor agents is steep for both toxic and therapeutic effects (1). In clinical practice, full utilization of the principle of dose escalation is limited by myelosuppression, particularly use of the alkylating agents. Autologous bone marrow support allows an increase in dose to the point at which nonmyelosuppressive toxicity becomes limiting. There is accumulating experience with several alkylating agents used in high doses with autologous bone marrow support (2). The data support the position that nonmyelosuppressive dose-limiting toxicity may differ substantially in man with differing alkylating agents, providing a rationale for their use in combination. Further, it has been established that selected combinations of alkylating agents possess significant therapeutic synergy and subadditive host toxicity when tested in a variety of tumor systems (3-5). For these reasons and others, we have undertaken a phase I trial utilizing combinations of cyclophosphamide (CPA), cisplatin (CDDP), and 1,3-bis-(2 chloroethyl-1-nitrosourea (BCNU) and CPA, CDDP, BCNU, and melphalan (PAM) with amelioration of myelosuppression by autologous bone marrow support. We report here preliminary experience with novel toxicities associated with the high-dose combination alkylating agents and autologous bone marrow support.

METHODS

Details of protocol design, patient selection, therapeutic regimen, dose escalation schema, and supportive care have been reported elsewhere (6). Twenty-four patients were treated with 26 treatment courses, all of which are evaluable for toxicity. The median age was 32; 12 were males and 14 were females. The mode performance status was 0 on the Eastern Cooperative Oncology Group scale (range, 0-2). Prior chemotherapy varied according to disease; melanoma (0/9), breast cancer (6/7), sarcoma (3/3), colon cancer (1/2), lung cancer (2/2), testicular cancer (1/1). After an autologous bone marrow harvest while the patient was under general anesthesia, each was treated at six dose escalations of CPA (1.5-7.5 g/m^2), CDDP (75-200 mg/m^2), and BCNU (150-750 mg/m^2) or at two escalations of CPA (5.6 g/m^2), CDDP (160 mg/m^2), BCNU (600 mg/m^2), and PAM (40-80 mg/m^2). Chemotherapy was administered over a 4-day period (days -6 to -3), and the marrow was reinfused (day 0), 3 days later. Daily hemograms were obtained. Daily serum creatinine levels and weekly creatinine clearance values were obtained to evaluate renal function. Hepatic function tests were followed by twice-weekly tests of serum glutamic-oxaloacetic transaminase (SGOT), lactate dehydrogenase (LDH), total and direct bilirubin, alkaline phosphatase, and serum albumin. Platelet support was provided as single-donor irradiated platelets to keep the platelet count above 20,000/mm^3.

RESULTS

Refractory Thrombocytopenia

Thrombocytopenia requiring platelet transfusion developed in all except one patient. Platelet counts fell below 50,000/mm^3 6 days after

the initiation of treatment, and recovery of hematopoiesis to a self-sustaining platelet count greater than 50,000/mm^3 occurred at a median of 25 days. The median platelet requirement was 249 units (range, 151-408 units) transfused per patient course. Platelet consumption appeared most prominent on days 14-19 following bone marrow reinfusion. Platelet counts often could not be kept above 20,000/mm^3 during this period. Although evidence of alloimmunization was detected by the lymphocyte cytotoxicity assay in five of 16 patients, human leukocyte antigen (HLA) matching did not improve platelet survival. The thrombocytopenia persisted despite leukocyte reconstitution until approximately 20 days following bone marrow reinfusion. At that time normal platelet increments were again attainable in all patients. The median time to white blood cell levels greater than 500/mm^3 was 12 days and the median time to polymorphonuclear leukocyte levels greater than 500/mm^3 was 19 days. Clinically significant bleeding occurred in four patients: two patients had gastrointestinal hemorrhage, one patient suffered a thalamic bleeding episode during a period of refractory thrombocytopenia and hypertension, and one patient had a hemorrhagic pericardial tamponade.

Hypertension

Hypertension developed in 14 of 24 (58%) of treated patients. The mean blood pressure for patients at the initiation of therapy was 120/82 mm Hg. By 30 days following bone marrow reinfusion, systolic and diastolic hypertension (mean, 150/100 mm Hg) had occurred despite treatment with antihypertensive medication. Diuresis, treatment with β-blockers and the use of spironolactone were ineffective in controlling the hypertension. The calcium channel blocker nifedipine provided partial control of the hypertension. By 60 days following bone marrow reinfusion, hypertension had resolved and antihypertensive medication was no longer required. Plasma renin values in six patients prior to and following chemotherapy were normal despite the development of hypertension.

Veno-occlusive Disease

Transient rises in liver function test values were seen in 22 of the 24 patients shortly following completion of the therapeutic regimen. Veno-occlusive disease (VOD) occurred in four of 26 treatment courses (15%), appeared dose related, and proved fatal in two patients. Two of six patients treated with CPA at 5.6 g/m^2, CDDP at 165 mg/m^2, and BCNU at 600 mg/m^2 developed VOD as a clinical syndrome, which resolved with conservative treatment. VOD limited doses in the three-drug trial, two VOD deaths occurred at doses of CPA of 7.5 g/m^2, CDDP 180 mg/m^2, and BCNU of 600 or 750 mg/m^2. No difference in previous treatment or pretreatment hematologic or hepatic chemistries could be discerned between patients who developed VOD and those who did not. However, at 7 days following treatment, differences in white blood cell count, platelet count, SGOT values, and bilirubin levels were seen between those patients who subsequently developed VOD and those who did not (Table 1).

Renal Dysfunction

Transient elevations of serum creatinine from a mean baseline of 0.6 mg/dl to a mean of 1.2 mg/dl occurred in 16 of 24 patients by day 0 and returned to baseline 8 days following bone marrow reinfusion. Creatinine clearance values decreased from a mean of 130 ml/min to 65 ml/min and again returned to a median of 100 ml/min by 7 days following bone marrow reinfusion.

However, addition of PAM to the therapeutic regimen led to a significant increase in the magnitude and duration of creatinine elevations following therapy. Table 2 presents data of patients who received CPA (5.6 g/m^2), CDDP (180 mg/m^2), BCNU (600 mg/m^2), and 0, 40, or 80 mg/m^2 of PAM. The response of patients receiving either 0 or 40 mg/m^2 differed

Table 1. Comparison of Patients with and without Veno-occlusive Disease Seven Days after Treatment with High-Dose Combination Alkylating Agents

Laboratory	VOD No.	VOD Mean	No VOD No.	No VOD Mean	P-Value
WBC	5	0.4	16	1.6	.04
HCT	5	43.5	16	42.9	
Platelets	5	50.8	15	94.9	.1
Total Bilirubin	5	1.9	16	1.10	.05
Direct Bilirubin	3	0.6	9	0.2	
GOT	5	73.2	16	49.8	.1
LDH	4	423	14	317	
PT	3	12	13	12	
PTT	3	30	13	32	

Note: *VOD, veno-occlusive disease; WBC, white blood cell count; HCT, hematocrit; GOT, serum glutamic oxaloacetic acid transaminase; LDH, lactate dehydrogenase; PT, prothrombin time; PTT, partial thromboplastin time.*

significantly ($P < .01$) from those who had received PAM at 80 mg/m^2. This statistical analysis excludes two patients, one receiving 40 mg/m^2 PAM and the other 80 mg/m^2 PAM, who developed acute renal failure following therapy and for whom the cause of acute renal failure was multifactorial. These data suggest that nephrotoxicity is a secondary nonmyelosuppressive effect of PAM when it is used in the combination employed in this study.

Table 2. Renal Function Following High-Dose Combination Alkylating Agent Therapy with Cyclophosphamide, Cisplatin, BCNU, and Melphalan

UPN	Dose L-Pam*	Cr_{in}§	Cr_{max}¶	Duration (days)	Sum of Cr Elevations
13	0	.7	1.6	9	3.8
14	0	.8	1.0	4	0.6
15	0	.8	3.2	10	9.0
16	40	.9	1.5	7	2.9
17	40	.7	1.0	5	1.3
18	40	.9	1.4	6	1.9
20	80	.9	3.1	21	29.3
21	80	.8	2.1	17	10.1

**In mg/m^2; all patients received 5.6 g/m^2 CPA, 165 mg/m^2 CDDP, and 600 mg/m^2 BCNU.*

§Baseline creatinine in mg/dl.

¶Maximum creatinine in mg/dl.

DISCUSSION

Phase I trials of single alkylating agents have been performed for CPA, CDDP, BCNU, and PAM. The predominant dose-limiting nonmyelosuppressive toxicity for CPA has been reported to be cardiac and urothelial damage (2,7,8). The dose of CDDP is limited by nephrotoxicity (9). High-dose BCNU produces pulmonary fibrosis and toxic hepatitis (10). PAM causes stomatitis and enterocolitis (11,12). The agents employed in this treatment program were selected because of the differing major nonmyelosuppressive toxicities. We have found unique and unexpected toxicities associated with high-dose alkylating agent combinations that differ from those seen with the single agents.

Refractory thrombocytopenia with evidence of this magnitude of platelet consumption is unusual in the practice of autologous marrow transplantation. The mechanism responsible for the excess platelet consumption is unclear, but endothelial damage related to the alkylating agent therapy has been demonstrated in experimental systems and may play a role in the excess platelet consumption. The importance of alloimmunization requires further study, but does not appear to be fully responsible for the refractory thrombocytopenia.

Chemotherapy-induced hypertension is also unusual. Systemic hypertension has been reported as a complication of intra-arterial CDDP infusion in four patients (13), and renal vascular lesions with the development of accelerated hypertension has been reported in one patient with testicular cancer being treated with vinblastine, bleomycin, and CDDP (14). The hypertension that occurred in 58% of patients treated in this program was not controlled by volume reduction, β-blockade, or treatment with spironolactone. The partial efficacy of the calcium channel-blocking agent nifedipine is intriguing and deserves further evaluation.

VOD developed in four patients and proved fatal in two. Toxic hepatitis but not VOD has been reported in 13% of patients treated with BCNU at high doses, fatal hepatic toxicity occurring at 1200 mg/m^2 in 7% of patients (10,15). Although VOD is seen as a complication in 21% of patients receiving high-dose CPA (120 mg/kg) and total body irradiation (TBI) as preparation for allogeneic or autologous bone marrow transplantation (16), it is rarely seen as a complication of CPA administration alone, even at doses of 200 mg/kg used as the preparative regimen for transplantation of aplastic anemia. The incidence of VOD in regimens combining CPA and busulfan has been estimated to be approximately the same as that seen with CPA and TBI (16). The occurrence of VOD in four of our patients suggests that combinations of alkylating agents may have overlapping toxicity in the production of this complication.

Primate studies inducing VOD have demonstrated that the early pathologic lesion causes endothelial damage and deposits platelets in the region of the endothelial damage (17). The data shown in Table 1 suggest that interpatient variation in drug bioavailability may be partly responsible for the development of VOD. The more rapid decline in the white blood cell count and platelets in the patients developing VOD and the evidence of more severe hepatic toxicity evidenced by the elevation of the SGOT and bilirubin levels suggest that an enhanced exposure or greater drug sensitivity in the patient may be responsible for disease development. Study of drug pharmacokinetics will be valuable in evaluating this toxicity.

Renal dysfunction has not been previously reported as a frequent nonmyelosuppressive toxicity associated with high-dose PAM administration and may reflect a secondary overlapping toxicity seen only in the combination employed in this trial. Nevertheless, the data suggest that the development of nephrotoxicity is possible with PAM and is relevant to the development of future combination therapeutic regimens.

In summary, the use of high-dose combination alkylating agents is associated with the development of novel and unexpected toxicities. The development of refractory thrombocytopenia and VOD suggests that a common mechanism of endothelial damage related to the alkylating agents may be responsible. Severe, persistent hypertension following chemotherapy has been a rare occurrence. The mechanism for this novel, frequent toxicity in this program needs to be studied further. Finally, the identification

of nephrotoxicity as a secondary nonmyelosuppressive toxicity associated with PAM administration has been previously unappreciated and has implications for the development of future therapeutic regimens. Further understanding of the underlying mechanisms may allow prevention or amelioration of the toxicity without compromising the administered dose.

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Panel Discussion: Session V

M. Lassus and J. A. Neidhart, Moderators

Dr. James Neidhart: I have to admit, as a noncardholding bone marrow transplanter, I'm fairly impressed by some of the data presented here in terms of response. Some of the questions are obvious, and I'll risk overstating the obvious. One question is, are we talking about a dose-response phenomenon? In fact, it's not so much a dose-response phenomenon as a toxicity-response phenomenon. We need to know where you are on that curve. It's not clear to me from hearing comments so far that we have defined that curve. It's fantastic that we are exploring it, but I'm a little bit bothered by the fact that there are really three modalities being tested concurrently in the very simplest studies being reported. One of them is the therapeutics of the anticancer drugs. The second two are supportive, supposedly without toxic effects and supposedly without therapeutic effects, but that remains unproved also.

It is fair to test three modalities at the same time in a phase I trial. Some reported studies have brought in another concept that certainly has to be tested--combination chemotherapy. In these studies you're testing as many as seven modalities at once. These trials clearly encompass new concepts in treatment that are quite promising. They are new concepts and approaches to phase I trials.

It seems to me that some discussion on acceptable toxicity is in order. We have heard of 10-30% mortality in some studies. The patients treated in the earliest leukemia studies had no real alternatives, and so transplantation clearly was of benefit and the risk justified. If we are getting into a group of patients with a better prognosis and less heavily treated disease or no prior therapy, what is acceptable toxicity? If a 10-30% mortality is warranted, should doses be tested in phase I trials that produce transient but severe myelosuppression that carries a risk of death? Does intensive supportive therapy or bone marrow transplantation allow you to raise further the dose of that drug or to decrease risk and morbidity? If so, is there a proportionate increase in response? The answers to those questions are not at all clear to me, but it seems the real question is whether multimodality or single-modality therapies should be tested. Mercedes, would you like to add to that?

Dr. Mercedes Lassus: Thank you. When Karel Dicke asked me to come, he asked me to discuss briefly the methodology in phase I trials and the escalations during them. At the beginning it seemed kind of a superfluous statement to make because everybody in the audience had had enormous experience with phase I trials and high-dose chemotherapy. However, looking back, it seems that because of the inherent toxicity of the high-dose chemotherapy trials, there are disagreements still about which is the best way to proceed to diminish the price of toxicity we have to pay. And because of that I would like to start the discussion by briefly summarizing the characteristics of the high-dose chemotherapy trials. Bear in mind that toxicity is inherent in phase I because we are already in the toxic part of the dose curve, since we are treating the patient with life-threatening marrow toxicity. Let's start by remembering that in conventional chemotherapy we select the drugs exclusively because of the clues we have from screening with animal models. In high-dose chemotherapy we are really selecting only drugs that have a dose-limiting myelosuppressive toxicity and that are therefore candidates for salvage therapy. And because we know that we are going to move in the toxic range, and in the lethal range as we have seen, we request that the drug have shown some activity at conventional doses to maximize the success of the treatment.

The starting dose in conventional chemotherapy is based on animal toxicology data, and so far we have been successful. In the high-dose range, we are basing our work on clinical experience, because there is no model for high-dose chemotherapy--the only model is the patient. Therefore, whenever a drug has been escalated in the clinical setting into the

leukemia dose range, which is already a higher dose range, we start the high-dose chemotherapy at the highest leukemic dose that has been tolerated without bone marrow transplantation. Even in the conventional chemotherapy range, the leukemia dose is usually at least two times the solid tumor dose, and it is very rare that we can escalate after the leukemia dose by twice again, which is telling us something regarding the caution with which we should escalate the doses.

When there are no leukemia data, then the point of reference is the solid tumor dose, and one starts by a multiple of that dose based on previous experience, usually 1.5 to 2 times the solid tumor dose, which is the "leukemic" dose. Above the leukemic dose, everybody seems more comfortable going with a much slower escalation in the sense of 25% or 30% at each dose level.

An interesting aspect of these trials has been the observation of delayed organ toxicity, which we will discuss more in the second session when Dr. Herzig presents his data. And we have learned through the model of the phase I high-dose chemotherapy trial that the best time interval for escalations is probably between 6 weeks and 8 weeks to make sure that we don't have delayed or severe organ toxicity.

Solid-organ toxicity has been the problem, which is to be expected because we are really exploring the dose toxicity curve in the region between marrow toxicity and solid-organ toxicity, since we all fervently believe that there, in that particular dose range, is going to be a steep dose-response curve. And as you see from these studies, the incidence of solid-organ toxicity is not negligible. For instance, in trials of 1,3-bis-(2 chloroethyl)-1-nitrosourea, where the target organs have been the lung, liver, central nervous system, and gastrointestinal (GI) tract, 17 deaths have been observed in a series of 114 patients.

I think that the good news of the problem of organ toxicity in high-dose chemotherapy is that fortunately, to a certain extent, the target for solid-organ dose-limiting toxicity is not uniform across the board for the drugs that have been explored. Even the alkylating agents, which are the ones with which we have more experience, do not have the same target-organ toxicity. This difference has been the basis of the trial that Dr. Peters presented. In conventional chemotherapy you usually try to explore each agent separately to determine toxic dose and eventually combine the drugs. My opinion here would be that high-dose chemotherapy should be explored in the same way even if we lose some time.

Dr. George Santos: Let's talk about escalating a new drug with a known drug. We studied it fairly well years ago for marrow transplantation. The maximum tolerated dose of cyclophosphamide was fairly well worked out. We next added increasing doses of busulfan, starting with 8 mg/kg. We found that to be an acceptable way. So I would make a plea that if you have one drug well studied, to start with the other at low doses and bring it up rather than escalate each one separately, which would not be efficient. At least that is my argument.

Dr. William Peters: I think that it is essential to establish the dose-limiting toxicity for each individual agent first. In fact, you may drastically compromise the dose of the second administrated drug because of overlapping toxicity. Dr. Santos, you were lucky that you didn't have substantial enhanced toxicity from having put busulfan and cyclophosphamide together. And in fact, that may have not been the best combination. To select the best combination you like to show that in fact the toxicities are not overlapping first and then put them together. If you take two GI toxic agents and have one at full dose, you are not going to get very far. I think that that was the sort of experience we have seen with our combination of adding melphalan, which resulted in excess nephrotoxicity and excess GI toxicity.

Dr. Emil Frei: I think we ought to remember the principles of successful chemotherapy in the past. You asked a very important question about going to complex combinations before you know all the variables about individual elements. That came up when we went from one drug to four drugs in Hodgkin's disease, but there were certain principles that we

had; that is, we knew that the agents were individually active, but we also knew that we could maintain full doses or nearly full doses when we put them together. And the history of chemotherapy, whether experimental or clinical, almost always is that if you can maintain the dose and put together agents that have qualitatively different mechanisms of action you get at least an additive effect.

Dr. Neidhart: Yes, but is it clear that conventional wisdom and lessons learned at low-dose chemotherapy will hold true when one gets into the range of extremely high dose chemotherapy. In other words, are concepts such as nonoverlapping toxicities, combination chemotherapy being better, etc., clearly valid in this new setting? My answer would probably be yes, but I'm not sure that I know that's true. It is one other area where questions exist. We must define the full dose-response curves and then determine if some of these concepts remain valid.

Dr. Herzig: I agree with Dr. Frei; that is, we would like to see as little toxicity as possible--none if we could. I think that we all recognize, though, that with the doses of a marrow transplantation setting, there is going to be about a 10% incidence of fatal, hematologic complications--sepsis and hemorrhage--during the 3 or 4 weeks of pancytopenia. The same is true for acute leukemia induction therapy. Since you are dealing with that background level of toxicity and since the purpose of that is to allow you to explore the maximum dose of an agent, then it seems to me that what you should be willing to accept, at least until you find out the activity of the agent, a similar degree of toxicity to the other in terms of the dose-limiting organ system. At the end of your study you would really answer the question, was there any increase in activity in this agent when it was given at its clearly maximum dose? If you stop short of that, then at the end of the study you may not have the answer to that question. Two years later somebody may feel he has to go back and find out whether that agent really was active.

Dr. Robert Epstein: I wonder if anybody is considering the contribution of the autologous marrow to the recovery in these patients. We are hearing a lot of different drugs and drug regimes being advanced. I wonder what some of our panelists thought about how one evaluates whether in fact the marrow is an ingredient of the treatment procedure.

Dr. Robert Souhami: I would like to address this question. What we did with cyclophosphamide was to give the marrow back at day 2 after the last dose of cyclophosphamide and then to sequentially delay, by 2-day increments, the return of the bone marrow in cohorts of patients. That way, particularly in an elderly patient group that you are worried about, you don't ever run the risk, in any patient, of causing more than a 2-day delay in the period of aplasia, which might be important. I think there is a general point to make about that, too, and that is the question to be asked is not whether it is absolutely necessary or not. It is not a question of whether a particular regimen is totally and irreversibly ablative, but whether a period of aplasia might be shortened by a period of 4 or 6 days, which might be very important from a toxicity point of view in a middle-aged or elderly population. So it isn't a question of all or none; it is a question of shortening the aplasia and whether it is all worthwhile.

Dr. Herzig: I actually like your idea about making conventional phase I studies go up to the maximum tolerated dose in the sense that I would use it for hematopoietic toxicity, being that dose that produces between 2 and 3 weeks of aplasia. Since a successful marrow transplant still leaves a patient with 2 or 3 weeks, sometimes 4 weeks, of severe pancytopenia, the dose that you would like to start with for the marrow transplant is one that you already know causes that level of aplasia without marrow. Then you'll have both answers--that is, how much escalation you can achieve and whether the marrow was protected from further hematopoietic toxicity. The only other alternative is of course to continue to escalate the dose until you reach some level of patients dying

of aplasia and see if you can prevent that with marrow, but I don't think any of us would be anxious to do that kind of study. Other than that our method of proceeding has been to follow the classical phase I-phase II studies for the reasons that have been put forward. In terms of the studies that we and others have done there is no direct answer about whether the autologous marrow is needed at the doses being used. The indirect evidence is that in the course of the dose escalations no further myelosuppression is seen over the last several dose escalations. For example, the data that Gary Spitzer presented suggests that when he finally got up to aziridinylbenzoquinone doses of 25 mg/m^2 x 5 he saw the level of myelosuppression that you would expect autologous marrow to be useful for. Going up further in the doses hasn't yet produced any extension in the degree of myelosuppression. So I think that is indirect evidence that the autologous marrow limited the degree of myelosuppression.

Dr. Philip: My opinion as a clinical oncologist is that the goal of phase I studies in solid tumors is not to produce myelotoxicity just because we want to use autologous bone marrow transplantation. The interest of phase I and II studies is to study the dose-effect relationship in the field of solid tumors. I want Drs. Frei and Peters to comment on how to use a drug. In your communication, Dr. Peters, you use cyclophosphamide in continuous infusion instead of using it as a bolus to produce a peak, and I think this is a very important point. Is it clear in the minds of the panelists that we have to produce peaks and then wait for myelotoxicity, or do we have to study how to use the drugs to obtain a dose-response relationship? In another example, yesterday, Dr. John Graham-Pole reported data on high-dose melphalan using 3 consecutive days of infusion instead of the 50 mg/m^2 bolus. Did he have data in vivo or in vitro to justify the rationale of his way of administration?

Dr. Peters: I think Dr. Philip's comment is very appropriate. The therapies that we have are going to be very schedule dependent in terms of their ability to produce responses, and I think that giving drugs bolus versus continuous infusion may markedly alter both the toxicity and the therapeutic response. Our choice of continuous infusion cisplatin was motivated by two considerations: 1) in our experience at the Farber we had found more myelosuppression and less nephrotoxicity by administering cisplatin by continuous infusion; 2) the continuous infusion administration of the drug was substantially better tolerated in terms of nausea and vomiting than by giving it by pulse. We reasoned, therefore, that these two parameters might allow us to deliver additional drug compared with what could be done by a bolus technique. That may or may not be true in the long run. The mode by which the drug is given may really affect the way the response occurs. Peak doses, particularly with cyclophosphamide, we believe may in fact contribute to toxicity. We will probably initiate a trial of continuous-infusion cyclophosphamide in an effort to reduce what we believe is endothelial damage related to peak doses of cyclophosphamide in an effort to increase the exposure of cells to the drug. To try to minimize the toxicity there are a variety of things that need to be explored. I think Dr. Philip was right on the nose.

Dr. Emil Freireich: I am one of those archeological relics who has never accepted the notion that the purpose of a phase I study is primarily to define toxicity. In a way, it sounds like an academic point, but I believe that as I listen to the discussion of people talking about phase I studies, it actually affects the design of the study in a fundamental way. In my opinion, the purpose of a phase I study, like the purpose of all clinical investigation, is therapeutic first and foremost. The secondary objective, which is far below that, is to define the toxicity and the dose-limiting biological effect. Now why do I feel that this is fundamentally important? Because, particularly in the bone marrow transplantation field where we are seeking enhanced effects by expanding the dose-response curve, we have to remember that what we are seeking is specificity, not mortality. The hypothesis that the escalation in toxicity will be at the same dose response as the escalation of antitumor

effect is unproved, and it should not be prejudged. So if your primary hypothesis being tested in phase I is seeking increased therapeutic efficacy, then the evaluation of the therapy-to-toxicity ratio is the fundamental purpose of the phase I studies. I think that as we gain experience in these studies, we are going to find inversions; that is, I think we are going to find at increasing doses drugs in combinations that diminish the therapeutic benefit rather than enhance it. It may not be essentially true that you always enhance therapy by increasing dose.

VI. Phase I Studies: Part Two

Phase I Studies with

Autologous Bone Marrow Transplantation

G. P. Herzig, R. H. Herzig, J. W. Fay,
S. N. Wolff, D. Hurd, H. M. Lazarus, and G. L. Phillips

Bone marrow transplantation limits the duration of pancytopenia produced by myelosuppressive agents and permits evaluation of high-dose regimens that would otherwise cause prohibitive marrow toxicity. The rationale for intensive cancer therapy is the dose-response relationship demonstrated in animal tumor studies and in assays of tumor cell sensitivity to drugs and radiation in vitro (1). The strongest clinical evidence supporting a dose-effect relationship in cancer treatment is the cure of refractory acute leukemia with intensive drug and radiation regimens followed by an allogeneic marrow transplantation (2). Unfortunately, allogeneic transplantation requires a donor whose major histocompatibility complex (MHC) is compatible with that of the donor, and it is associated with serious morbidity and mortality (approximately 20% of patients contract fatal graft-versus-host disease [GVHD]; another 20%, interstitial pneumonitis) that prevent its widespread application. Further, in order to prevent graft rejection, allogeneic transplant regimens must include potent immunosuppressive agents that add to toxicity but may not be effective antitumor therapy.

The use of autologous marrow avoids most of the problems of allogeneic transplantation. For this technique, a portion of the patient's marrow is withdrawn and stored for reinfusion after treatment has been completed. Marrow can be stored for at least 4 years with current methods of cryopreservation. Because the marrow cannot be rejected, the treatment regimen is not immunosuppressive and GVHD does not occur. With autologous marrow transplantation the problem of greatest concern is the possibility of occult tumor in the stored marrow. It is encouraging that recent animal studies have demonstrated the feasibility of eliminating tumor cells from marrow without damaging normal hematopoietic stem cells by treating marrow in vitro with drugs or tumor-specific antibodies (3,4). Another deficiency of using autologous marrow is lack of a "graft-versus-tumor" immunologic reaction that contributes significantly to the antitumor effect of allogeneic marrow transplantation (5). Long-term remission is possible, though, without a graft-versus-tumor reaction, as has been shown by transplants between identical twins (6).

With the ability of cryopreserved autologous marrow to restore hematopoiesis firmly established, clinical studies have begun to define its role in cancer therapy (7). One approach is to devise intensive combinations of agents empirically, using marrow transplantation to limit the anticipated (additive) myelosuppression. The lack of information concerning toxicity and effectiveness of most agents in high doses makes this approach unsatisfactory because extrapolation from experience with conventional doses is not reliable. As an alternative, autologous marrow transplantation can be used to extend phase I (dose-escalation) studies of single agents that are limited by myelosuppression. Beginning at the maximum used in conventional trials, physicians increase the dose until severe toxicity occurs in another organ system. Although few patients are likely to achieve prolonged disease-free survival with single agents even in high doses, these trials permit the design of high-dose combinations using agents that share antitumor activity but differ in dose-limiting nonhematologic toxicity.

The selection of agents for high-dose evaluation is based on myelosuppressive potency, antitumor activity, and nonhematologic toxicity at conventional doses. Special consideration may also be given to agents with unusual mechanisms of action or desirable pharmacologic behavior (e.g., central nervous system penetration). The most important feature, however, is the production of prolonged, severe myelosuppression without other significant toxicity. Lack of demonstrated antitumor activity

should not exclude an agent from study since dose escalations of tenfold or greater may reveal significant responses when none was apparent at lower doses.

We have completed the phase I evaluation of three single agents (1,3-bis-(2 chloroethyl)-1-nitrosourea [BCNU], mitomycin C, and melphalan) and two combinations (cyclophosphamide and fractionated total body irradiation [TBI]); melphalan and BCNU. As summarized in Table 1, prevention of prolonged myelosuppression by autologous marrow transplantation permits an increase of up to six times the usual dose, and up to two times the maximum dose tolerated without bone marrow transplantation. The dose-limiting nonhematologic toxicities encountered were hepatic (found with BCNU and mitomycin C), pulmonary (BCNU, mitomycin C, and TBI), cardiac (mitomycin C), and gastrointestinal (melphalan).

Studies of high-dose BCNU were begun at a dose of 600 mg/m^2 (200 mg/m^2/day for 3 days), the highest dose previously given without marrow transplantation (8). Dose escalation was carried out in successive groups of patients up to a dose of 2850 mg/m^2. The maximum tolerated dose was 1200 mg/m^2. At this dose fatal toxicity levels were reached in eight patients (two in the liver, six in the lung) for an incidence of 13% in the 60 patients at risk for at least 2 months. Doses greater than 1500 mg/m^2 resulted in prohibitive liver toxicity, though lung toxicity was not increased. Fatal encephalomyelopathy appeared at the highest doses (2250-2850 mg/m^2). Hematologic toxicity was severe but neutrophil recovery (>500/μl) occurred in a median of 17 days, and platelets recovered (>10,000/μl) in a median of 18 days after marrow infusion. Delayed myelosuppression was not seen. Both liver toxicity (centrilobular necrosis) and lung toxicity (diffuse interstitial pneumonitis) were delayed in onset, typically appearing 6-8 weeks after transplantation. The incidence of lung toxicity is similar to that reported after comparable cumulative BCNU doses administered in a conventional schedule (9). An unexplained finding is the association of fatal liver toxicity with the presence of primary or metastatic brain tumor in nine of ten instances. The association is not due to the concomitant use of dilantin, glucocorticoids, or other medications.

Studies with mitomycin C began at a dose of 60 mg/m^2 (20 mg/m^2/day for 3 days). Veno-occlusive disease of the liver occurred in two of four evaluable patients (one patient died) at 90 mg/m^2, the highest dose tested (10). During subsequent treatment with declining doses fatal toxicity occurred in three of nine patients treated with 75 mg/m^2 (one because of hepatic veno-occlusive disease, two because of pleuropericarditis). A total of 20 patients were then treated at the starting dose. Severe toxicity developed in three patients (in one fatally)--one instance was due to hepatic veno-occlusive disease, one to pleuropericarditis, and one to interstitial pneumonitis. No renal toxicity or hemorrhagic enterocolitis was observed. Because of the development of severe toxicity involving multiple organ systems at the 60-mg/m^2 dose, it appears that no significant increase in the mitomycin-C dose above the maximum tolerated level (40 mg/m^2) without marrow transplantation is possible.

Thirty-three patients were treated in the phase I evaluation of high-dose melphalan (11). Dose-limiting mucositis (stomatitis and diarrhea) occurred in all nine patients who received a dose of 225 mg/m^2 (75 mg/m^2/day for 3 days), the highest dose tested, although no case was fatal. Reduction of the dose to 180 mg/m^2 resulted in a 15% incidence of severe mucositis, and this dose is recommended for phase II studies. All patients experienced severe hematologic toxicity; however, neutrophils recovered (>500/μl) in a median of 19 days, and platelets recovered (>20,000/μl) in a median of 24 days after marrow transplantation.

A phase I study was carried out to determine the maximum tolerated dose of melphalan and BCNU in combination. Interest in this combination followed the demonstration of substantial activity of both agents against melanoma and their apparent lack of overlapping toxicities. The starting dose was 50% of the maximum tolerated single-agent dose (melphalan, 90 mg/m^2; BCNU, 600 mg/m^2), and doses were increased stepwise to the full single-agent dose of each (melphalan, 180 mg/m^2; BCNU, 1200 mg/m^2). At the highest dose level, prohibitive pulmonary toxicity occurred with two severe and three fatal episodes for 10 patients. Twenty patients have

Table 1. Phase I Studies
with Autologous Marrow Transplantation

Agents (Units)	DOSE			Limiting Toxicity
	Usual	Maximum Without AMT	Maximum With AMT	
BCNU (mg/m^2)	200	600	1200	Hepatic, Pulmonary
Mitomycin C (mg/m^2)	20	40	40	Hepatic, Pulmonary, Cardiac
Melphalan (mg/m^2)	35	140	180	GI
Cyclophosphamide (mg/kg)/TBI (rad)	---	---	120/1400	Pulmonary
Melphalan (mg/m^2)/BCNU (mg/m^2)	—	—	180/900	Pulmonary

Note: *AMT, autologous marrow transplantation; TBI, total body irradiation (2-Gy fractions twice daily).*

been treated at the maximum tolerated dose (melphalan, 180 mg/m^2; BCNU, 900 mg/m^2), resulting in one case of severe and one case of fatal pulmonary toxicity. Other severe (but not fatal) toxicities at this dose include protracted nausea and vomiting (one patient), and abnormal liver enzymes (one patient).

A phase I study of cyclophosphamide (120 mg/kg) and TBI was carried out to determine the dose of fractionated TBI with toxicity equivalent to the usual transplant regimen of 10 Gy TBI in a single dose (at dose rates of 5-10 Gy/min). In our previous experience involving 13 patients with refractory leukemia and lymphoma treated with a single TBI dose of 10 Gy, there was one episode (7.7%) of fatal interstitial pneumonitis. The split-dose TBI was administered in 2-Gy fractions (.45 Gy/min) twice daily with a minimum of 6 h between fractions. The advantages of the split-dose technique are improved patient comfort because of the short treatment sessions and a more uniform dose distribution achieved with high-energy photons. Fatal pulmonary toxicity occurred in all four patients treated with 16-Gy TBI, the highest dose given. Well-tolerated doses of 12 Gy and 14 Gy produced fatal pulmonary toxicity in only 1/14 (7.1%) and 2/25 (8%) patients, respectively.

These phase I studies suggest that autologous marrow transplantation will only permit modest dose escalation for most myelosuppressive agents before limiting toxicity to other organ systems appears. The increment achieved may be up to 10 times the usual dose, but rarely will it be more than twice the maximum possible without marrow transplantation. Changes of this magnitude are not likely to improve the response of resistant tumors to single agents significantly. However, such studies are needed to define the spectrum of antitumor activity and extramedullary toxicity since neither can be reliably predicted from the results at conventional doses. In this way it will be possible to identify agents that share activity against a specific tumor but differ in nonhematologic toxicity for use in high-dose combinations. Based on the results of conventional therapy, combinations of three or four active agents may be expected to have curative potential.

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High-Dose Chemotherapy with Autologous Bone Marrow Transplantation in Solid Tumors: A Wayne State University Experience

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Over the past 4 years, the Autologous Bone Marrow Transplant Program at Wayne State University, Detroit, Michigan, has focused its research effort on three phase I-II studies: (*a*) use of high-dose mitomycin C (MMC); (*b*) use of high-dose cyclophosphamide, VP-16-213, and cisplatin in treating refractory germ cell tumors; and (*c*) use of high-dose L-phenylalanine mustard (L-PAM) and dibromodulcitol (DBD) in treating a group of patients with metastatic malignant melanoma with poor prognosis. In this report, we wish to summarize our results thus far and discuss the potential use of animal toxicology models in selecting the optimal drugs or combination of drugs in this setting.

HIGH-DOSE MMC

Twelve patients with various refractory malignancies were entered into this study; one of the patients received two courses. All patients except one received autologous bone marrow rescue. Marrow had been harvested and cryopreserved 1-26 weeks prior to the administration of MMC. The remaining patient received transplanted marrow from her identical twin sister. We selected a fixed total dose of 60 mg/m^2 based on the starting dose of 20 mg/m^2/day for 3 days used in another study (1). Four dose schedules were studied: (schedule A) 60 mg/m^2 by single intravenous infusion over 60 min (one patient); (schedule B) 30 mg/m^2/day for 2 days, 15-min infusion (five courses in four patients); (schedule C) 30 mg/m^2/day for 2 days, 60-min infusion (three patients); (schedule D) 15 mg/m^2/day for 4 days, 60-min infusion (four patients). Timed plasma and urine samples from nine courses of therapy were subjected to high-performance liquid chromatography assay and pharmacokinetic analysis (2). All patients have since died, and autopsies were performed in nine cases.

Four partial remissions were seen in patients with breast, colon, lung, and malignant fibrous histiocytoma, but none were of significant clinical benefit. Correlation of the dose schedule, pharmacokinetics, and extramedullary toxicities are summarized in Table 1.

In this small series of patients, we have made two important observations. First, the peak concentration of MMC appeared to be in direct proportion to the dose and infusion time. Furthermore, we could not confirm the existence of saturation kinetics as suggested by other investigators because the terminal half-lives were not increased over fourfold of dose escalation (3). Second, dose schedule was the major determinant of extramedullary toxicities (i.e., hemorrhagic colitis was encountered in six out of six courses in schedules A and B and two out of seven in schedules C and D); hepatic veno-occlusive disease occurred in four out of five in schedules A and B, and two out of four in schedules C and D.

Because of the toxicities seen at threefold of the conventional dose, we concluded that MMC is unlikely to be a useful single high-dose agent in the autologous bone marrow transplant (ABMT) setting.

ANIMAL TOXICOLOGY MODEL FOR DRUG SELECTION

The severe extramedullary toxicities of high-dose MMC exemplified the major problem of chemotherapeutic cytoreduction in ABMT. On the other hand, a supralethal dose of total body irradiation (in leukemia and lymphoma) has been successfully applied in clinical bone marrow transplan-

Table 1. Dose Schedule, Pharmacokinetics, and Extramedullary Toxicities

Schedule	Peak MMC (ng/ml)	AUC (ng.h/ml)	t1/2 (min)	HC	VOD[a]
A	1965	2712	48.9	1/1	1/1
B	2035 ± 303	1998 ± 514	47.9 ± 3.7	5/5	3/4[b]
C	792 ± 447	1082 ± 710	41.3 ± 26.5	1/3	1/2
D	346 ± 151	582 ± 397	48.6 ± 5.9	1/4[c]	1/2[b]

Note: *AUC, area under the curve; t1/2, terminal half-life; HC, hemorrhagic colitis; VOD, veno-occlusive disease.*

[a]*As determined at autopsy in nine patients.*

[b]*One of the patients in this group had documented extrahepatic biliary obstruction at autopsy.*

[c]*At autopsy, patient was found to have extensive infiltration of tumor in the submucosa and lymphatic channels throughout the entire colon and segments of small bowels.*

tation, resulting in some cures, and the toxicities to the nonhematopoietic tissues are predictable and tolerable. The difference in the pattern of toxicities of these two modalities is likely owed to the mechanisms of cytotoxicity of radiation (a physical agent) and chemotherapy (involving a complex metabolic process), thus resulting in difficulty in predicting organ toxicities. Furthermore, bone marrow transplantation has been consistently demonstrated to be effective in rescuing animals from marrow-ablative doses of radiation, but the similar rescue of mice treated with high-dose chemotherapy has not always been possible. At least in one murine model, Drs. F. H. Schabel and T. H. Corbett (unpublished data) could not demonstrate a clear improvement in survival of animals receiving a high-dose single intravenous injection of 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU), L-PAM, or MMC with syngeneic bone marrow transplantation, since the animals still died from other organ toxicities not protected by the stem cell rescue. An animal model for high-dose chemotherapy and ABMT is obviously needed for identifying ideal drugs, drug combinations, and dose schedules in this setting.

Despite the lack of a suitable animal model for high-dose chemotherapy with ABMT, animal toxicology models can potentially be very helpful in selecting agents or a combination of agents that is less likely to produce cumulative toxicities and perhaps delayed organ toxicities other than the bone marrow. To quantitate the degree of cumulative and delayed toxicities, researchers have proposed the ratio of total lethal dose (LD_{10}) for multiple-course treatment and single-dose treatment (M/S ratio). Some of the results are shown in Table 2. Therefore, the drug that gives the highest M/S ratio will be the one with the best therapeutic margin and the one least likely to produce cumulative or delayed organ toxicities.

Another potential clinical application of an animal toxicology model in ABMT is in the area of screening drug combinations. Ideally, one would choose the most active, non-cross-resistant single agents with the fewest overlapping toxicities to be used in combination to result in significant potentiation of antitumor effect. The degree of overlapping toxicity in animals can be measured by the "combination toxicity index" (CTI), defined by Skipper (4) as the sum of the fraction of LD_{10} for each agent used in combination to produce equivalent toxicity of LD_{10} when an agent is used alone. In ABMT, it may be important to determine the CTI for both single- and multiple-course treatments. Some of the CTI values for two alkylating

Table 2. M/S Ratio of Selected Agents

Agents	Mouse	Total LD_{10} Value (mg/kg)		
		Single	Multiple (30 days)	M/S
Piperazinedione	CDF1	14.4	55	3.8
AZQ	CDF1	15	54	3.6
L-PAM	BDF1	14	44	3.1
DBD	C3H	478	1200	2.5
Cyclophosphamide	BDF1	253	530	2.1
Cisplatin	CDF1	13	25	1.9
BCNU	BDF1	36	65	1.8
Mitomycin C	C57B1/6	4.2	Unknown	?<1.5

Note: *These data are mostly from single unconfirmed trials and abstracted from "LD_{10} Summary. Toxicity Testing of Anticancer Drugs in Small Experimental Animals; Total Experience May 16, 1960-August 23, 1980," published by the Southern Research Institute. M/S, multiple-course treatment/single-dose treatment; LD, lethal dose; AZQ, aziridinylbenzoquinone.*

combinations are the following: 1.3 for L-PAM and DBD, 1.2-1.3 for cyclophosphamide and BCNU, 1.2 for cyclophosphamide and L-PAM, and 1.1 for L-PAM and BCNU.

PHASE I-II STUDY OF L-PAM AND DBD IN MELANOMA

A combination of L-PAM and DBD was chosen for study because of its high CTI value and its individual antitumor activity in melanoma. The attractiveness of L-PAM is the magnitude of dose escalation that one can achieve (approximately tenfold the conventional dose) and the report by Selby et al of the response rate of 46% and 50% in metastatic melanoma of the lung and liver. At the conventional dosage, DBD has been found to be active, and five (two complete remissions and three partial remissions) out of 25 cases refractory to treatment with dacarbazine (DTIC) and nitrosourea responded (5). This combination is synergistic in advanced mammary adenocarcinoma 18/c in C_3 H/He mice (tumor weight at treatment, 200-800 mg), and the results are shown in Table 3. The combination of L-PAM and DBD was also potentiating against advanced stage mammary adenocarcinoma 16/c (100-600-mg tumor) with a single injection schedule. Results are shown in Table 4.

In the clinical study we selected patients with visceral involvement who had been either previously untreated or whose disease had failed to respond to first-line therapy. Two patients were treated at the step 1 dose, which consisted of L-PAM 30 mg/m^2/day for 3 days and DBD 200 mg/m^2/day for 5 days. Patients who had stable disease or responded after the first course of treatment were retreated with the same dose schedule provided that the marrow for the second rescue was available. The interval between the first and second course was at least 4 weeks from the resolution of toxicities so that delayed or cumulative organ toxicities could be avoided. The tumor response in these two patients was less than a partial remission. Hematologic toxicity is summarized in Table 5.

PILOT PHASE I-II STUDIES IN RELAPSED OR REFRACTORY GERM CELL TUMORS

The possibility of using high-dose chemotherapy and ABMT as salvage treatment for patients with refractory germ cell tumors is suggested by the reports of Spitzer et al and Ozols et al (6,7). In addition to high-

Table 3. Combination of L-PAM and DBD in Mammary Adenocarcinoma 18/c

Drugs	HNTD (mg/kg/dose)	ILS (%)	Growth Delay (days)	Log_{10} Cell Kill	CRs
DBD	240	46	15	1.07	2/12
L-PAM	3.8	31	16.5	1.18	0/12
L-PAM + DBD	140/2.3	91	30	2.14	4/12

Note: *Tumors were implanted subcutaneously by trocar as 30-60-mg fragments on day 0. First treatment was delayed until day 18 (200-800 mg). DBD was administered orally, Q7d x 7. L-PAM was administered intravenously, Q7d x 7. Dosages above those listed were greater than LD_{30}. The tumor volume doubling time was 4.2 days. The day of death (median) for the control group was day 50. HNTD, highest nontoxic dose.*

Table 4. Combination of L-PAM and DBD in Mammary Adenocarcinoma 16/c

Drugs	Route and Schedule	HNTD (mg/kg)	Median Day of Death	ILS (%)	Median Tumor Size on Day 14	T/C (%)	PRs	CRs
Control	-	-	19	-	2551	-	-	-
DBD	po, day 8	270	19.5	3	582	23	1/12	0/12
L-PAM	i.v., day 8	4.8	20	5	1582	62	0/12	0/12
DBD	po, day 8	200	23	21	63	2	8/12	3/12
L-PAM	i.v., day 8	3.4						

Note: *Tumors were implanted subcutaneously by trocar as 30-60-mg fragments on day 0. A single treatment schedule was used (one time only, day 8). Dosages above those listed were greater than LD_{20}. The tumor volume doubling time was 1.5 days. The CTI was 1.3. There were no cures in any of the treatment groups. HNTD, highest nontoxic dose; ILS, increased life span; T/C, treated/control; PRs, partial remissions; CRs, complete remissions; po, orally.*

Table 5. Toxicities of High-Dose L-PAM and DBD in Treating Melanoma

Patients	Cell Dose (x 10^8/kg)	Number Days with Platelets <20,000/mm³	Number Days with Granulocytes <500/mm³
LH (course 1)	1.96	2	10
LH (course 2)		4	10
GM (course 1)	1.90	7	23

Note: *Other toxicities included grade 1 diarrhea (day -3), grade 2 nausea/vomiting (day -3), and grade 2 stomatitis (day -1).*

Table 6. Schedule of High-Dose Chemotherapy and ABMT in Germ Cell Tumors

Drugs	Day of Treatment							
	1	2	3	4	5	6	7	8
Cyclophosphamide (1.5 g/m²/day)	X	X	X					
VP-16-213 (200 mg/m²/day)	X	X	X					
Cisplatin (40 mg/m²/day)	X	X	X	X	X			
ABMT[a]								X

[a] *ABMT, autologous bone marrow transplantation.*

Table 7. Hematopoietic Recovery in Five Patients with Germ Cell Tumors after High-Dose Chemotherapy and ABMT

Patients	Cell Dose (x 10^8/kg)	Number of Days with	
		Platelets <20,000/mm³	Granulocytes <500/mm³
RT (course 1)	2.7	2	15
RT (course 2)	2.7	11	16
GH	1.45	4	19
MC	0.25	24	24
BB	1.57	22	18
TK	1.30	10	16

dose cyclophosphamide and VP-16-213 at the previously established dose schedule, we added high-dose cisplatin, which has been shown to have a steep dose-response relationship in this tumor (8). The treatment schema for the first dose level of cyclophosphamide, VP-16-213, and cisplatin are shown in Table 6.

In this pilot, we had given six courses of treatment to five patients. All patients had documented progressive disease following induction therapy with VBP (velban, bleomycin, and cisplatin) and surgical cytoreduction and had undergone treatment with a salvage combination of VP-16-213 and cisplatin prior to entry into this study. Complete remissions were obtained in three patients with normalization of the beta-subunit of human chorionic gonadotropin; the remaining patients had only partial remissions. The duration of unmaintained complete remissions ranged 2-5 months. One patient (RT) relapsed after 3 months of complete remission and achieved another complete remission with the second treatment. Nonhematologic toxicities encountered were two episodes of renal failure and hyperbilirubinemia; both were reversible. All patients developed severe nausea and vomiting, which were manageable with intravenous fluid and antiemetics. Kinetics of hematopoietic recovery are shown in Table 7. The low marrow cell dose used in the rescue treatment for patient MC resulted in a longer duration of myelosuppression and also suggested that the dose of chemotherapy employed in this study is not marrow ablative.

CONCLUSION

In this review of our experience with high-dose chemotherapy and ABMT, we have shared with other investigators the difficulty in drug selection for this setting. We hope to be able to prevent such difficulty with the animal toxicology models, which may possibly help us to select the most active and least toxic combination. The development of the combinations such as L-PAM and DBD may be the beginning of this effort. We believe the ultimate success of ABMT may depend on the development of drug combinations that allow a greater magnitude of dose escalation and thus overcome tumor resistance. Until then, the role of ABMT will probably be most promising in the treatment of patients with chemotherapy-responsive tumors who are unlikely to achieve a durable remission with conventional doses of chemotherapy; nonetheless, this subset of patients remains to be identified.

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High-Dose Chemotherapy with Autologous Bone Marrow Transplantation for Primary Tumors of the Central Nervous System: Phase II and III Studies of the Southeastern Cancer Study Group

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INTRODUCTION

Routine treatment of malignant primary neoplasms of the central nervous system (CNS) is partial surgical resection followed by radiation therapy. Recently, the addition of chemotherapy to this standard approach has been evaluated. Although slight improvement in survival has been demonstrated, the outlook still remains dismal for many patients (1). Glial cell tumors, which make up the majority of all primary CNS tumors, have an especially poor prognosis. Treated with combined modality therapy, including surgery, postoperative radiotherapy, and adjuvant chemotherapy, half the patients with high-grade gliomas survive less than 12 months from diagnosis, and there are virtually no long-term survivors (2). The inability of chemotherapy to substantially alter the course of these tumors is, in part, due to the lack of active agents, the inadequate exposure of the tumor to drugs, inherent tumor cell resistance, and poor patient tolerance of cytotoxic therapy.

Recently, administering high-dose myelosuppressive chemotherapy and radiotherapy has become feasible when restoration of hematopoiesis is assured by the technique of autologous bone marrow transplantation (ABMT) (3). In particular, as a method of improving drug exposure for tumors of the CNS, high-dose chemotherapy is an attractive experimental model. This report details the experience of the Southeastern Cancer Study Group (SECSG) with high-dose chemotherapy for the treatment of malignant gliomas.

MATERIALS AND METHODS

VP-16-213 and 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU) have previously been evaluated by the SECSG in suprastandard doses with ABMT, redefining the maximally tolerated levels of high-dose therapy well beyond standard doses (4,5). These agents, at their new maximally tolerated dose, were then studied in phase II programs for tumors of the CNS since each at standard dose had demonstrated activity against CNS tumors. Patients eligible for these phase II studies had to have measurable recurrent or progressive CNS tumors, no prior treatment with the intended agent, be suitable candidates for the rigors of high-dose chemotherapy and ABMT, and give informed consent. After recovery from therapy, patients underwent no further therapy and were observed until disease progression occurred. Corticosteroids and mannitol were administered as necessary to control cerebral edema and were frequently given during the period of chemotherapy administration.

BCNU, having demonstrated in standard doses activity against gliomas and having produced encouraging results during the high-dose phase II trial, was administered to patients in a phase III trial. Patients eligible had to have a documented high-grade glioma, no prior chemotherapy with BCNU, and have received standard radiotherapy usually consisting of 55-60 Gy using standard fractionation. In order to avoid selection bias, BCNU was scheduled to be given within 1 month of completing radiotherapy and thus given adjuvantly before tumor progression. After completion of BCNU therapy, no further cytotoxic treatment was given until progression.

VP-16-213 was administered over 3 days at daily doses of 700-800 mg/m^2. One to two courses of chemotherapy each followed by ABMT were accomplished. BCNU was administered over 3 days at daily doses of 350-400

mg/m^2. Because of concern about excessive extramedullary toxicity, patients treated adjuvantly were scheduled to receive a total dose of BCNU of 1050 mg/m^2 (daily doses of 350 mg/m^2).

Bone marrow harvest, cryopreservation, and reinfusion were performed as previously reported (3). The minimum mandatory dose of nucleated marrow cells per transplant was required to be greater than 1.0 x 10^8/kg body weight.

Evaluation included a detailed neurological examination and contrast-enhancing brain computerized tomographic (CT) scan before and at 1 and 3 months after therapy and then every 6-12 months. Patients were treated in single rooms without specialized isolation procedures. Hyperalimentation, broad-spectrum antibiotics, and irradiated (25 Gy) blood component support were used as indicated.

The response criteria used in this study have been reported previously (6). Tumor regression was defined as improvement in neurological findings and a definite decrease in contrast enhancement on CT scan while patients were taking constant or decreasing doses of glucocorticoids; progressive disease was anything else. Patients who died of nonmalignant causes without proof at necropsy of malignant glioma were considered to have progression at that date. Survival and response duration were calculated from the day of transplantation for the phase II studies and from diagnosis for the phase III studies. Actuarial survival was calculated by the method of Kaplan and Meier (7).

RESULTS

As of July 1984, 27 patients had been treated. There were 18 males and 9 females. Patients ranged in age from 8 to 68 years (median, 39 years). According to histological findings, 20 patients had glioblastoma multiforme and seven had anaplastic astrocytoma. Five had undergone prior chemotherapy. The time from diagnosis to treatment with BCNU ranged from 0 to 60 months (median, 7 months). Fifteen patients were classified in the Eastern Cooperative Oncology Group (ECOG) 0-1 performance status category, four in ECOG 2, four in ECOG 3, and four in ECOG 4. Therefore, most patients had a diagnosis of glioblastoma multiforme, and almost one-half of the patients had a poor performance status. Twelve patients (44%) demonstrated a response with a median duration of 4 months (range, 1-71+ months). Two of these patients are now long-term survivors at 43 and 71 months. The first of these two patients is now exhibiting symptoms of relapse, but the second patient remains completely well. Hematological toxicity was acceptable, only two patients (7%) had a fatal outcome owed to neutropenia-associated sepsis. As previously described, nonhematological toxicity occurred in three major organ systems--liver, lung, and brain. In these 27 patients, two episodes of fatal hepatic necrosis, one episode of fatal interstitial pneumonia, and one episode of fatal encephalomyelopathy occurred.

As of July 1984, eight patients (four males, four females) with progressive or recurrent tumor received high-dose VP-16-213 with ABMT. Patients ranged in age from 14 to 52 years (median, 40 years). Histological analysis showed five patients to have glioblastoma multiforme and three to have anaplastic astrocytoma. Of the eight, six had undergone prior chemotherapy. The time from diagnosis to treatment ranged from 4 to 70 months (median, 22 months). One patient was classed in the ECOG 0-1 performance status group, four in ECOG 2, one in ECOG 3, and two in ECOG 4. Four patients responded to therapy that lasted from 4 to 8 months. Compared with the BCNU phase II patients, these patients have poorer performance status and were more resistant to therapy because of previous extensive chemotherapy exposure. All eight patients tolerated therapy without any episodes of severe toxicity.

As of July 1984, 12 patients (eight men, four women) received high-dose BCNU as part of initial treatment and were all evaluable. These patients ranged in age from 26 to 64 years (median, 35 years). Histological analysis found all 12 to have glioblastoma multiforme. Two had undergone prior chemotherapy with hydroxyurea during radiation therapy. The time from diagnosis to first treatment with BCNU ranged from 3 to 12

months (median, 4 months). Ten patients were classed in the ECOG 0-1 performance group, one in ECOG 2, one in ECOG 3, and four in ECOG 4. (Four of these have not yet received transplantation and are not evaluable.) Eleven patients had BCNU administered within 1 month of completing radiotherapy and therefore approximately 3-4 months after diagnosis. One patient, because of an undefined severe cardiomyopathy, had the administration of BCNU delayed until 12 months after diagnosis. This patient, the only patient to receive BCNU at a dose of 1200 mg/m^2, developed fatal interstitial pneumonia. Five patients have died: three of tumor progression at 5, 16, and 20 months after diagnosis, one of a second malignancy without full postmortem examination at 5 months, and one of fatal pulmonary toxicity at 15 months. Seven patients are alive at 7, 11, 15, 15, 19, 39, and 59 months postdiagnosis. Six of these patients are well without tumor progression. One patient, at 15 months has required a repeat craniotomy for recurrent and progressive tumor. Actuarial survival is shown in Fig 1.

DISCUSSION

ABMT is a mechanism for administering suprastandard doses of cytotoxic therapy. Presumably, for many tumors, augmentation of cytotoxic activity will occur with escalation of dose. The CNS, normally regarded as excluding adequate penetration of certain drugs, is an ideal system in which to evaluate the potential dose-response relationship. In the three studies reported here, the SECSG has evaluated in phase II studies the drugs BCNU and VP-16-213 with ABMT. Both of these agents at standard doses have demonstrated activity against primary CNS tumors and therefore might be expected to demonstrate increased activity when administered at high-dose levels (8,9).

BCNU given at 1050-1200 mg/m^2 to patients with recurrent CNS tumors was associated with a response rate of 44% and long-term survival in 7% of patients. In previous phase II studies using BCNU for recurrent gliomas, response rates as high as 50-60% have been reported, though in general the criteria for response were not both clinical and roentgenographic improvement (8). In the study reported here, although the overall response rate is not substantially different from that found in other studies, the observation of long-term survivors (with glioblastoma multiforme) suggests improved efficacy of the high-dose therapy.

The effectiveness of VP-16-213 against CNS tumors has not been previously carefully studied. In a recent report, 3 of 18 (17%) evaluable patients demonstrated an authentic response to VP-16-213 given at 50-100 mg/m^2 for 5 days every 3 weeks (9). Additionally, six patients demonstrated clinical improvement without change in CT scan findings. Pharmacologic investigation of high-dose VP-16-213 therapy has demonstrated adequate CNS penetration in both cerebrospinal fluid and tumor fluid that was not associated with standard-dose studies (10). In our eight patients treated--there were four responses, but the median duration of response was only 4 months. Further accrual to this study is ongoing to truly define the effectiveness of high-dose VP-16-213.

In data not reported and essentially identical to that reported in previous phase I studies, both high-dose BCNU and VP-16-213 produced a degree and duration of cytopenias that are tolerable for patients with CNS tumors. Only 7% of the BCNU patients died of neutropenia-associated sepsis, and none of the VP-16-213-treated patients died during therapy. BCNU and VP-16-213 at these doses produce about an average duration of severe cytopenias (platelets <20,000/μl and neutrophils <500/μl) of about 7 days. Severe nonhematologic toxicity occurred only in the BCNU-treated group (39 patients): fatal interstitial pneumonia occurred in two patients, fatal hepatic necrosis occurred in two, and leukoencephalopathy occurred in one.

With the encouraging results obtained in the BCNU phase II study, BCNU was administered shortly after primary treatment with radiotherapy of high-grade gliomas. This study design was similar to that of previous studies using BCNU as a standard dose. At latest evaluation, two patients have no evidence of tumor by CT scan and are well at more than 3 years

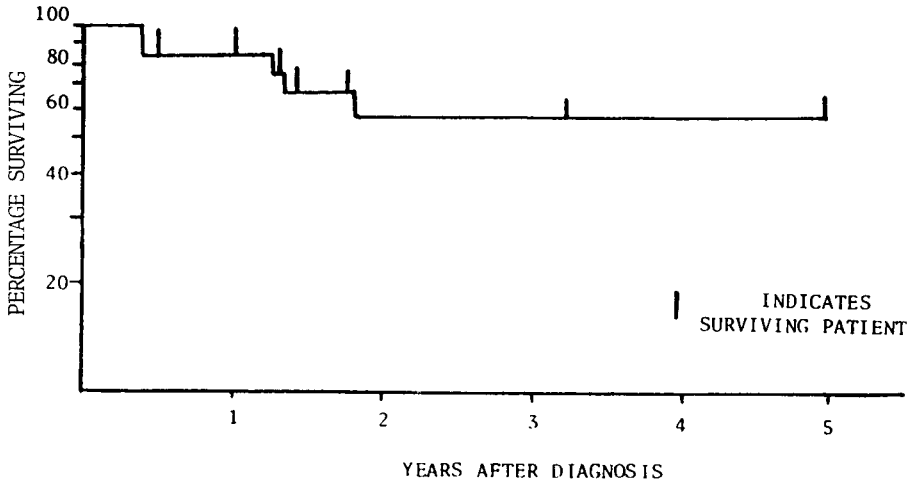

Figure 1. High-dose BCNU as adjuvant therapy for primary tumors of the central nervous system.

after diagnosis. The median survival of this series cannot yet be determined (but will be greater than 11 months) because this ongoing study has not accrued an adequate number of patients. Again, as in the phase II study, the observation of long-term survivors with glioblastoma multiforme is quite uncommon.

Further studies are suggested by this report. After the completion of the high-dose adjuvant study, we plan to combine both high-dose BCNU and high-dose VP-16-213 as adjuvant treatment if phase II study of these two agents in combination demonstrates tolerable hematopoietic and extramedullary toxicities. However, as in other high-dose studies, high-dose combination therapy must produce substantial survival benefit to counterbalance the anticipated formidable toxicity.

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Panel Discussion: Session VI

E. Frei III and G. W. Santos, Moderators

Dr. Robert Souhami: We have treated 24 patients with relapsed glioma with VP-16-213, and I should point out what a hard road this is to travel, because you only have a very short time after relapse before performance status becomes very poor and all treatment quite impossible. There may only be a couple of weeks, so I think that Dr. Wolff has every reason to be congratulated on persisting with these studies. However, I must tell you that our own results with VP-16-213 have been very disappointing. We treated our 24 patients with VP-16-213 at a slightly lower dose than Dr. Wolff did. We infused it at a dose of 1200 mg/m^2 over 48 h to produce plasma concentrations of about 5-10 times higher than those normally reported. Unfortunately, we have only seen one patient with stable disease out of the 24; all the rest have progressive disease. The patients had all had prior chemotherapy with procarbazine, vincristine, and nitrosourea, and so it is not a very favorable group. Nonetheless, our results have been disappointing.

Dr. Steven Wolff: In one of the oncological journals, there was a report evaluating VP-16-213 at standard dose for gliomas. The authors reported about a 20% authentic response rate and about double that for stabilization. Therefore, we may not be able to detect a true response rate if your patients with recurrent tumor were heavily pretreated with chemotherapy and had poor performance status.

Dr. Gary Spitzer: Steve, these long-term survivors are very impressive. Could you give us a little more information about them? Could a local infusion of drugs have been effective?

Dr. Wolff: I'm not sure I know how to answer you. The patients treated in the phase II trials had recurrent progressive tumor that produced neurological impairment and had computed tomography scan-enhancing lesions. Generally speaking, long-term survivors of that group are quite rare. Again, the number of patients treated is small, so that one cannot unequivocally state the increased benefit of high-dose chemotherapy. These long-term survivors might have survived as long with other interventions such as second craniotomies. Nevertheless, we are seeing responses and long-term survivors that are rarely observed with other interventions for recurrent gliomas.

In the adjunctive therapy study, most of the patients had good performance status and were ambulatory without much neurologic impairment. In other adjunctive studies, typically less than one-half of the patients survive 1 year and few survive 2 years. Again, observation of long-term survivors in our series suggests that therapeutic intervention is the reason they survived so long, and not patient selection.

Dr. Spitzer: I was just asking you if the patients that had done well were ones, particularly ones with unilateral hemisphere lesions, who could have had alternative therapy?

Dr. Wolff: The two long-term survivors in the adjuvant study had unilateral lesions, but some of the other long-term survivors had quite extensive tumors. We did not exclude any patient because of tumor extent.

Dr. Karel Dicke: Dr. Wolff, I appreciate the difficulty in the study, but what are you going to use as your end point to evaluate the study, and how many patients do you think you need to establish the therapeutic benefits in the absence of a control arm?

Dr. Wolff: Well, I think that in this tumor the use of historical controls is acceptable. The Brain Tumor Study Group has treated hundreds

Table 1. Survival of BDF1 Mice Challenged with
B16 Melanoma or P388 Leukemia Followed by Busulfan Treatment and Syngeneic Marrow Rescue

	B16 Melanoma					P388 Leukemia		
	Busulfan (mg/kg)[a]							
Variable	0	100	150	180	200	0	100	180
No. Received Bone Marrow	--	--	20	28	14	--	--	20
No. Evaluable	20	15	13	22	10	28	17	18
Survival Days, Median (Range)	29.5 (25-38)	22 (17-24)	46 (28-68)	55.5 (28-75+)	49 (27-75+)	10 (7-11)	10.5 (10-11)	14 (13-16)
T/C Ratio[b]	--	75	150	188[c]	166[c]	--	105	140
No. Tumor-Free Survival	0	0	0	1	4	0	0	0

[a] *Cumulative busulfan doses given 20-25 mg/kg/day.*

[b] *(Survival days of treated / survival days of control) x 100.*

[c] $p < .05$.

of patients and produced a 2-year survival rate of approximately 20%. Statistically, for our study to demonstrate a 20% improvement, we are going to need to treat 20-30 patients. Therefore, we are continuing the 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU) adjuvant study to accrue another 12-15 patients. We will complete the VP-16-213 phase II study with a total of about 20 patients and then evaluate BCNU with VP-16-213 for patients with recurrent gliomas. Perhaps an adjuvant study would follow. Other possibilities include evaluating other agents such as aziridinylbenzoquinone with BCNU and VP-16-213.

Dr. Herbert Kaizer: This is addressed to the comment that Dr. Lassus from the National Cancer Institute made in the previous session about the need for selecting drugs that have antitumor activity of "acceptable levels of toxicity." Busulfan produces profound myelotoxicity and is one of the few drugs that can consistently produce lethal aplasia in experimental animals. Its only clinical use outside the transplantation setting is for chronic myelogenous leukemia. We have been studying busulfan in BDF1 mice. When given at 25 mg/kg/day, cumulative doses of 120 mg/kg were lethal to all animals not rescued by syngeneic marrow. As shown in Table 1, significant prolongation in survival was obtained at doses of 150 mg/kg and above in mice with the B16 melanoma. Even more striking is the fact that 4 of 10 evaluable mice receiving 200 mg/kg of busulfan have survived for more than 75 days and may be cured. The situation shown in Table 1 for P388 lymphoma is quite different. In this tumor significant prolongation of survival is not seen, even at doses of 180 mg/kg.

VII. Freezing Methods and Studies

Postcryopreservation Growth of Human CFU-GM: Analysis of Technical Variables Unrelated to Freezing Rate Studies in Nonfrozen Cells

R. A. Abrams, M. Saggio, and L. Polacek

INTRODUCTION

Cryopreserved autologous bone marrow collections are currently being used in man to affect hematopoietic reconstitution following hematologically ablative levels of systemic antineoplastic therapy (1). Although the techniques used to affect cryopreservation have been validated in various animal models (canine, primate) (2), it is known that there is risk of cell death during cryopreservation. Since there is currently no recognized assay for self-replicating pluripotent human hematopoietic stem cells, the reconstitutive potency of human marrow collections before or following in vitro manipulation cannot be measured directly. In vitro assays of human hematopoietic progenitor cells are of interest in this regard and multiple groups have studied the postcryopreservation growth of human granulocyte-monocyte progenitors (CFU-GM) (3,4). Although largely unreported, postcryopreservation growth of human CFU-GM is often technically less consistent than precryopreservation growth of these progenitors. However, we recently reported (5), and others have confirmed (6), that measurement of human CFU-GM following cryopreservation of bulk marrow collections may provide insight into technical variations in freezing that may be associated with delayed hematopoietic recovery. Therefore, we believe it is important to study and characterize technical issues other than freezing rate relevant to the measurement of human CFU-GM following cryopreservation. Our results from exposing cells to separation, processing, Me_2SO (dimethyl sulfoxide), Me_2SO dilution, and plating *without* freezing are summarized in this chapter.

METHODS

Following appropriate consent, 38 bone marrow samples were obtained from patients of the various Hematology/Oncology services (adult and pediatric) of the Medical College of Wisconsin.

Marrow samples were aspirated into preservative-free heparin. Upon arrival in the laboratory (within 4-6 h), they were diluted 3:1 with Hanks' balanced salt solution (HBSS) without calcium or magnesium, layered over Ficoll-Hypaque solution (Ficoll-Hypaque, Pharmacia, Piscataway, NJ) s.g. 1.077 $\pm$ 0.003, and centrifuged at 400 x g for 30 min at room temperature. Light density marrow cells were aspirated, washed twice in HBSS with 10% fetal bovine serum (FBS), and resuspended in Iscove's modified Dulbecco's media (IMDM) with 20% FBS to a final count of 1 x 10^7/ml + 10%.

CFU-GMs were plated as previously described (7) using IMDM conditioned by human placenta (HPCM) as a source of colony-stimulating activity (CSA). Colonies (>30 cells) were scored by inverted phase microscopy after 7-10 days of high humidity incubation in 5% CO_2 at 37°C. Prior to incubation, plates were routinely screened to exclude the presence of cell clumps or other confounding artifacts. Colony numbers were normalized per 10^6 cells, although the number of cells per plate was 2 x 10^5 (7,8).

The following manipulations were investigated to assess their impact on human CFU-GM growth:

1. Maximal Nontoxic Final Concentration of Me_2SO. Cell suspensions were plated in the presence of 0.01%, 0.05%, 0.10%, 0.50%, 1.0%, 2.0%, or no Me_2SO in IMDM. Concentrations of other reagents were not altered, and this methodology avoided protein denaturation or exposure of cells to unacceptable temperature changes due to the release of solution heat.

2. Sham Freeze. Bone marrow cells suspended in IMDM plus 20% at room temperature were mixed with an equal volume of freshly prepared "freezing media" consisting of 20% Me_2SO and 20% FBS in IMDM, which resulted in a final Me_2SO concentration of 10%. The freezing media were prepared by adding undiluted Me_2SO to IMDM, allowing for equilibration to room temperature, and then adding FBS. Following equilibration at room temperature for 5-10 min, Me_2SO was diluted by a slow or rapid technique (*vide infra*) and CFU-GM plating was completed without further manipulation or washing.

3. Dilution Techniques. After adding freezing media, dilutions were made at room temperature using IMDM plus 20% FBS. Slow dilutions were made by decreasing Me_2SO concentration by 1% or 5%, with subsequent plating as necessary to result in a final postplating concentration of less than or equal to 0.1% Me_2SO. Fast dilutions were prepared in one of two ways. Cells were slowly diluted to a concentration of 5% Me_2SO (as described above) and then instantaneously diluted to 1% Me_2SO with further IMDM plus 20% FBS, or simply diluted directly from 10% Me_2SO to 1% Me_2SO with IMDM plus 20% FBS. Cell plating was then undertaken, as with slow dilution cells, such that the final postplating Me_2SO concentration was less than or equal to 0.1%.

4. Effect of α-Thioglycerol (α-TG). Varying concentrations of α-TG were incorporated into final plating suspensions to assess the impact of this reagent on human CFU-GM growth. This was done both with and without subsequent exposure to Me_2SO. The Me_2SO used was chemically pure (Spectranalyzed, Fischer).

5. Statistical Methods. Each marrow plated in standard fashion served as its own control. Significant results (*P* values) are two-sided. No assumptions regarding data distribution were utilized. Accordingly, the nonparametric Wilcoxon rank sum and matched pairs, signed rank tests were used to assess the significance. Results are reported as means, ± 1 standard error (SEM).

6. Cell Counting and Staining. These techniques were accomplished by standard methods. Cell differentials (100) on Wright-Giemsa-stained cytopreparations are reported in terms of mononuclear marrow cells (blasts, lymphocytes, and monocytes) versus "other" for ease of comparison since hematopoietic progenitor cells and stem cells are believed to be mononuclear in morphology.

Table 1. CFU-GM Colony Number vs Final Me_2SO[a] Plating Concentration

Me_2SO (%)	Colony Number/10^6 Light Density Marrow Cells
0.00	452 ± 131[b]
0.01	521 ± 138
0.05	579 ± 159
0.10	491 ± 124
0.50	437 ± 121
1.00	101 ± 49
2.00	None

[a] Me_2SO = Dimethylsulfoxide.

[b] ± SEM.

Table 2. CFU-GM Colony Number vs Final α-TG Plating Concentration

α-TG (mMolar)	Colony Number/10^6 Light Density Marrow Cells
NONE	566 ± 157
0.3	573 ± 137
0.6	584 ± 154
1.2 [a]	421 ± 101

[a] 1.2 value significantly lower than other 3 ($0.02 < P < 0.05$).

RESULTS

Effects of Me_2SO and α-TG on CFU-GM Growth

As shown in Table 1, final plating concentrations of Me_2SO in excess of 0.5% appear to inhibit CFU-GM growth. Lower concentrations (e.g., 0.01-0.05%) may be mildly stimulatory of CFU-GM growth. Similarly, as shown in Table 2, thioglycerol is also inhibitory of CFU-GM growth at a concentration of 1.2 mmol/l. Lower concentrations (0.3 and 0.6 mmol) did not appear to influence CFU-GM growth.

Effect of Sham-Freeze on CFU-GM Growth and Marrow Differentials

As shown in Table 3, exposure to 10% Me_2SO, subsequent dilution to 1% or 5%, and then further dilution through the plating manipulation for a final Me_2SO concentration of less than or equal to 0.1% did not significantly impair CFU-GM growth. In addition, the presence of 0.3 mM α-TG or 0.6 mM α-TG did not alter CFU-GM growth in this context. CFU-GM growth was also not altered by doing the initial dilution rapidly or at 1%/min (data not shown).

Finally, the percentage content of our marrow preparations with regard to the number of potentially immature cells (blasts, mononuclear cells) was also unaltered by sham-freeze exposure (22 ± 5% vs. 23 ± 2% before and after sham-freeze, N=5).

DISCUSSION

The possibility of monitoring changes in CFU-GM numbers before and after cryopreservation of human marrow as an indirect indicator of hematopoietic stem-cell survival following cryopreservation has been extensively considered (2-5). However, before one can attribute changes in CFU-GM growth before and after freezing to cryoinjury per se, potentially confounding variables should be examined. In this chapter, we have considered several nonfrozen variables and found them to be *unimportant* in the absence of cryoinjury when a constant batch of HPCM is used as a source of CSA.

Since others (9) have suggested that α-TG augments the postcryopreservation growth of human CFU-GM, especially when HPCM is used to provide CSA, we chose to examine the effect of α-TG on nonfrozen cells to eliminate the possibility of unexpected, nonspecific effects unrelated to

Table 3. CFU-GM Colony Number vs Sham-Freeze [a]

Manipulation	Colony Number/10^6 Light Density Marrow Cells (mean ± SEM)
	n = 10
A. Sham-Freeze, Slow Diluton	660 ± 228
Dilution to 5%	
Control [b]	560 ± 144
Addition of:	
0.3 mM α-TG	605 ± 162
0.6 mM α-TG	561 ± 147
B. Sham-Freeze, Fast Dilution	n = 8
Dilution to 1%	638 ± 104
Control [b]	676 ± 130
Addition of:	
0.3 mM α-TG	670 ± 174

[a] Similar data produced by fast dilution to 5%, slow dilution to 1% Me_2SO with or without α-TG.

[b] Control = Colony number with routine plating without Me_2SO exposure.

cryoinjury. Unlike the results described by Schlunk and colleagues (9), we did not observe improved growth of CFU-GM in fresh marrow specimens and found that 1.2 mM α-TG was moderately toxic to unfrozen marrow cells. Our studies show many points of difference in technique (colony definition, modification of HPCM, cell separation, cells per plate, number of marrows examined), which may account for these differences and underscore the complexity of interpreting CFU-GM data from different laboratories.

Other work in our laboratory, which is currently in progress, suggests that following cryopreservation, CFU-GM growth is severely impaired in the absence of extensive manipulation. However, the results reported here, using nonfrozen cells, indicate that cryoinjury must play a significant (although not necessarily exclusive) role in impaired postcryopreservation growth of human CFU-GM. Moreover, in nonfrozen cells: rate and extent of preplating dilution of Me_2SO are unimportant if final Me_2SO plating concentrations are less than or equal to 0.5%, sham-freeze does not appear to alter the percentage of mononuclear cells present, and α-TG concentrations of 0.3 mM and 0.6 mM do not impact on CFU-GM growth in the presence of unfractionated HPCM.

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Studies of Cryopreservation Methods for Human Bone Marrow

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INTRODUCTION

When marrow is cryopreserved in small (2 ml) ampoules, marked viability differences between samples result (1-3). Marrow function after cryopreservation in bags may be more consistent (4). Analyses of factors affecting bone marrow function after freeze-thawing are scant (5). Measurable parameters of freezing do not predict for subsequent in vitro cell viability and there is usually no relationship between progenitor cell survival assayed by in vitro culture and nucleated cell recovery (6). On the basis of indirect evidence from studies of committed hematopoietic precursors and other mammalian cells, marrow stem cell death during freeze-thawing has generally been attributed to the interaction of the following three factors: 1) ill-defined "chemical" injury from dehydration or solute concentration, 2) changes occurring intracellularly at ice crystallization, and 3) "osmotic" injury occurring during the post-thaw dilution procedures (7-10).

There is still no general agreement about the optimal method of reconstitution of bone marrow after thawing. Slow freezing at $1^{o}C$/min and slow stepwise post-thaw dilution have been advocated for marrow cryopreservation in most reports in animals and man (3,5,11).

We observed that bone marrow, cryopreserved in differently designed freezing cabinets that give identical cooling curves, showed strikingly different viability. This report presents data that both implicate the mode of liquid-nitrogen (LN_2) delivery as a potentially important factor affecting cell viability and indicate that improved recovery of cells can be obtained from bags as compared to ampoules. In these studies, slow stepwise dilution after thawing provided no better cell viability than rapid dilution.

MATERIALS AND METHODS

Freezing Systems for Bone Marrow Cryopreservation

A freezing instrument (A) that comprises a cylindrical freezer cabinet with internal dimensions of 42.0 cm (diameter) and 42.0 cm (height) was constructed. The walls were constructed of stainless steel, externally sheathed with fiberglass, with an external wall thickness and air cavity of 1 cm. The lid and base were made of 1 cm of stainless steel. LN_2, which entered the cabinet through the base, was circulated by an electric fan triggered by a solenoid valve. The cooling rate was maintained through a thermister probe placed in air inside the cabinet and connected to a Planar PTC2000 programmable temperature controller. The cabinet was designed for two purposes: 1) to optimize the delivery and droplet dispersion of LN_2 to the cells, and 2) to explore the efficacy of an air space in place of conventional insulation in the cabinet walls. The Planar microprocessor was preprogrammed to maintain a cooling rate of $1^{o}C$/min. A second thermocouple, inserted into a control ampoule or bag, recorded the cooling curve on a Fisher series 3000 recorder.

A second instrument (B) included a fully insulated freezer cabinet, as described previously (12). Cells were placed in an alcohol bath in the freezer cabinet. The mode of LN_2 delivery and temperature monitoring was the same as for system A.

Instruments A and B had similar cooling curves and differed primarily in the mode of LN_2 delivery. The air cavity proved inadequate as insulation for freezer A and failed to prevent major temperature loss. To maintain a precise programmed cooling rate, LN_2 delivery was frequently

and erratically triggered. In contrast, the fully insulated freezer B permitted more regular and infrequent delivery of LN_2 to maintain the same cooling rate.

A conventional air-cooled freezer instrument (C) was used as the "control" with the following components: a Linde freezer chamber, model BF-4/2; a manually controlled temperature controller, Linde model BF-6, which delivered LN_2 at a fixed rate; and a Honeywell single pen chart recorder, model OPT-F300. In initial studies (see Tables 1 and 2, Fig 1), the cooling rate of 1^oC/min was manually maintained from a temperature readout of the thermister placed in air inside the cabinet. For later studies (see Fig 2), the thermister was placed directly into a control bag and the cooling rate was manually maintained from the temperature curve of the bag.

No attempt was made with instruments A, B, or C to compensate for the latent heat of ice crystallization.

Bone Marrow Harvest and Processing

Marrow was collected and processed from normal donors for allogeneic transplantation or from autologous marrow harvested for storage from patients with malignant lymphoma (3,6).

Freezing and Reconstitution of Marrow Cells

Bone marrow or buffy-coat (60-100 ml) was placed into freezing bags, or 1 ml of marrow aliquots was placed into plastic ampoules. All specimens were cooled to 4^oC and mixed with precooled Me_2SO to a final concentration of 10%. Samples were placed in the freezer cabinet, which was precooled from $+2^oC$ to $+4^oC$ and freezing was carried out at a rate of -1^oC/min to -60^oC, then plunged directly into the gas or liquid phase of LN_2.

Table 1. Progenitor Cell Recovery Comparing Cryopreservation Methods

Method	Nucleated Cell Count	CFU-C	BFU-E	CFU-E	N
Rapid dilution[a]					
Instrument A[b]	76.5[c]	1.5	2.2	2.3	4
Instrument B	67.7	37.0	32.8	50.3	6
Instrument C	93.7	52.2	50.2	22.7	4
Slow dilution					
Instrument A	6.2	0.3	1.0	0	3
Instrument B	4.6	58.3	38.7	41.0	3

Note: *CFU-C, colony-forming units in culture; BFU-E, erythroid burst-forming units; CFU-E, colony-forming units of erythrocytes.*

[a] *Describes reconstitution method.*

[b] A, B, *and* C *refer to instruments described in Materials and Methods section.*

[c] *Mean percentage of fresh recovery.*

Table 2. Bone Marrow Function after Cryopreservation by Two Systems with Comparable Cooling Curves

	Donor 1		Donor 2	
Units	Instrument A	Instrument C	Instrument B	Instrument C
CFU-C	3.7[a]	60.7	54.1	78.7
CFU-E	8.7	118.5	46.3	32.1
BFU-E	5.5	33.8	30.6	47.0

Note: *CFU-C, colony-forming units in culture; CFU-E, colony-forming units of erythrocytes; BFU-E, erythroid burst-forming units.*

[a]*Mean percentage recovery of fresh marrow.*

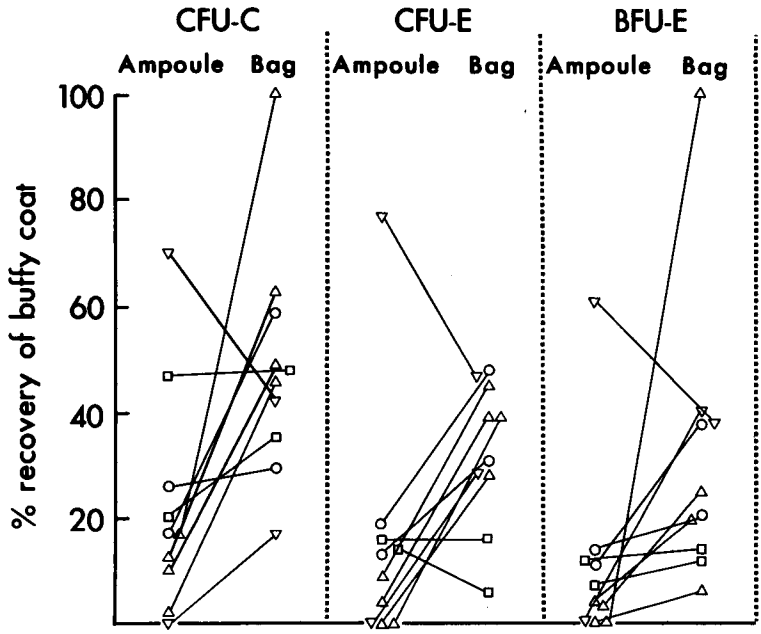

Figure 1. Comparison of in vitro progenitor cell growth in cryopreserved marrows comparing ampoule and bag freezing. The triangle, inverted triangle, square, and circle represent four patients. Lines join single marrow samples.

Cell Reconstitution

Bags or ampoules were removed from LN_2 and immediately thawed by immersion with continuous agitation in a 37°C-water bath. Test samples were reconstituted by either rapid or slow stepwise dilution (3). Comparisons of fresh and cryopreserved marrow progenitor cell growth were done using comparable volumes to fresh marrow progenitor cells (3,6).

Culture of Marrow Cells

Nucleated marrow cells were cultured in vitro for colony-forming units in culture (CFU-C), colony-forming units of erythrocytes (CFU-E),

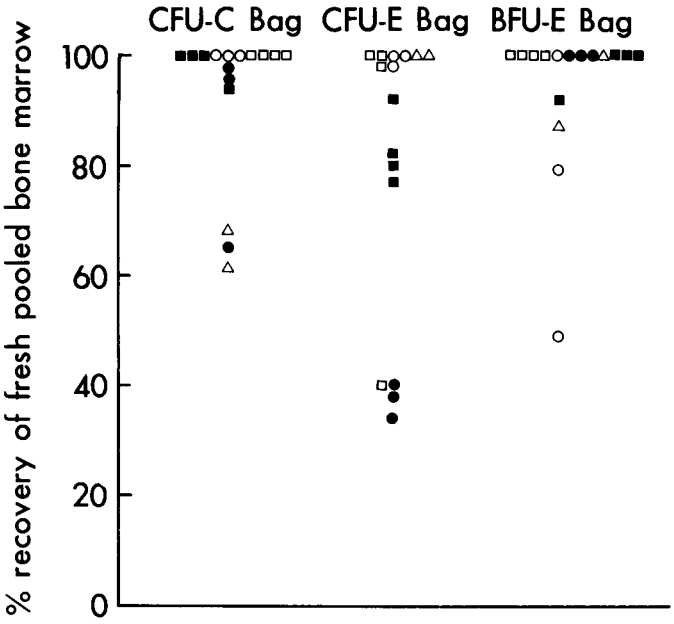

Figure 2. In vitro recovery of marrow progenitor growth from bone marrow cryopreserved in bags. The open square, circle, and triangle refer to marrow from three patients. The open square with solid square represents separate marrow harvests from the same patient.

and erythroid burst-forming units (BFU-E) growth, as described previously (13,14). Paired in vitro data were statistically analyzed using the Student's t-test.

RESULTS

To explore the effect of different modes of LN_2 delivery to cells, the in vitro viability of marrow, cryopreserved in 2-ml ampoules in the two test instruments (A and B) and in the manually operated control instrument C, was evaluated. Cryopreserved marrow consistently showed more growth of progenitors when it was cooled in instruments B and (control) C than when it was cryopreserved in instrument A (Table 1). *P* values were as follows: BFU-E, <.05; CFU-E, <.01; CFU-C, .1>*P*>.05; nucleated cell counts, .1>*P*>.05. No significant differences in marrow availability were observed between instruments B and C.

Figure 3 shows the cooling curves of two comparable marrows from normal donors 1 (instrument A) and 2 (instrument B). The freezing curves have been superimposed for comparison and are essentially identical. However, the marrow viability monitored as growth of progenitors (CFU-C, BFU-E, CFU-E) in culture was strikingly different for the two instruments (Table 2). In vitro data for the same marrows after cryopreservation in instrument C (control) are given for comparison. The cell recoveries, after cryopreservation for instrument A as mean percentage of fresh marrow samples, were as follows: CFU-C, 3.5%; BFU-E, 5.5%; and CFU-E, 8.7%. This contrasted with 54.1% CFU-C, 30.6% BFU-E, and 46.3% CFU-E for instrument B. The loss of function of marrow stored in cabinet A was substantial (*P*<.05) for each assay and consistent finding, independent of the characteristics of the freezing curve.

To compare viability in ampoules and bags, in vitro growth of progenitors was measured in buffy-coat obtained from patients who were previously treated for chronic granulocytic leukemia (CGL) and cryopreserved in ampoules and bags using instrument C. The thermistor was

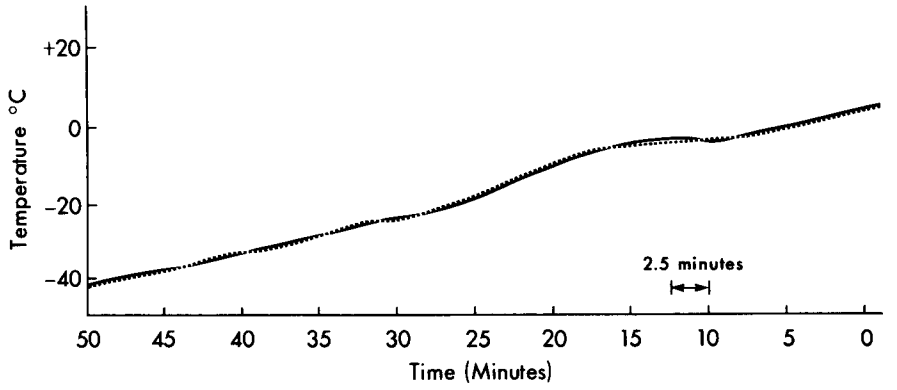

Figure 3. Cooling curves for marrow from two donors, donor 1 (Cabinet A) (solid line) and donor 2 (Cabinet B) (dashed line) frozen in ampoules.

again placed directly in the air and the cooling rate was manually maintained at 1°C/min from the temperature readout. The percentage of recoveries of CFU-C, CFU-E, and BFU-E are shown in Fig 1 with lines connecting corresponding fresh and cryopreserved samples. The differences, which were significant ($P<.05$) for all three assay systems, favored cryopreservation in bags. Very wide disparities in ampoule and bag viability for individual marrows were apparent.

Marrow samples from patients with treated Hodgkin's disease were assayed for in vitro progenitor cell growth after cryopreservation with instrument C. In contrast to the previous studies, the single thermistor was placed directly into a control bag and the cooling rate was manually maintained at 1°C/min from the bag readout, the aims being to provide a fixed and constant LN_2 delivery and to optimize cell cooling. Data points represent assays of individual bags. The results, which are shown in Fig 2, are expressed as percentage of recovery of pooled fresh bone marrow. Equal or greater than 100% progenitor cell recovery was obtained from 28 of 48 individual assays, including 12 of 16 BFU-E, 8 of 16 CFU-E, and 10 of 16 CFU-C. For 42 of 48 assays, data points performed on individual bags from a single aspirate from the same patient were closely clustered.

DISCUSSION

Marrow, cryopreserved in freezing instruments of different design with comparable cooling curves, show markedly different post-thaw viability. No attempts were made with these instruments to compensate for the latent heat of ice crystallization, and there is no evidence to show that the events of ice crystallization or subsequent viability are favorably influenced by such manipulations. The data presented suggest that the mode of LN_2 delivery to cells during cryopreservation may be important and that attention paid to this aspect of freezing may permit more consistent in vitro recovery of marrow processed in bags. Current studies are aimed at designing and testing a cryopreservation instrument, which both provides a constant temperature gradient in the freezer so that cells can be cooled at predetermined rates and eliminates irregularities and fluctuations in LN_2 delivery to cells.

Dog marrow can be cryopreserved without resulting in significant stem cell injury (11). However, comparable studies are not possible in man. Because of the lack of consistency in the published results of in vitro viability for both fresh and frozen human marrow with available techniques (2,3,6), the contribution of actual cryogenic injury to in vitro and in vivo stem cell failure remains conjectural. The difficulties of evaluating cryogenic injury in human marrow are further compounded by the absence of a satisfactory direct assay for bone marrow stem cell activity

and the scant data available for the cryogenic susceptibility of the CFU-MEGG (2,15).

Most reported studies have cryopreserved cells in ampoules or test tubes in air at a controlled rate of 1°C/min (1-3,5,16,17). Few reports have documented progenitor cell viability from bags (4,6,18); and comprehensive data that compare bags and ampoules are lacking. Our results confirm the improved survival of CGL cells cryopreserved in bags rather than ampoules. The unpredictability of ampoule freezing is highlighted in the study shown in Fig 1. The reason for the wide differences in ampoule viability remains unexplained, but may derive from differences in the biologic events of ice crystallization unique to individual samples. The use of large-volume bags or an absolute alcohol bath may modify these events and improve overall survival.

ACKNOWLEDGMENT

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Enhancement of Recovery of Gut-Associated Lymphoid Tissue in the Murine Syngeneic Bone Marrow Transplant Model

G. S. Leventon, L. Huynh, B. M. Levy, and A. R. Zander

INTRODUCTION

Sepsis, a major risk of bone marrow transplantation procedures, is caused by pathogenic microorganisms that often enter the bloodstream through mucosal portals (1,2). These sites are protected by the "local" or mucosal immune system, which is distinguished from the systemic immune system by a number of characteristics, including the optimal route of antigen presentation (3-5), the homing ability of lymphoid cells (6-10), and the major immunoglobulin product (11). Gut-associated lymphoid tissue is comprised of the Peyer's patches and mesenteric lymph nodes, lamina propria plasma cells and lymphocytes, and intraepithelial lymphocytes (IELs). IELs are comprised of 36% null, 29% B, and 35% T lymphocytes. Both Ly-1 and Ly-2 are present on 87% of the T cells (12).

Previous studies demonstrated a period of profound depression of jejunal plasma cells and IELs in mice exposed to 9 Gy of ^{60}Co and rescued by a minimal-survival syngeneic bone marrow dose (GS Leventon, L Huynh, BM Levy et al., unpublished observations). When Zander et al (13) examined marrow engraftment in the same model, a more rapid recovery of IELs and jejunal plasma cells occurred when infused marrow was increased or when buffy-coat cells were added to the innocula. The purpose of this study was to determine if recovery of mucosal membrane immune system components could be potentiated by increasing the bone marrow dose or by adding buffy-coat cells or IELs to the minimal-survival bone marrow innocula. We also sought to gain insight into the in vivo role of the IEL. Recovery of the mucosal membrane immune system was determined by quantifying IELs and lamina propria plasma cells. To assess epithelial damage, we monitored the integrity of the gut epithelium and assayed the number of crypts.

MATERIALS AND METHODS

After receiving a single 9-Gy dose of ^{60}Co total body irradiation (TBI), conventional 8-week-old male BDF-1 mice were prepared for syngeneic transplantation (GS Leventon, L Huynh, and BM Levy et al., unpublished observations). IELs were obtained from the small bowel (12), buffy-coat cells from the retroorbital space, and bone marrow from aspirated femurs. Groups of 40 mice received 2.0×10^5 bone marrow cells (LBM), 1.0×10^6 bone marrow cells (HBM), 2.0×10^5 bone marrow cells plus 2.0×10^5 buffy-coat cells (LBM-BC), or 2.0×10^5 bone marrow cells plus 2.0×10^5 IELs (LBM-IEL) as a 0.5-ml aliquot injected into the tail vein. Twenty animals, who had sham bone marrow transplants, received TBI only. An untreated control group was also included in the experiment. Groups of six animals from each treatment group were killed on days 7, 11, 14, 21, 28, and 35 after bone marrow transplantation, and cross sections of jejunum were prepared (GS Leventon, L Huynh, and BM Levy et al., unpublished observations). Crypts (14) and lamina propria plasma cells (GS Leventon, L Huynh, BM Levy et al., unpublished observations) were quantified by the number per cross section, and IELs on the number in 100 epithelial cells (15). Student's t-test was used to analyze data sets between different experimental treatment at each time interval (cross-sectional data) and within the same treatment at different time intervals (longitudinal data).

RESULTS

All of the animals receiving bone marrow transplants survived through day 35, and all of the irradiated mice not receiving transplantation died

by day 14. The number of crypts were not examined in mice receiving the buffy-coat cell- or IEL-augmented marrow treatments. All three quantitated sites underwent an initial decrease with all treatment modalities, as did the cellularity of the Peyer's patches. By day 7 the physical integrity of the epithelium was restored. Recovery of plasma cell levels began by day 11 and IEL levels by days 14-21, whereas the reduced number of intestinal crypts remained constant (Fig 1).

Seven days after irradiation, jejunal crypts with surviving epithelial stem cells completely regenerated and crypts without surviving stem cells had totally involuted. The number of crypts per cross section between days 7 and 35 never increased after the initial reduction from the control level of 158 ± 13.8 (SD) ($P<.05$); crypt numbers ranged from 69.6 ± 7.3% to 82.3 ± 5.5% of control levels after TBI, LBM, and HBM treatments, generating interlacing subsets as determined by Student's t-test procedure.

The pretreatment control level of plasma cells was 365 ± 35.8 (SD) cells per cross section. By day 7 mean plasma cell values dropped to 19.6 ± 5.9%, 18.5 ± 7.0%, 25.6 ± 10.9%, 27.7 ± 8.0%, and 38.1 ± 6.0% of mean control levels for the TBI, LBM, HBM, LBM-BC, and LBM-IEL treatment groups, respectively. The TBI and LBM treatment groups were significantly more depleted than the HBM and LBM-BC treatment groups ($P<.05$), and the LBM-IEL group was the least depleted ($P<.05$). All of the transplantation treatments by day 11 produced a significant elevation in the number of plasma cells from their day 7 levels ($P<.05$), whereas the plasma cell levels in the TBI treatment group continued to decline ($P<.05$). Cell repopulation approached the control level by days 28, 21, 21, and 14 for the LBM-, HBM-, LBM-BC-, and LBM-IEL-treated mice, respectively.

IELs decreased after irradiation by day 7 to 27.1 ± 5.4%, 15.5 ± 10.1%, 62.8 ± 19.4%, 53.5 ± 9.3%, and 63.6 ± 10.1% of normal mean control values for the TBI only, LBM, HBM, LBM-BC, and LBM-IEL treatment groups, respectively. The LBM treatment group's IEL counts did not fluctuate significantly from day 7 through day 14, and by day 14 the HBM treatment group had reached maximal decline in IEL level that approached the day 14 level of the LBM treatment group. The LBM-BC-treated mice also experienced their IEL level nadir on day 14, but it was higher (35.7 ± 16.3%) than the day 14 level of the LBM- and HBM-treated animals ($P<.05$). Maximal decline of IELs for the LBM-IEL-treated group was observed on day 7 at 38.9 ± 10.9% of the mean control level. On day 21 the IEL levels for the HBM, LBM-BC, and LBM-IEL treatment groups formed one subset; the LBM treatment group formed lower and the control higher populated subsets ($P<.05$). The LBM treatment group achieved peak levels of IEL repopulation on days 28 and 35 at 60.5% to 63.6% of pretreatment values, and the HBM treatment group peaked on days 21 and 35 at 77.5% to 86.1%; both were different from each other and significantly short of the pretreatment level ($P<.05$). The LBM-BC and LBM-IEL treatments produced levels of IELs that approached control levels by day 35 for the former and days 28 through 35 for the latter ($P<.05$).

DISCUSSION

In this study, syngeneic bone marrow transplantation was able to reverse plasma cell and IEL depletion in mice caused by irradiation with 9 Gy of ^{60}Co. In animals rescued with bone marrow alone, the mean IEL level was reduced to between 13 and 16% of the mean control level by day 14. The depopulation was not as severe in transplants augmented by buffy-coat cells or IELs. In the LBM-IEL treatment group, declines in IELs were reversed earlier, and significant repopulation was achieved by day 14. Bone marrow infusion without augmentation by other cell populations demonstrated a positive dose response, increasing levels of IELs, plasma cells, and crypts, but control levels were not achieved. Recovery plateaus occurred on day 28 at 64% of the mean control level for mice receiving the LBM treatment and at 86% for those on the HBM regimen. Normal IEL levels were achieved by day 28 for the IEL treatment group and by day 35 for the LBM-BC treatment group.

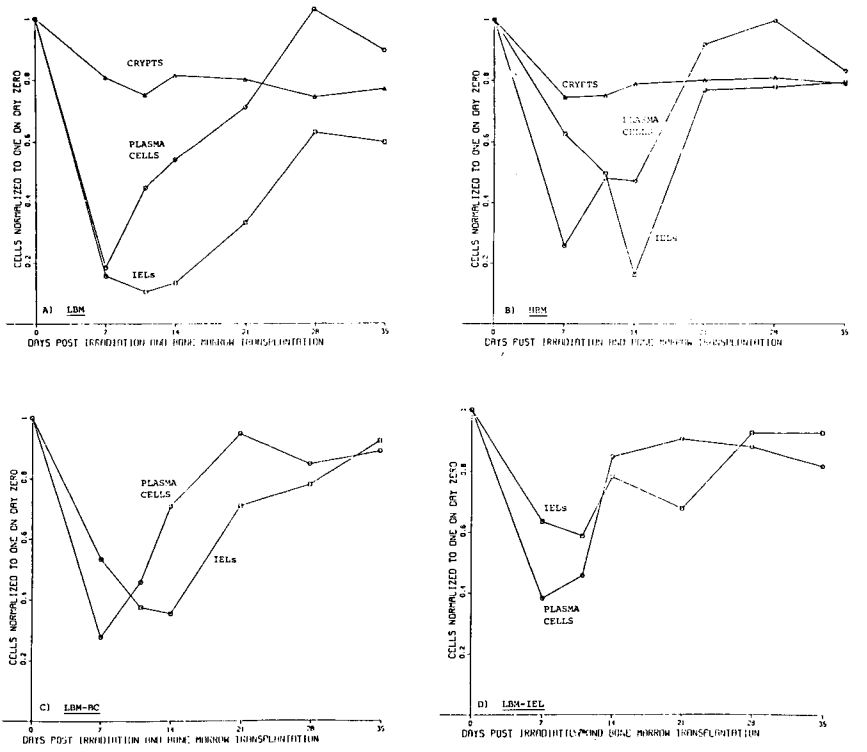

Figure 1. Levels of intestinal plasma cell, intraepithelial lymphocyte, and crypts following 9 Gy of ^{60}Co total body irradiation and transplantation of (A) 2.0 x 10^5 bone marrow cells (LBM), (B) 1.0 x 10^6 bone marrow cells (HBM), (C) 2.0 x 10^5 bone marrow cells and 2.0 x 10^5 buffy-coat cells (LBM-BC), (D) 2.0 x 10^5 bone marrow cells and 2.0 x 10^5 intraepithelial lymphocytes (LBM-IEL).

Increases in plasma cell recovery were affected by all four transplantation modalities, listed as follows in order of least severe initial depletion and most rapid recovery: LBM-IEL, LBM-BC, HBM, and LBM groups. In all treatment groups, the lowest plasma cell levels occurred on day 7: 38% of mean control value for the LBM-IEL treatment group, 28% for the LBM-BC group, 26% for the HBM group, and 19% for the LBM group. Control levels were approached by days 14, 21, 21, and 28 in these groups, respectively.

Curves of gut plasma cell and IEL levels over time following TBI and syngeneic bone marrow transplantation showed that these cell populations followed different time courses (Fig 1). Differences in the degree of initial loss, rates of recovery, and maximal repopulation levels demonstrated positive dose responses to bone marrow. Augmenting bone marrow at a 1:1 ratio with buffy-coat cells, and especially IELs, proved beneficial in promoting morphologic recovery. It is unknown what shifts in IEL subpopulations or functional defects remained after normal morphology was regained.

IELs are mitotically active migratory cells containing a high proportion of immature T cells, suggesting the intraepithelial site as a maturational location for the mucous membrane immune system. A progenitor-effector cell relationship between IELs and plasma cells provides a possible explanation for the observed differences in cell loss and recovery between the IEL and plasma cell populations in these studies. If the IELs are progenitor cells for gut plasma cells, there may be a feedback mechanism between them governing production. When effector cell levels are low, the control mechanism favors differentiation of progenitor

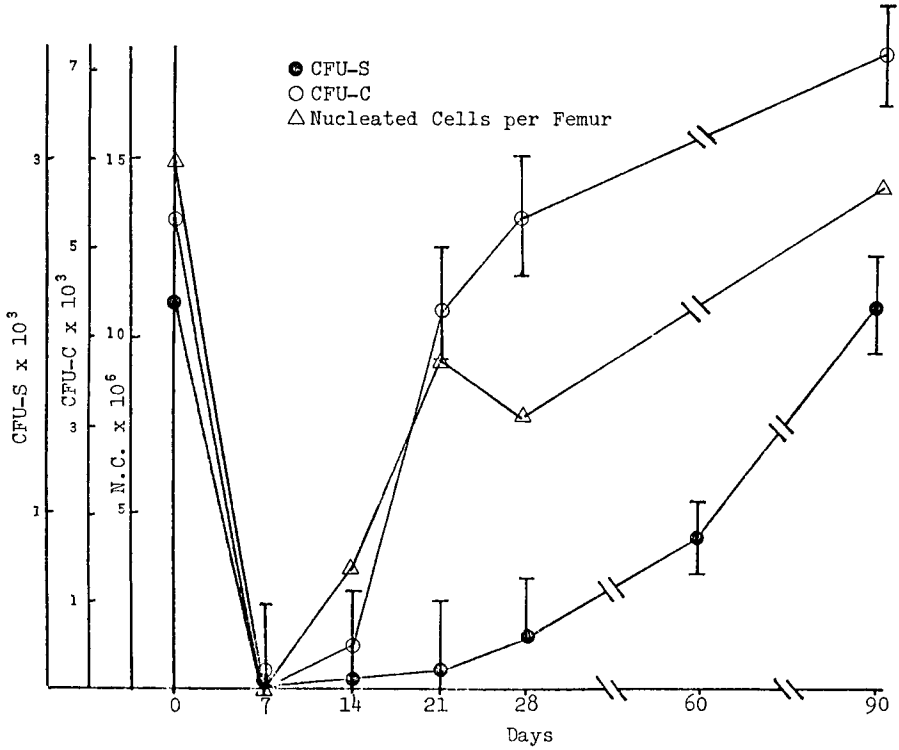

Figure 2. Hematopoietic recovery after transplantation with a minimal cell dose of 2.0 x 10^5 bone marrow cells following irradiation with 10.5 Gy. Stem cell colony-forming units (CFU-S), effector cell colony-forming units (CFU-C), and nucleated cells (N.C.) were measured per femur. Reprinted with permission from Blood: *Zander et al, Enhancement of hematopoietic recovery after bone marrow transplantation with the addition of nucleated blood cells in mice, Blood 1980;56:1132-5.*

cells and maintenance of levels for self-replication. When the threshold level of effector cell is reached, the size of the progenitor cell pool increases. In the transplant groups, the IEL pool demonstrated increases when the number of plasma cells had achieved approximately 50% repopulation.

A similar mechanism has been observed in the bone marrows and spleens of mice receiving TBI and syngeneic bone marrow transplantation (13) (Fig 2). Effector cell repopulation was found to be favored over stem cell repopulation in the face of a severe effector cell deficit. The course of bone marrow and gut effector cell recovery in the BDF-1 mouse has been found to be similar. Zander et al (13) found that effector cell and stem cell repopulation and nucleated cells per femur increased rapidly, and the effector cell repopulation reached control levels by days 21 to 28 post-transplantation. Stem cell repopulation was slower; an initial increase in numbers was achieved on day 28 and control levels were attained on day 90.

Studies have associated a poorly functioning mucosal immune system with susceptibility to gut infection and malabsorption syndromes (16,17). Present studies on the fluctuations in gut plasma cells and IELs after TBI and syngeneic marrow transplantation in the mouse show that after this treatment a severe loss of gut-associated lymphoid tissue occurs over a prolonged period of time. This loss can be abated and recovery potentiated by augmenting the transplantation regimen with buffy-coat cells or IELs. The fact that mucosal immunoincompetency may occur in humans after TBI and bone marrow transplantation may explain the high rate of associated mucosal ABMT membrane infection.

ACKNOWLEDGMENT

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Autologous Bone Marrow Transplantation in Aged Dogs

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In many instances the treatment of hematologic and some other malignant diseases is limited by the toxicity of chemotherapy and radiotherapy effective against hematopoietic stem cells. This limitation may be overcome by autologous bone marrow transplantation (ABMT). Little is known about the influence of a patient's age on the restitution of hematopoiesis following autologous transplantation. In the mouse, various studies with bone marrow of aged animals have provided controversial evidence for the senescence of hematopoietic stem cells. Serial transfer experiments indicated that hematopoietic stem cells from aged mice were equally as capable as those of young mice to repopulate irradiated recipients (1-3). In contrast, long-term cultures in vitro showed an early decline in the self-renewal capacity of aged marrow as compared to young marrow (4,5).

We investigated hematopoietic restitution following total body irradiation (TBI) and autologous marrow transplantation in aged and young dogs. With two exceptions, initial recovery of blood counts was similar in old and young dogs; however, several months after transplantation, white blood cell counts and lymphocyte counts were lower in aged animals.

MATERIALS AND METHODS

Beagles and Labradors were bred in the kennels of the Gesellschaft fur Strahlen- und Umweltforschung; they were vaccinated against canine hepatitis, distemper, and leptospirosis, and were dewormed and healthy. All dogs used were fully grown. The 10 young dogs were between 15 and 22 months old, and the seven aged dogs between 8 and 11 years old. Marrow was obtained under general anesthesia between 1 and 3 months before transplantation, as previously described (6). The cryopreservation and thawing procedures have also been described previously (6). The minimal number of mononuclear marrow cells (MNC) obtained from each dog was 2×20^8/kg body weight. After thawing, 1×10^8 viable MNC were infused per kg body weight. Of nucleated cells, 22 $\pm$ 7% stained positive with trypan blue.

The growth of colony-forming units in culture (CFU-C) was determined in semisolid agar according to the method of Pike and Robinson (7) adapted for the dog (6). To stimulate colony activity, we used 20% of serum from a pancytopenic dog treated with busulphan. Colonies (aggregates containing 50 or more cells) and clusters (aggregates containing between 10 and 50 cells) were counted after culturing for 8 days. The absolute values of CFU-C ranged from 39 to 265 per 10^5 MNC with a mean of 109 and a standard deviation of 64. Recovery of CFU-C after thawing was 75 $\pm$ 54%.

TBI was applied from two opposing cobalt-60 sources to each dog placed in an aluminum cage midway between the sources. The body midline dose was 9 Gy as calculated from dosimetry studies using condenser chambers placed in the esophagus and colon in separate experiments. The dose rate in the body midline was 4.2 cGy/min. Marrow cells were infused the day after irradiation. Dogs were kept off food and water from the day of irradiation until 5 days after transplantation. They received parenteral fluids and intravenous antibiotics as indicated. Dogs were infused twice daily until the levels of leukocytes and platelets had recovered.

After hematopoietic restitution, blood counts were taken daily until 30 days after transplantation. Constituents measured included white blood cells, platelets, hematocrit, reticulocytes, and differentials.

RESULTS

Leukocyte counts continued to rise following the postirradiation nadir after reaching levels of 350/μl and platelet counts continued to

rise after reaching levels of 40,000/μl. One dog (N16) died 45 days after transplantation with persistent thrombocytopenia and hemolysis. A complete autopsy was carried out. Episodes of diarrhea postirradiation were observed within 7 days of irradiation. Two phases of elevated temperatures (greater than 39.5°C) were observed, the first phase between days 5 and 9 during the period of severe granulocytopenia and the second phase between days 10 and 12.

Days with fever or diarrhea per total number of days evaluated were compared in the two groups. The chi-square test was used for statistical comparison of days with fever and with diarrhea, the number of dogs with documented infections, and the number of transfusions. The number of days required to reach leukocyte counts of 350/μl, 700/μl, 1400/μl, and 2800/μl and platelet counts of 40,000/μl, 80,000/μl, and 160,000/μl were compared using a one-sided analysis of variance (F-test). Leukocyte, lymphocyte, and platelet counts at day 100 were also compared, using a one-sided analysis of variance. Levels of $P \leq .05$ were considered significant.

DISCUSSION

Larger volumes of marrow had to be aspirated in aged dogs than in young dogs in order to obtain sufficient cells for autologous transplantation ($P \leq .004$) (Table 1). This result was expected, since the cellularity of bone marrow decreases with age in dogs as in humans. The concentration of hematopoietic precursors (CFU-C) per 10^5 MNC was not significantly lower in aged dogs than in young dogs, suggesting that the cellular composition of marrow cells may not be different in aged dogs from that in young dogs. Therefore it may not be necessary to preserve more MNC in aged individuals than in young ones.

Following infusion of the same amount (1 x 10^8 MNC/kg body weight) of autologous marrow cells, the recovery of leukocyte values in peripheral blood was not different in aged dogs from that in young dogs (Fig 1). One dog (N278) showed an exceptionally slow recovery of leukocyte and platelet counts. She had received the lowest amount of CFU-C. However, considering all dogs, a correlation could not be demonstrated between the leukocyte recovery and the amount of CFU-C. After the initial rise in blood counts, the leukocyte values of aged dogs remained subnormal ($P \leq .01$) (Fig 2). Leukocyte and lymphocyte values (Fig 3) of aged dogs did not reach the values of young dogs nor their own pretreatment values. This was not true for platelet values (Fig 4). Dog N16 could not be evaluated for platelet recovery, since she developed hemolytic anemia and possibly secondary thrombocytopenia. Autopsy revealed calcifying endocarditis, active erythropoiesis in marrow and spleen, and the presence of megakaryocytes.

Table 1. Number of Mononuclear Cells and CFU-C[a] in Aspirates of Bone Marrow from Aged and Young Dogs

Bone Marrow	MNC[b] x 10^9/100 ml	CFU-C/1 x 10^5 MNC
Aged dogs 8-11 yrs	1.1 ± 0.6 (N = 20)	98 ± 57 (N = 14)
Young dogs 1-2 yrs	1.7 ± 0.8 (N = 30)	118 ± 71 (N = 16)
P value	.004	.41

[a] *CFU-C, colony-forming units in culture.*

[b] *MNC, mononuclear marrow cells.*

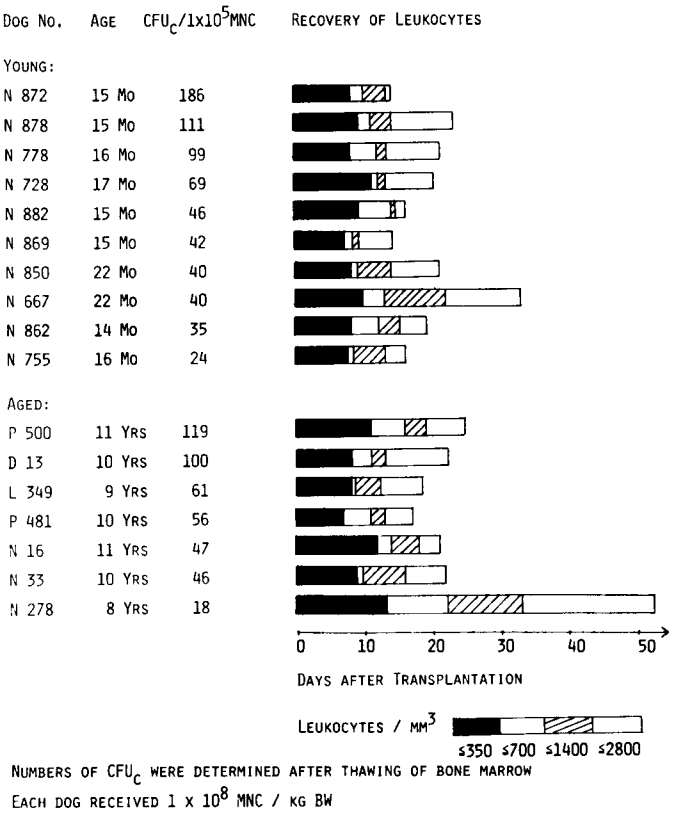

Figure 1. Doubling time of leukocyte values in peripheral blood after autologous bone marrow transplantation compared between aged and young dogs. (CFU-C, colony-forming units in culture; MNC, mononuclear marrow cells.)

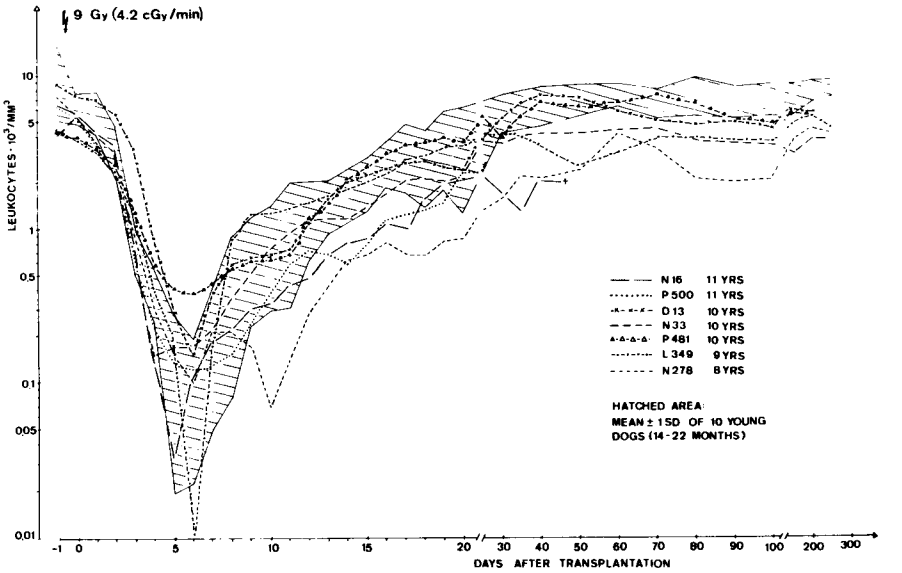

Figure 2. Recovery of leukocytes in aged dogs after total body irradiation and autologous bone marrow transplantation (1 x 10^8 MNC/kg). (MNC, mononuclear marrow cells.)

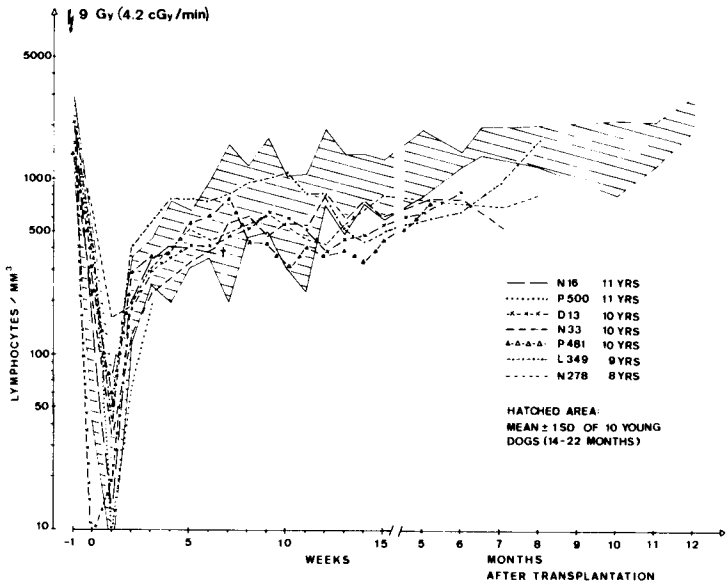

Figure 3. Recovery of lymphocytes in aged dogs after total body irradiation and autologous bone marrow transplantation (1 x 10^8 MNC/kg). (MNC, mononuclear marrow cells.)

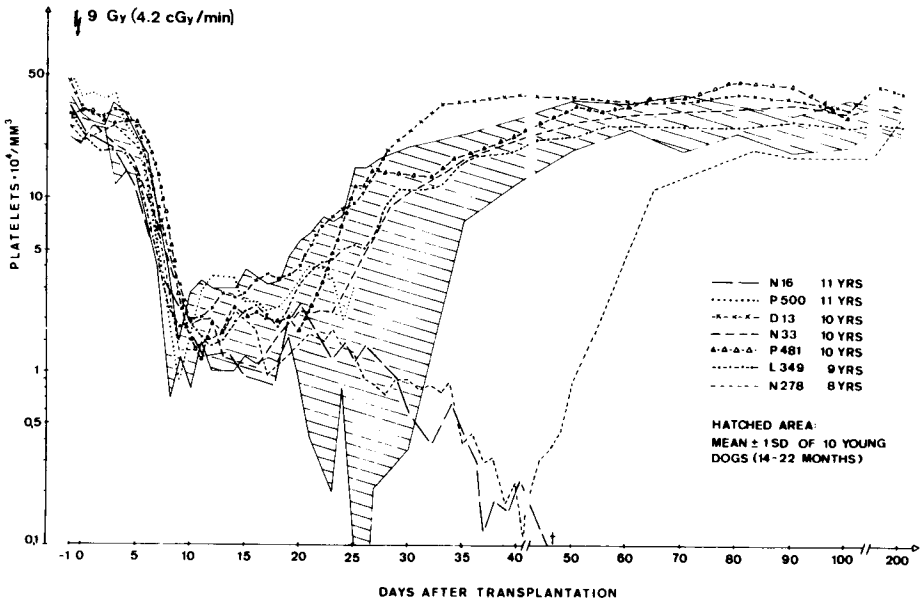

Figure 4. Recovery of platelets in aged dogs after total body irradiation and autologous bone marrow transplantation (1 x 10^8 MNC/kg). (MNC, mononuclear marrow cells.)

Aged dogs had more infections after transplantation (Table 2). In three dogs minor localized infections--blepharitis, external otitis, and interdigital cellulitis--were preexisting and flared up after TBI. Moreover, aged dogs had more days with diarrhea and fever during the first 10 days after transplantation. Thus, in aged individuals, side effects such as diarrhea, fever, and infections may be more limiting for ABMT than incomplete hematopoietic restitution.

Table 2. Side Effects after 9 Gy (4.2 cGY/min) Total Body Irradiation and Autologous Bone Marrow Transplantation Compared between Aged and Young Dogs

Side Effect	Category of Dog: Young (N=10)	Category of Dog: Aged (N=7)	P Value
Days with diarrhea (mean), days 1-7	1.2	2.4	$P \leq .05$
Days with fever (>39.5°C)			
First episode (mean), days 5-9	1.3	2.3	$.1 > P \geq .05$
Second episode (mean), days 19-21	3.5	2.7	N.S.
Number of dogs with documented infections	1	5	$P \leq .05$
Number of transfusions required (median per dog)	1.5	2	N.S.

It is particularly important that safe levels of leukocyte and platelet counts are reached as rapidly in aged as in young dogs. However, the restitution of leukocyte counts remains incomplete in aged dogs. It may be speculated that early recovery is forced by maximal stimulation early after transplantation. Only after the immediate requirements have been fulfilled are subnormal values of leukopoiesis sustained. This may be secondary to a minor defect of hematopoietic stem cells. The higher capacity of proliferation and differentiation and relatively lower capacity of self-renewal of hematopoietic stem cells of aged individuals has been discussed on the basis of experiments in mice (5,8). The maximal capacity of self-renewal determined in serial transfer experiments remains the same with increasing age, but the proliferative capacity, measured as the repopulation of hematopoietic stem cells with 14 days of transfer, is increased (8). In long-term cultures in vitro, the production of hematopoietic stem cells by marrow from aged mice is increased during the first weeks and reduced later on as compared to that from young mice (5).

The persistence of subnormal leukocyte values may also be due to a regulatory change. Either the precursor cells of aged individuals become less sensitive to regulatory factors or less regulatory factors are produced.

In summary, early hematopoietic restitution is similar in aged and in young dogs, but the restitution of leukocyte values in aged dogs is incomplete, for reasons that remain to be determined.

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Source and Processing of Stem Cells for Transplant: Comparative Studies

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INTRODUCTION

The reconstitution of blood cell formation after myeloablative therapy and subsequent autologous stem cell transfusion may well be considered as a consequence of the migration of transfused stem cells to sites of hematopoietic tissue that are capable of inducing stem cell replication and differentiation. From embryologic studies it becomes evident that the physiologic establishment of hematopoiesis is also the result of the migration of hematopoietic stem cells through the blood stream from sites of stem cell production to sites of stem cell replication and differentiation in the bone marrow (1). This is the reason why it is tempting to use, for autologous stem cell transfusion, stem cells not only from the marrow but also from the circulating blood.

The purposes of this chapter are to review briefly means and ways of collecting and storing stem cells from either the marrow or the peripheral blood of humans, and to consider possible ways of monitoring the stem cell viability that will not only apply to in vitro methods but also consider their limitations in predicting the hematopoietic reconstitutive potential.

POTENTIAL STEM CELL SOURCES

Since Thomas and associates first published the techniques for human marrow grafting almost 15 years ago (2), the bone marrow has classically been considered the source of stem cells for hematopoietic reconstitution after myeloablative treatment. Although still experimental, blood-derived hematopoietic stem cells gathered by cytapheresis offer a different, viable, and perhaps in some instances preferable alternative option.

There are both practical and theoretical advantages to collecting stem cells from marrow over peripheral blood and vice versa. On one hand, except when patients have chronic myelogenous leukemia (CML), the marrow contains the highest hematopoietic stem cell concentration and therefore is considered the primary stem cell source for clinical bone marrow transplantation. On the other hand, harvesting stem cells from the blood rather than the marrow offers these advantages:

- no anesthesia is needed;
- the harvest procedure can be repeated easily;
- cytapheresis is performed routinely in blood banking;
- at least in patients with CML, the ratio of stem cells to tumor cells in peripheral blood after induction chemotherapy seems to favor stem cells (3).

COLLECTION OF STEM CELLS

The total stem cell yield per multiple marrow aspiration depends on, among other factors, the marrow stem cell concentration at the time of harvest. This can be influenced by the patient's previous irradiation or chemotherapy. As shown in Table 1, we have analyzed marrow collections from 31 patients to determine the relationship between stem cell concentration and the patient's preharvest clinical status. Because of the high standardization of induction and reinduction cytoreductive therapy for patients with acute myelogenous leukemia (AML), we have chosen to study groups of these patients in first and second or subsequent remission. Compared to the marrow of normal donors and patients in first remission,

Table 1. Correlation between the Patient's Preharvest Clinical Status and the Number of Hematopoietic Precursor Cells Harvested per 10^5 Mononuclear Marrow Cells

Clinical Status of Patients before Harvest	Stem Cells per 10^5 MNC Harvested (Mean ± SD)		
	CFU-GM	BFU-E	CFU-GE
No treatment (Normal marrow donors) (N = 36)	56 ± 21.8	16 ± 7.9	69 ± 20.0
AML (first remission) (N = 24)	84 ± 21.2	23 ± 11.5	60 ± 18.9
AML (Second remission) (N = 7)	54 ± 16.7	5 ± 4.1	36 ± 12.2

Note: *MNC, mononuclear marrow cells; CFU-GM, granulocyte-macrophage colony-forming unit; BFU-E, erythroid burst-forming unit; CFU-GE, granulocyte-erythroid colony-forming unit; AML, acute myelogenous leukemia.*

Table 2. Number of Hematopoietic Precursor Cells in Marrow versus Peripheral Blood

Cells	Concentration	
	Marrow (per 10^5 cells)	Blood (per 10^6 cells)
CFU-GM	20-100	10-100
BFU-E	50-200	2-10
CFU-GEMM	10-100	5-100

Note: *CFU-GM, granulocyte-macrophage colony-forming units; BFU-E, erythroid burst-forming units; CFU-GEMM, human pluripotent stem cells.*

the harvested marrow stem cell concentration from patients in second or subsequent remission was found to be significantly reduced for the more immature erythroid burst-forming unit (BFU-E) and granulocyte-erythroid colony-forming unit (CFU-GE).

The major disadvantage of using blood-derived stem cells for transplantation purposes is their low concentration, only about 10% of that in the marrow (Table 2). Compared to the stem cell yield per marrow harvest, the mean total number of blood-derived stem cells collected by a single leukapheresis run is only about one-seventh (Tables 3 and 4).

FREEZING AND STORING

Most clinical protocols for autologous bone marrow transplantation (ABMT) studies impose a long enough delay between the time of stem cell procurement and reinfusion that cryopreservation must be employed. Some variables suggested as having a significant effect on stem cell viability are the cryoprotective agent, freezing rate, exact compensation for the heat of fusion during phase change, storage containers, and method of

Table 3. Mean Yield (± SD) of Human Blood Cells Collected by Continuous-Flow Leukapheresis

MNC x 10^9	CFU-GM x 10^5	CFU-GEMM x 10^5
12.0 ± 3.1	8.7 ± 4.3	3.2 ± 2.9
(*N* = 35)	(*N* = 35)	(*N* = 6)

Note: *MNC, mononuclear marrow cells; CFU-GM, granulocyte-macrophage colony-forming units; CFU-GEMM, human pluripotent stem cells.*

Table 4. Stem Cell Yield: Marrow versus Peripheral Blood

Source	MNC x 10^9	CFU-GM x 10^5
Multiple marrow aspiration	7 - 10	10 - 80
Single stem cell apheresis	5 - 19	1 - 6

Note: *MNC, mononuclear marrow cells; CFU-GM, granulocyte-macrophage colony-forming units.*

thawing--rapid or stepwise. However, these technical aspects are no longer controversial. In our experience, the most convenient, reliable, and efficient way of freezing a mononuclear cell suspension is the following:

- use 10% dimethyl sulfoxide (DMSO) as a cryoprotective agent;
- freeze at 1-2°C/min to -40°C; then 7°C/min to -100°C;
- store in 200-ml polyolefin bags;
- store in the liquid phase of nitrogen;
- thaw rapidly in a 42°C water bath.

Removing DMSO prior to reinfusion is not necessary.

A more critical question that is still controversial concerns the temperature at which stem cells are stored. Data from the dog model show that the minimum dose of cells required to 100% rescue lethally irradiated dogs does not change under vapor-phase storage up to 5 months (4). After this, the minimum dose begins to increase. Both our preclinical and clinical data strongly favor storage in the liquid phase of nitrogen, suggesting that the consistency of ultra-low temperature is the most important factor for keeping cells viable (5).

EFFECT OF GRAFT MANIPULATION ON CELL NUMBERS

Particular attention must to be paid to the effect of purging methods on the autologous stem cell graft. Very subtle differences in the proliferative kinetics between normal and tumor stem cells, and therefore similar sensitivities to cytotoxic drugs or antigens that are shared on the surface of normal and tumor stem cells, the specific target of monoclonal antibodies (MAb), might risk severely damaging the normal stem cell pool. Since we still lack a suitable in vitro assay for the human pluripotent stem cell, the question arises as to what extent the usual in vitro colony-forming unit (CFU) assay really represents the presence or absence of pluripotent stem cells. As shown in a phase I autologous transplant study performed by the Baltimore group, despite the absence of detectable

Table 5. Number of Hematopoietic Precursor Cells (CFU-GM, BFU-E, CFU-GEMM) per 1 x 10^5 MNC Remaining after Short-Term Incubation of Normal Human Marrow Cells Cultured through Day 35

	Days after Treatment					
Cells	0	7	14	21	28	35
CFU-GM	0	44	80	41	36	11
BFU-E	0	7	10	14	5	0
CFU-GEMM	0	63	71	48	ND	0

Note: *Marrow cells were treated with an active cyclophosphamide derivative—ASTA Z 7557, 100 μg/ml cell suspension. CFU-GM, granulocyte-macrophage colony-forming units; BFU-E, erythroid burst-forming units; CFU-GEMM, human pluripotent stem cells.*

hematopoietic progenitor cells in a marrow graft treated with high-dose cyclophosphamide, all cell lines including the immunologic pathway had a complete hematopoietic recovery (6). This indicates a lack of correlation between in vitro progenitor cell content and in vivo marrow repopulation ability after treatment. This might be explained by selective inhibition of committed hematopoietic stem cells, leaving the pluripotent stem cells mostly unharmed. Even the CFU-GEMM assay, believed to represent a human pluripotent stem cell by combining different cell lines in one mixed colony, reacts like the granulocyte-macrophage colony-forming unit (CFU-GM) and BFU-E assay with cyclophosphamide treatment. This suggests that a more immature pluripotent stem cell is responsible for hematopoietic reconstitution after myeloablative treatment (7).

IN VIVO HEMATOPOIETIC RECONSTITUTION

It is believed that the kinetics of hematopoietic reconstitution reflect the size and integrity of the transfused pluripotent stem cell pool, although a strong correlation between transfused CFU and the pattern of hematopoietic reconstitution has not been clearly established in humans as it has in the dog model. Considering the above-mentioned selective destruction of the various stem cell pools by chemoseparation as an example, other in vitro systems need to be established to foresee hematopoietic reconstitution after myeloablation. In our experience, the long-term marrow culture system, a modified Dexter system, guarantees the best evaluation of the replication and differentiation capability of the pluripotent stem cell pool (8) (Table 5).

Besides the function of the transfused hematopoietic stem cells, other factors that might interfere with hematopoietic reconstitution are the microenvironment and autoimmune phenomena. Severe isolated thrombocytopenia has been observed by several groups after ABMT. There is some evidence that this is caused by autoantibodies, a result of a transient immune-system imbalance in the early posttransplant period. The extent to which these autoantibodies affect the peripheral blood counts probably depends on the ability of the engrafted marrow to compensate for the rate of antibody-mediated cell destruction (9).

Hematopoietic reconstitution does not depend on the source of stem cells, as we could show in preclinical studies. We compared the kinetics and hematopoietic recovery after supralethal total body irradiation in dogs transfused with cryopreserved autografts derived from either peripheral blood or marrow (10). The grafts were adjusted to contain equal numbers of committed progenitor cells (1 x 10^5 CFU-GM/kg body weight). Dogs transfused with blood-derived cells showed significant granulocyte

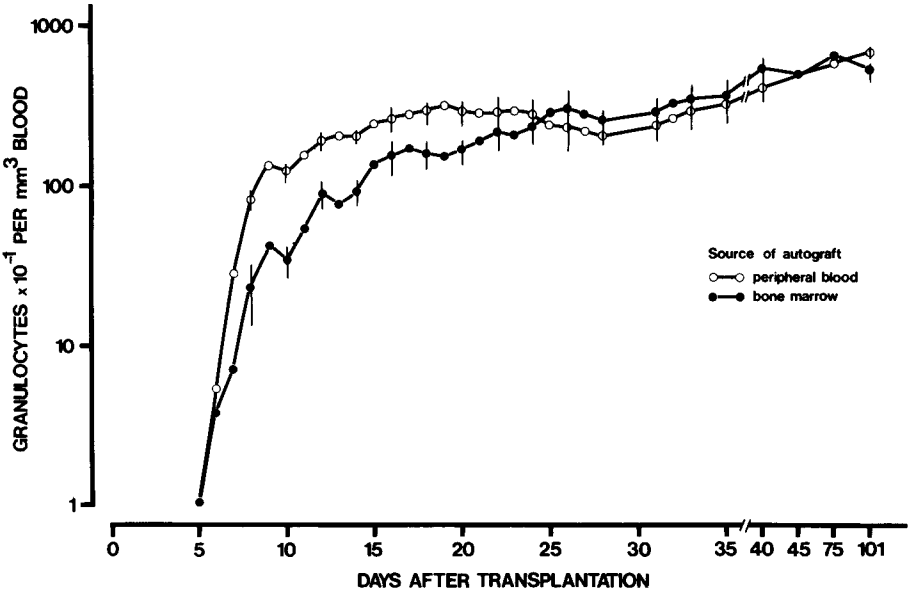

Figure 1. Pattern of granulocyte reappearance in the peripheral blood of dogs after myeloablative treatment and transfusion of either blood-derived (open circles) *(N=4) or marrow-derived* (closed circles) *(N=4) hematopoietic stem cells. Error bars represent SEM.*

(Fig 1) and lymphoid regeneratory advantages, respectively, during the first 20 and 14 days after transplantation, while the regeneration patterns of platelets and reticulocytes revealed no significant differences (data taken from reference 10).

In humans, the ability of blood-derived stem cells to restore hematopoietic function was extensively investigated by McCarthy and Goldman (11) in patients with CML. They reported the successful reversal of blast crisis in 50 patients with CML after myeloablative treatment and transfusion of leukapheresis-derived autologous white blood cells. However, due to the retransfusion of mainly Philadelphia chromosome (Ph^1)-positive clonogenic cells, the median duration of the second chronic phase was only 13 weeks.

Hematopoietic reconstitution following the transfusion of "normal" blood-derived stem cells from identical twins failed in two instances (12,13). A further attempt was made to prove the reconstitutive ability of peripheral blood stem cells in a patient with CML. This situation differed from others in that blood-derived CFU-GM were collected after initial therapy with cyclophosphamide (CY_3) that induced a complete, albeit short, remission, leaving the bone marrow free of Ph^1-positive cells (14). When these leukapheresis-derived cells were infused later after myeloablative treatment, there was evidence of recovery of hematopoiesis: all elements were present in a cellular bone marrow containing normal numbers of CFU-GM. That the patient became free of Ph^1-positive marrow cells is evidence that the blood stem cell graft contained Ph^1-negative clonogenic cells, perhaps with preferential growth (Fig 2).

CONCLUSIONS

We have been able to draw the following conclusions:

- Blood-derived hematopoietic stem cells represent an alternative stem cell source for autologous transplantation.

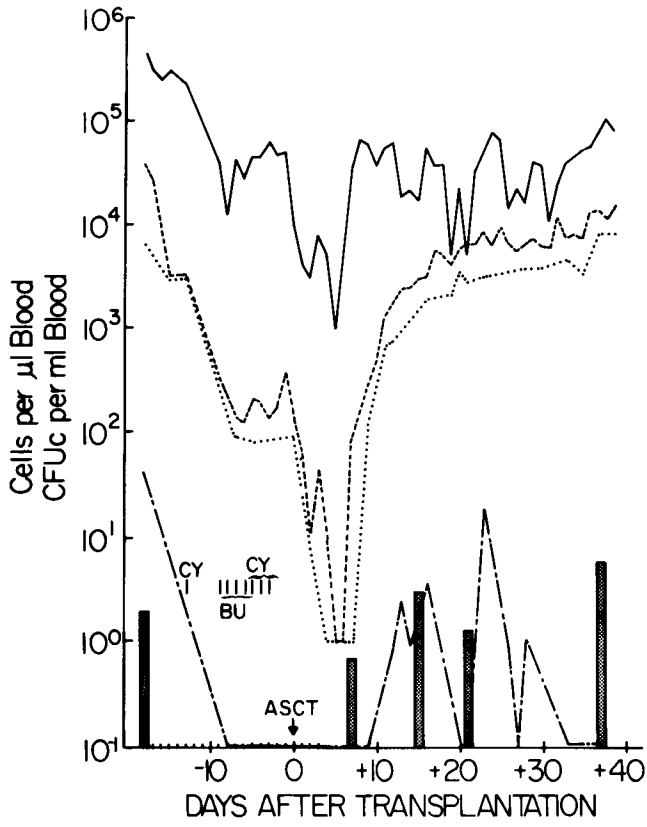

Figure 2. Platelet (solid line), *WBC* (dashed line), *granulocyte* (dotted line), *and CFU-C/ml peripheral blood* (dashed-dotted line) *and in bone marrow (per 2 x* 10^5 *mononuclear marrow cells, stippled bars) before and after autologous blood stem cell transplantation (ASCT). WBC, white blood cells; CFU-C, colony-forming units in culture.*

- In vivo cytoreductive pretreatment and chemotherapeutic purging have an inhibitory effect on CFU formation, although the CFU do not necessarily represent the pluripotent stem cell when cytoreductive treatment has occurred.
- Freezing and storing time do not significantly affect the stem cell viability.
- The long-term marrow culture is the most suitable in vitro system for evaluating the integrity of the pluripotent stem cell pool.

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Relevance of Natural Killer Cells in Autologous Bone Marrow Transplantation

E. Lotzova

INTRODUCTION

Autologous bone marrow transplantation (ABMT) represents a vital therapeutic approach to the treatment of various types of malignancies, especially leukemia. However, this procedure requires the conditioning of the prospective bone marrow recipients by total body irradiation or drug treatment or both for two main reasons: (*a*) to eradicate the malignant cells from the host and (*b*) to provide an optimal microenvironment in the bone marrow compartment (represented by sufficient space and stimuli) for transplanted bone marrow cells to grow and differentiate. Furthermore, bone marrow cells used for transplantation are, as a rule, cryopreserved. All of these treatments and procedures are harmful for functional properties of the immune system, the intact function of which is essential for successful growth and prompt recovery and maturation of hematopoietic and lymphoid cells.

IMPORTANCE OF IMMUNE SYSTEM IN ABMT

The immune system is of importance in ABMT for several reasons. First, immunocompetent cells play a role in defense against residual leukemic cells, frequently present in the bone marrow recipient, in the bone marrow graft, or in both. Second, these cells are involved in resistance against microbial infections, one of the major problems associated with bone marrow transplantation. These infections may be fatal for the patient receiving a bone marrow transplant. Third, lymphocytes, via cell-to-cell interaction and production of soluble factors, contribute to the hematopoietic and lymphoid recovery and perhaps also to the speed of such recovery.

NATURAL KILLER CELL FUNCTIONAL, THERAPEUTIC, AND PROGNOSTIC PROFILE IN ABMT

I shall restrict my subsequent comments to the relevance of natural killer (NK) cells to ABMT. I do not wish by any means to dismiss the possible role of other lymphocytes in this phenomenon; NK cells are preferred in this context because they have been shown to mediate a multitude of functions directly related to autologous transplantation.

The original function proposed for NK cells was their role in immune surveillance against leukemias. This premise was later substantiated by observations in experimental animals that mice with high NK cell activity had low incidence of lymphohematopoietic malignancies and mice with low NK cell activity experienced these types of malignancies more frequently (1). Further evidence in support of NK cell antileukemic activity is the exquisite sensitivity of leukemia and lymphoma cell lines to NK cell lysis in vitro (1-3). Moreover, high susceptibility of patients with NK cell defects to leukemia or lymphoma (4,5) and defective NK cell activity in patients with leukemia and preleukemic disorders (6,7) demonstrated the role of NK cells in defense against hematolymphoproliferative malignant diseases in man.

The above examples indicate that NK cells may be of great importance in ABMT. For instance, NK cells, because of their prompt, uninduced antileukemic activity, may play a crucial role in eliminating the residual leukemic cells from the immunoincompetent bone marrow recipient. In fact, the recent observations that NK cell activity is directed not only against leukemia cell lines but also against freshly derived leukemic cells, such

as acute myelogenous and lymphocytic leukemias and chronic myelogenous leukemia in blast crisis (7,8), support this possibility.

In addition to their antileukemic activity, NK cells display resistance against viral, bacterial, fungal, and parasitic infections (3,9), another function directly related to bone marrow transplantation. Thus, optimal NK cell functions may be of crucial importance for the patient receiving a bone marrow transplant to assure defense against leukemia and against life-threatening infections before hematopoietic and lymphoid recovery.

Recent data also indicate that NK cells are involved in the regulation of granulopoiesis (10), erythropoiesis (11), and lymphopoiesis in man (12) and collectively suggest that these cells may be involved in control of the homeostasis of hematopoiesis and lymphopoiesis.

Another important functional NK cell activity is the production of several soluble factors, such as colony-stimulating factor, various types of interferons, T-cell growth factor, B-cell growth factor, and cytotoxic, tumor-directed factor (3). These substances, specifically T-cell and B-cell growth factor, may contribute to the quality and the speed of hematopoietic and lymphoid recovery after bone marrow transplantation; the cytotoxic factor may aid in anticancer activity.

Since it appears that NK cells play a multitude of elementary functions in ABMT, transfusions of autologous NK cells before or before and together with the bone marrow transplant may be beneficial for the patient. This approach is technically quite feasible since the NK cell cloning technology allows the propagation of NK cells in vitro. Encouragingly, such in vitro-propagated NK cell clones have been found to retain their functional activities in vivo (13).

It is important to emphasize another potentially relevant NK cell function in ABMT and in leukemia. We and other investigators have shown (7,14) that NK cell cytotoxicity is restored in leukemia patients in remission; however, a decline in NK cell activity reappears in leukemia patients suffering relapse. Thus, a decline in NK cell activity may be indicative of recurrent leukemia prior to its clinical diagnosis. Such early detection of recurrent leukemia may lead to more-timely bone marrow transplantation or less-vigorous therapy or both.

The above-mentioned studies clearly demonstrate the potential importance of NK cells in ABMT. I hope this discussion will initiate clinical considerations for NK cell use as well as further research in NK cell activity.

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T. M. Fliedner and A. R. Zander, Moderators

Dr. Robert Gale: Dr. Korbling, I wonder if you are aware of any human who has received what is believed to be a marrow lethal dose of a chemotherapy combination and whose marrow was reconstituted with peripheral blood cells from a patient other than one with chronic myelogenous leukemia (CML).

Dr. Martin Korbling: We did a transplant in a patient with CML whose graft was obtained after an in vivo "purging." This patient underwent conventional chemotherapy that was followed by a transient Philadelphia chromosome (Ph^1)-negative disease phase, when several consecutive leukaphereses were performed. After myeloablative treatment and retransfusion of the Ph^1-negative blood stem cell graft, the patient's hematopoietic system was completely reconstituted without further evidence of Ph^1 recurrence. But that is all I know about such blood stem cell transfusion studies other than the studies in which Ph^1-positive cells were used.

Dr. Gale: I think we should keep in mind that reconstitution with peripheral blood cells other than Ph^1-positive ones has yet to be proved successful in man. I think one would have to be very careful and choose a clinical situation in which there is some backup before trying this.

Dr. John Goldman: I think I'd like to agree with what Bob Gale said. Martin Korbling's case is absolutely fascinating, but I think there is a possibility that those Ph^1-negative stem cells that reconstituted his patient may have had leukemic kinetic potential.

Dr. Ross Abrams: I think there are two other issues that need to be addressed. One is the possibility of augmenting the number of circulating hematopoietic progenitors and hematopoietic stem cells during the period of postchemotherapy recovery from nadir counts. This is an area that we have explored rather extensively in the dog model. The other possibility, which has been explored in animals by Dr. Zander's group, is the possibility of combining peripheral blood stem cells or peripheral blood mononuclear cells with marrow, particularly if you have marginal amounts of marrow. In an anecdotal case of ours, a gentleman had been extensively pretreated for Hodgkin's disease and had hypocellular marrow. We collected mononuclear cells from his peripheral blood, which had essentially no granulocyte-macrophage colony-forming units (CFU-GM) or erythroid burst-forming units (BFU-E), and combined that with a limited amount of marrow. We got brisk hematopoietic reconstitution after high-dose cyclophosphamide, 1,3-bis(2 chloroethyl)-1-nitrosourea (BCNU), and VP-16-213.

Dr. Herzig: I think all of us would like to have an assay of bone marrow recovery that we were confident in. You made reference to your belief that the Dexter system was useful. Do you have data? And how do you quantitate that system?

Dr. Korbling: Yes, we have such data, ones that show the presence of hematopoietic precursors in cultured marrow cells over a period of almost 4 weeks despite the fact that, owing to in vitro treatment with 4-hydroperoxycyclophosphamide (4-HC), all CFU had initially been eliminated. That shows that the replication potential of the pluripotent stem cell pool is intact, the most important factor for the establishment of a complete and permanent hematopoietic reconstitution.

Dr. Abrams: My question for Dr. Hill is, what do you put in your control bag? Normal medium or medium with cells? Do you put your thermocouple in your control bag or is it attached to the bag from the outside?

Dr. Roger S. Hill: In those earlier studies we used the same chronic granulocytic leukemia (CGL) specimens in the control and test bags. We were able to collect enough CGL buffy coat for the total experimental system. For the Hodgkin's disease bone marrow studies, the control bags contained whole peripheral blood and 10% dimethyl sulfoxide (DMSO).

Dr. Karel Dicke: Are you using other reagents like AB serum or just DMSO?

Dr. Hill: There are convincing data to indicate that protein, as serum or plasma, is an essential ingredient to add to marrow cells before freezing. Of course, if you are freezing whole marrow, it already contains plasma. Marrow prepared as buffy coat using the IBM 2991 also has 5-10% plasma remaining. The real problem arises with bone marrow samples prepared as mononuclear cell concentrates on Ficoll. It is essential to resuspend the mononuclear cells in plasma or serum, preferably autologous, before cryopreservation. I have data that I haven't shown that indicate that mononuclear cells frozen without plasma are less viable than those frozen with plasma added.

Dr. Richard Shadduck: If I understood you correctly, you stated on several occasions that you did not make any corrections in the freezing program for the heat of fusion.

Dr. Hill: That's correct.

Dr. Shadduck: The curves you showed had a rather long plateau of perhaps 5 or 6 min, yet I did not see a 6^{o} or 8^{o} temperature rise at the time the heat of fusion was applied. Was there indeed a plunge in chamber temperature or some other correction? You weren't showing the temperature of the chamber per se; you were simply showing the temperature of the material you were freezing.

Dr. Hill: The two superimposable freezing curves that I showed for those normal donors were from thermocouples placed inside the control ampoules. A major problem of cryopreservation is that there is no measurable parameter of the cooling curve that allows one to predict for cell recovery. The shape of the cooling curve, temperature of onset of the latent heat of ice crystallization, and the characteristics of the freezing plateau are extremely variable from ampoule to ampoule even in the same frozen batch. One ampoule will produce a curve with very little temperature elevation and the next adjacent ampoule in the freezer will produce a temperature elevation of 4^{o} or more. There is no consistency in our experience with ampoules, including the recovery of cell viability. So I think there is a real problem with the continuing use of ampoules for analysis of marrow viability.

Dr. Shadduck: My experience is more limited to bag freezing, in which there is a rather regular increase of 6^{o} or 8^{o} if you don't make corrections in the freezing program. But, if I am hearing you correctly, if you don't bother to correct the freezing for the heat of fusion you do retain the progenitor cells and do retain the ability to engraft someone with those cells?

Dr. Hill: Yes, absolutely. The temperature elevation reflects the magnitude of ice crystallization. By the time the freezing instrument has responded to the temperature elevation, ice crystallization is already proceeding. Compensation produces a cosmetic effect by eliminating the temperature elevation but does not necessarily favorably influence the biologic events of ice crystallization that are already in progress. There are no data that I am aware of for human marrow to indicate that by compensating for the latent heat of ice crystallization one is favorably affecting cell survival. In fact, there is some evidence from other mammalian cell studies to suggest that one may produce supercooling and actually adversely affect cell survival.

Dr. Robert Epstein: I wonder if you could comment on any particular differences in the freezing biology between different subsets of mononuclear cells. For example, do various tumor cells that can also be cloned show differences in their curves for freezing tolerance?

Dr. Hill: I have no experience with that. I might just make one comment about Dr. Korbling's cryopreservation method. He freezes at 2^{o}/min down to -20^{o}, then increases the rate to 7^{o}/min for further cooling. This program is controversial. In studies reported by Herzig and coworkers in the late 1970's, dog marrow samples were cooled at a rate of 1^{o}/min down to -50^{o} and then placed directly into the gaseous phase of liquid nitrogen. Viability as measured by engraftment potential was comparable to fresh. These remain probably the best studies in the literature dealing with the evaluation of freezing injury to marrow cells. I would be interested to hear of any other data that suggest that one has to evolve a more complicated freezing technique in order to get better results. I don't believe the data are there.

Dr. William Robinson: I would be interested in whether you think controlled-rate freezes are really necessary. In fact, a few times when my freezer has been broken down I have simply tossed the bags in a cardboard box and put them in the -4^{o} refrigerator and then the -20^{o} and then the -70^{o} and hauled them out and dumped them in the liquid nitrogen. It seems to me that the viability has been just as good that way, although I haven't done a comparative study with the controlled-rate freezer.

Dr. Hill: I'm pleased you asked that. The point I want to make briefly is that marrow viability appears to improve when a fixed rate of liquid nitrogen delivery is provided. Taking this idea further, one may speculate that an optimal freezing system might be a fixed temperature gradient, cold at the bottom and warm at the top, with the marrow cells set at a predetermined level or moved in the gradient to cool at the appropriate fixed cooling rate. We are now testing a freezer design that has liquid nitrogen in the bottom and a computer-controlled program that adjusts the level at which marrow cells sit in a gradient and are cooled. I believe a study is being published to show that cells also survive satisfactorily if they are placed in the top of a liquid nitrogen freezer under appropriate conditions in a fixed liquid nitrogen gradient. Freezing systems seem to be getting simpler rather than more complicated, and the mode of liquid nitrogen delivery to the cells may be an important factor affecting viability.

Dr. Subhash Gulati: I just wanted to agree with what you just said. We do not use controlled-rate freezing. We use a mixture of 6% hydroxyethyl starch, 5% DMSO, and 4% albumin. And we have easily 80-95% recovery of viability of the cells including CFU-C and BFU-E. We have had no trouble with engraftment of our patients. We just put it in the freezer at -80^{o} for 2 days and then transfer the bags into liquid nitrogen. This is the method you mentioned that is being published.

Dr. Hill: Are these bags or ampoules? I would be skeptical if you said ampoules.

Dr. Gulati: These are plastic bags, and what we do is we keep small aliquots in plastic ampoules for assessment of CFU-C and BFU-E function as a prescreen before we use the marrow for infusion into the patients.

Dr. Elliott Winton: Just in support of these last few comments, I'd like to say that we routinely, in our experimental situation, put marrow in small ampoules and put them in a Styrofoam container and put it in the -80^{o} freezer and then put it in the liquid nitrogen. We have routinely been getting 90+% recovery after thawing of all progenitors, including the mixed colonies.

Dr. Hill: My studies are relatively recent, and survival times are short. With cryopreservation in a Linde-type freezer, not dissimilar to

instrument C with the fixed rate of liquid nitrogen delivery, viability has been maintained for as long as 12 years. I think it is ironic that in 1984 we still don't know the degree of cryogenic injury that occurs to human marrow stem cells, and we don't know the contribution of that injury, if present, to engraftment. The information is available for dogs but not for humans. It's a sobering thought.

Dr. Goldman: The maximum duration of storage of cryopreserved cells in our hands is now 13 years, and we have been unable to demonstrate any loss of viability during storage in the liquid phase of nitrogen over a 13-year period. But I wanted to ask Roger Hill a question. For the last 10 or so years, we have been storing cells in small ampoules, and the reason we have been doing that is because you, Roger, introduced the system at the Hammersmith when you worked with us. I seem to remember that when we did reconstitution studies with ampoules, we got figures higher than the ones you showed on the slides today. I would like your comments.

Dr. Hill: You have made a very good point. There wasn't time to discuss these aspects. The CML data I presented were from heavily pretreated patients given busulphan, and they raise several interesting points. One of the proposed questions for the panel to answer was whether previous therapy affected cell recovery. Our recovery data for fresh CGL cells show 90-100% viability. By contrast, cell viability in the heavily pretreated patient group of about 30-40% most likely does reflect prior chemotherapy's effect on cell survival. Norman and coworkers have recently reported similar findings with CML cells. As far as the ampoules are concerned, there is a consistent and significant difference between cell viability from ampoules and bags as I showed. However, we get engraftment in the majority of patients whatever we do. This is not to say we shouldn't worry about the data we get and strive to improve our techniques. It may be that as viability improves, correlations not previously obtained become evident between in vitro and engraftment data. Ampoules, because of wide variability in cell recovery, are not helpful when striving to establish useful in vitro correlations.

Dr. William Peters: Could you speculate on why ampoules and bags are different?

Dr. Hill: Yes, I can. There appears to be a relationship between the cell volume being frozen and the characteristics of crystallization. If one were to freeze a single water molecule, all the energy generated as latent heat of ice crystallization is liberated as a single event, as the water molecule transforms to an ice crystal. Ice crystallization, as we deal with it, is a random event, and as the freezing volume increases, the number of water molecules crystallizing relative to the total volume, at any instant of time, becomes progressively lower. This means that ice crystallization, registering on the freezing curve at any single time point as latent heat of ice crystallization, diminishes overall as the volume increases. The temperature elevation is probably lost by dissipation through the larger volumes. What I am trying to say is that in a small ampoule, ice crystallization monitored as latent heat tends to be explosive, occurring as a single event, whereas if you increase the freezing volume, ice crystallization, as monitored, becomes a more gradual event occurring over a longer time frame.

Dr. Peters: Can you look at big ampoules?

Dr. Hill: That's a good point. If the hypothesis is correct there should be a critical volume, and perhaps a critical bag thickness, above which the latent heat may no longer be regularly detected. Large ampoules may be as satisfactory as bags.

Dr. Epstein: What is the correlation between recovery and number of bone marrow cells transfused?

Dr. Spitzer: We have published on this in the past in *Blood*. The cell numbers infused never correlated. In the second set of data they also didn't correlate.

Dr. Epstein: You know it correlates in every experimental system that has been investigated.

Dr. Spitzer: But those were not similar systems to the marrows being treated here, which have had chemotherapy, freezing, and so on. I don't know what the numbers are like in dog marrows after freezing, but they don't undergo so many manipulations.

Dr. Dicke: We need to keep in mind that in man cell dose initiation studies are not possible. Most likely, in man the number of cells used for transplantation are from above the minimum number of cells required for engraftment.

Dr. Axel Zander: We have a few more contributions by Dr. Gorin, Dr. Herve, and Dr. Robinson. I hope we will come to the most important question concerning freezing--What do you do if you have zero CFC? Can you use the bone marrow for rescue in an ablative situation? Did you see engraftment failure? Can engraftment failure be predicted by in vitro assays?

Dr. Norbert Gorin: First, I would like to say that the quality of cryopreservation is probably much more important than we can think of in terms of pure engraftment. Well-cryopreserved marrow reduces delays of recovery of leukocyte and platelets. But more than that, it is conceivable, for instance in the setting of autologous transplantation in acute leukemia, that reinfusion of numerous, well-preserved, normal stem cells may somehow account for a longer duration of remission and possible cure of leukemia. One may postulate, for instance, that because of interrelations between the normal stem cell population and the leukemic population, it might be beneficial to reinfuse the biggest dose of noninjured, normal stem cells. Therefore, I would not say that collecting much marrow somehow compensates for medium or even poor cryopreservation techniques. And I believe that we must infuse the highest dose of marrow possible as evaluated after thawing and after a very nice cryopreservation technique.

Second, although it is not yet really proved that the heat of fusion must be compensated for, we have to remember that numerous animal experiments in the past have clearly demonstrated that the longer the transition, the higher the destruction of the frozen cells. Over the years, I have discovered that we forget all these previous experiments, and we are starting to forget that maybe it is beneficial to compensate for the heat of fusion.

And finally, I would like to say for Dr. Vellekoop, for instance, that CFU-Cs evaluated on day 14 are different from CFU-Cs on day 10. CFU-Cs evaluated on day 14 probably are stem cells that are more immature. And in our experience CFU-Cs evaluated on day 10 are the best reflection of the cryopreservation technique as compared to BFU-E. I believe that the time has come now to address the question of the quality of cryopreservation in terms of CFU megas rather than CFU-C.

We have had some failures of autologous engraftment and some delayed kinetics of recovery, especially in platelets, and we started to try to correlate the freezing rate after the heat of fusion (the so-called third slope) to the quality of recovery in terms of CFU-C on day 10. We decided that good recoveries were above 50% of recovery, and poor recovery below 50%. What appears to be the case (and the same has been shown by Abrams in *The Lancet*) is that if the third slope is too steep then the percentage of good recovery decreases. On the other hand, if the third slope is very shallow, then you have 100% good recoveries.

This result was shown very well when we thawed the 71 bags of cryopreserved bone marrow, and we correlated the freezing rate on the third slope to the quality of CFU-C recovery. The probability of having a good cryopreservation is related to the freezing rate. The slower the freezing

Table 1. KINETICS OF ENGRAFTMENT ACCORDING TO THE METHOD FOR CONCENTRATING STEM CELLS IN 25 CHILDREN

HAEMONETICS M-30 (N = 14)			IBM 2991 (N = 11)		
WBC	PMN	PLTS	WBC	PMN	PLTS
$\bar{X}_1$	$\bar{X}_2$	$\bar{X}_3$	$\bar{X'}_1$	$\bar{X'}_2$	$\bar{X'}_3$
17±5.5d	24±10d	33±17d	19±6d	25±5d	29±9.4d
			NS	NS	NS

d, days; NS, not significant. (P. Hervé et al Besancon 1983)

rate, the higher the probability of getting a good recovery. We studied human marrow by the technique that we worked on with Geoff Herzig and Roger Herzig at the NIH in 1974 for dog marrow. The temperature goes down at a rate of -1^{o} or -2^{o} until the heat of fusion is released, and then we compensate. If you try to compensate too much, the third slope goes down, and if it goes down too quickly, then CFU-C are destroyed. In other words, the best curve would be this one with a constant decrease of -1^{o} or -2^{o} and the smallest transition phase.

Now there are some other situations in which the cryopreservation technique cannot be very satisfactory and this, in our experience, occurred in patients with CML. Differing, for instance, from John Goldman, we tried to do autologous bone marrow transplantation (ABMT) in a patient with CML using bone marrow instead of peripheral blood stem cells. And as I have stated in the past many times, from time to time we had failures of engraftment. Here again we tried to understand why. And we found that this recovery was especially poor when the amount of CFU-C per milliliter of frozen marrow was very high. There was an inverse correlation between the quality of freezing and the richness of the marrow in terms of CFU-C and not in terms of nucleated cells.

We tried to compare the recoveries in CFU-C in all our patients. The first group consisted of patients with acute leukemia, the second group of patients with non-Hodgkin's lymphomas, and the third group of patients with CML. (The question of purging procedures is not addressed here.) We noticed good recoveries in patients with acute leukemias, good recoveries in patients with non-Hodgkin's lymphomas, and worse recoveries in CML. There were only 21 good recoveries out of 41 frozen marrows.

If you want to have a very good recovery in neutrophils to 1000 white cells per cubic millimeter in less than 14 days, you need to infuse more than 5000 CFU-C/kg. On the other hand, below this threshold there is no difference. The same result appears in the survey of the European data I presented yesterday.

Dr. Patrick Herve: I would like to show you the difference in the cell recovery between two cell separators (Haemonetics model 30 and IBM 2991). Using the Haemonetics 30, the granulocyte contamination was between 40 and 60%, the CFU-GM recovery higher than 80%, and the hematocrit 5-10%. On the other hand, after IBM 2991 separation, the hematocrit was

Table 2. KINETICS OF ENGRAFTMENT ACCORDING TO THE METHOD FOR CONCENTRATING STEM CELLS IN 32 ADULT PATIENTS

HAEMONETICS M - 30 (N = 17)			IBM 2991 (N = 15)		
WBC	PMN	PLTS	WBC	PMN	PLTS
$\bar{X}_1$	$\bar{X}_2$	$\bar{X}_3$	$\bar{X'}_1$	$\bar{X'}_2$	$\bar{X'}_3$
16±4.7d	19±8.9d	27±21d	22±9.8d	24±10d	28±9d
			$0.02 < p < 0.05$	NS	NS

d, days; NS, not significant. (P. Hervé et al Besançon 1983)

lower than 1%, the granulocyte contamination lower than 20%, and the CFU-GM recovery 70-80%.

Engraftment data of Haemonetics- and IBM-processed marrow infused in children (Table 1) and adults (Table 2) have been documented. In adults, the white blood cell recovery was slightly delayed after grafting of bone marrow processed with the IBM 2991. However, in terms of granulocytes and platelet recovery the delay was not significantly different after IBM and Haemonetics separation. In children, there were no significant differences.

Dr. Robinson: I would urge you to consider, after looking at my data this morning, the use of noncryopreserved bone marrow cells for relatively short-term chemotherapy. I'm not going to show you the data again, but briefly, what we have shown is that at 7 days after storage in heparin at 10^0 of whole blood bone marrow, one has about 50% viable stem cells. Even out to 12 days you have about 20%. Using the controlled-rate freezer, after all the manipulations we wind up with only 10% of stem cells; thus, I think you should consider strongly the use of noncryopreserved marrow.

VIII. Tumor Cell Detection Methods

Leukemic Cell Colony Assay

K. A. Dicke, G. Spitzer, A. R. Zander, S. Jagannath, L. Horwitz, and L. Vellekoop

INTRODUCTION

The potential exists for leukemic cells to contaminate remission marrow. We would like to develop an efficient procedure for separating normal hematopoietic progenitor cells from leukemic cells, but to do this we need monitor systems for both cell populations. For normal hematopoietic progenitor cells, we use the following in vitro clonogenic assays: granulocyte-macrophage colony-forming cells (CFC-GM), erythroid burst-forming units (BFU-E), and pluripotent hematopoietic stem cells (CFU-GEMM). However, an adequate colony assay for leukemic cells in remission marrow does not yet exist; all available assays also allow normal hematopoietic progenitor cells to grow, so that a mixture of abnormal and normal colonies may be present in the same culture dish. Furthermore, it is at the moment not yet possible to identify leukemic cell colonies, in other words to differentiate between normal cell-derived and leukemic cell-derived colonies. The reason is that, except for cytogenetic abnormalities, markers specific for leukemic cells do not yet exist, although the availability of monoclonal antibodies (MAb) against leukemic cells may open new perspectives in this field.

In this chapter we review the methods of growing leukemic cells and discuss the experimental design we have developed to detect leukemic cells in remission and to monitor the separation of remission marrow cells.

IN VITRO LEUKEMIC CELL COLONY ASSAY

Two basic types of assays are available: cell culture in agar and cultures in methylcellulose. In agar, human placenta-conditioned medium (HPCM) leukocyte underlayers are used as sources of colony-stimulating factor. In methylcellulose, conditioned medium of cultures of mononuclear peripheral blood cells stimulated by phytohemagglutinin (PHA) is used. Colonies are usually counted 7-14 days after initiation of culture. In the agar system, aggregates containing greater than or equal to 50 cells are considered as colonies, aggregates containing 20-40 cells as macroclusters, aggregates containing 8-20 cells as medium clusters, and those containing 2-8 cells as microclusters. In methylcellulose cultures, colonies are considered to be aggregates if they contain greater than or equal to 20 cells.

Proliferation of leukemic cells is measured by a liquid culture system procedure when cloning in agar or methylcellulose and by recloning colonies in secondary plates after picking them from purging cultures.

When we want to use an assay as a monitor system, we need to determine the biologic value of in vitro colony formation. Several published studies indicate that leukemic cell growth in vitro is a useful predictor of remission induction, sensitivity of leukemic cells to chemotherapeutic agents, and recurrence of leukemia after remission. The size and number of aggregates are inversely correlated with remission induction, as has been demonstrated (1-3). The combination of in vitro growth patterns and cytogenetic analysis predicts induction of remission significantly better than either parameter alone, as Kirshner et al (4), Gustavsson et al (5), and Preisler et al (6) have shown. Francis et al (7) found that the higher the colony-stimulating activity threshold is for growing leukemic cells, the better the chances are of achieving remission.

Our group did not find a correlation between the in vitro growth patterns and remission induction. Nor could we correlate in vitro growth with remission duration (see results for patients with acute myelogenous leukemia [AML], Table 1). Not many patients were studied in the M4 subcategory, which may be a reason for our failure to find a significant

Table 1. Correlation between In Vitro Growth of Untreated Marrow Cells and Remission Induction Rate in Patients with Acute Leukemia

Disease Variant	Complete Remission	In Vitro Growth Rate Low[a]	High[b]	Total
AML (M2)	No	4	9	
		NS (P=.347)[c]		
	Yes	8	10	
CR rate		66.7%	52.6%	58.1%
AMML (M4)	No	0	8	
		NS (P=.085)		
	Yes	4	7	
CR rate		100.0%	46.7%	57.9%

[a] *Low growth: number of colonies, clusters ≤ 50.*

[b] *High growth: number of clusters > 50.*

[c] *Fischer's Exact Test.*

Note: *AML, acute myelogenous leukemia; NS, not significant; CR, complete remission; AMML, subcategory of AML.*

correlation. At least 25 more patients are needed before the answer can be conclusive.

In Fig 1, the survival data for patients with M2 AML according to in vitro pattern have been documented in a Kaplan-Meier curve. As can be seen, no correlation exists between in vitro growth and survival. Buick et al (8) and Preisler and Epstein (9) demonstrated a correlation between in vitro and in vivo sensitivity of leukemic cells to anthracyclines. Spitzer et al (2) and Moore et al (1) found that sequential in vitro growth of remission marrow and peripheral blood cells, measured by an increase of the colony:cluster ratio, predicts for relapse.

Kaneko and Motomura (10) and our group found a correlation between patterns of in vitro growth of marrow cells from untreated patients with AML and the morphologic structures of distinct AML subclassifications. Our results are listed in Table 2. Different patterns of in vitro growth have emerged for different morphologic types; specifically, acute lymphocytic leukemia has a low growth rate, and AMML bone marrow cells grow in a large number of clusters. In 15 of 19 instances, more than 50 clusters per 10^5 plated cells could be grown.

Several groups have studied the proliferation of leukemic cells in vitro and have attempted to correlate this with their chemosensitivity in vitro. Only Buick et al (8) found such a correlation, although Preisler and Epstein (9) found some instances of additional cluster growth. We did not find differences in the growth patterns of cells increased by delayed plating and T_0 plating.

An optimal monitor system for leukemic cells in remission marrow must meet the following criteria:

1. A detectable clonogenic leukemic cell subpopulation.
2. High plating efficiency.
3. Specificity.

If specificity is zero, a method should be developed to distinguish leukemic colonies from normal colonies.

Clonogenic leukemic cell subpopulations are only detectable when they have a high plating efficiency. A high plating efficiency can be achieved when a specific growth factor (or factors) for leukemic cells can be

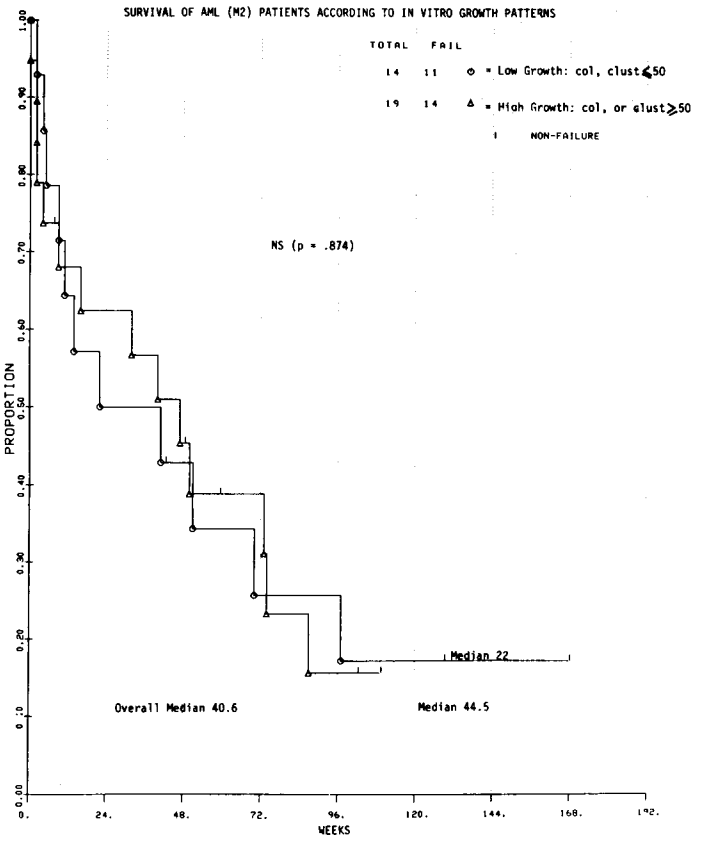

Figure 1. Survival rate of patients with AML (M2) according to in vitro growth patterns. The horizontal axis represents the time in weeks and the vertical axis the proportion of surviving patients. The patient population with a high growth pattern (colonies or clusters ≥50) is indicated by triangles; the patient group with a low growth pattern (colonies and clusters < 50) is indicated by circles.

Table 2. Patterns of In Vitro Growth of Marrow Cells from Untreated Acute Leukemic Patients

Culture Growth		Disease		
Colonies	+ Clusters	AML	AMML	ALL
≤ 50	≤ 50	14	4	40
≤ 50	> 50	15	12	0
> 50	≤ 50	0	0	0
> 50	> 50	4	3	0

Note: *AML, acute myelogenous leukemia; AMML, subcategory of AML; ALL, acute lymphocytic leukemia.*

found. At present, we are investigating sources for such a growth factor, including:

1. HPCM.
2. Peripheral-blood marrow mononuclear cells.
3. Irradiated K562, HL60, and Raji cells.
4. Conditioned media (CM) from K562, HL60, and Raji cells.
5. CM from Raji cells co-cultured with PHA and peripheral-blood marrow mononuclear cells from healthy persons.
6. CM from T-cell lines from healthy persons, from acute leukemia patients both untreated and in remission, and from hairy cell leukemias.

Preliminarily, we believe that different growth factors are needed for different leukemias. In certain situations we have found that growth is considerably enhanced by growth factors other than HPCM and peripheral leukocytes.

In case conditioned media can be found that stimulate leukemic cell growth, these media will be biochemically analyzed. Table 3 lists results of two experiments to test the activity of various conditioned media. As a target, we used bone marrow cells from patients with untreated acute leukemia. The percentage of leukemic cells in the marrow of these two patients was higher than 70%.

In another area of our research, we continue to look for specific leukemic cell markers so that, in remission marrow cultures, colonies derived from leukemic cells can be distinguished from colonies of normal hematopoietic progenitors. An example of such a marker is the human malignancy-associated nucleolar antigen (HMNA) (11). Heterologous antibodies against nucleolar antigens of leukemic cells and other tumor cells could be prepared by immunizing rabbits with nucleoli of HeLa cells. However, in only a few rabbits could antibodies specific against nucleoli of tumor cells be produced, which renders this procedure impractical. Despite this, because its significance is so far reaching, a search will be continued. Other markers will be examined, such as MAbs against specific antigens on the surface of leukemic cells; we need to see whether such antigens are expressed in sufficient enough quantities to make in vitro detection possible.

Outlined below are the steps we like to follow when using the in vitro colony-formation assay to monitor the in vitro purging procedure. The purpose is to eliminate leukemic cells from a remission marrow cell suspension, as discussed by Poynton et al (see the chapter "Colloidal Immunomagnetic Fluids for Cell Separation").

Materials:

1. Perform assay with methylcellulose.
2. Select a growth factor before remission induction.
3. Select a MAb as the leukemic marker before remission induction.

Technique:

1. At the time of marrow harvest and purging in remission, culture marrow cells with the previously selected growth factor(s). Determine the proportion of MAb-positive colonies before and after purging.
2. At the time of marrow harvest and purging, concentrate the MAb-positive cell population by performing fluorescence-activated cell sorting (FACS) before and after purging; then culture the cells.

DISCUSSION

Data are available in the literature suggesting a biologic relevance for leukemic cell growth in vitro. The reason that we could not find any correlation between in vitro cell growth and remission induction may be our use of HPCM as the colony-stimulating factor in our culture system.

Table 3. Results of Growth Factor Screening
Using Marrow Cells from a Patient with Untreated AML as Target

Growth Factor	No. of Colonies and Clusters/10^5 Cells	
	Patient 1	Patient 2
No conditioned medium	0/0	0/25
HPCM[a]	102/1113	0/97
Raji + nMN	1160/6140	0/121
nMN + HL60	109/4060	0/79
nMN + K562	153/3787	0/62
nMN + U937	48/1910	0/88
U937 + TPA	0/0	0/0
nMN + CCRFSB	7/139	0/237

Note: *The percentage of blast cells in the original bone marrow suspension was 78%.*

[b]*HPCM, human placenta-conditioned medium.*

Other groups have used leukocyte under layers and conditioned media of a giant tumor cell line (6). It is not known whether different sources of colony-stimulating factor turn on different leukemic cell subpopulations to form colonies in vitro.

A high plating efficiency of leukemic cells in vitro is essential to maximize the sensitivity of the in vitro colony-forming assay. However, even with a 100% plating efficiency of the clonogenic leukemic cell population, the assay is not likely to be able to detect one leukemic cell per 10^6 normal marrow cells in remission. One of the ways to detect such a small number of cells is to amplify the leukemic cell population before cloning the cells. Amplification or expansion of a cell population is possible, as Ruscetti et al (12) have demonstrated. They expanded T lymphocytes in cultures, after which cloning was possible. In the initial phase of these cultures, T-cell growth factor and PHA were essential to expand the cells. We have not yet identified such factors for leukemic cells; however, our continuous search for leukemic-cell growth factors may give us some leads in the near future.

The search for tumor markers is another essential part of our program. Such markers allow us to differentiate between leukemic cell-derived colonies and colonies from normal hematopoietic progenitors. We can then determine a ratio between the numbers of normal and leukemic colonies, which can be used as a monitor of the efficacy of marrow purging. Unfortunately, specific antibodies against the nucleolar antigens of tumor cells are very hard to obtain; only two batches of heterologous antibody raised in rabbits by immunization with nucleoli of HeLa cells were specific enough for our purpose.

Although we will continue our attempts to search for a "specific" HMNA antibody, we will use MAbs against leukemic cells as a marker in the meantime. A drawback of these cell surface markers is the relatively weak identification of antibody-reactive cells in agar. Agar interferes with immunofluorescence and makes the test laborious and cumbersome. Despite

these shortcomings, detection is possible by plucking the colonies out of the agar and staining the cells on slides. In contrast, the HMNA antibody can be applied directly to the colonies in the dish.

The experimental design as outlined in Table 3 allows us to use in vitro clonogenic leukemic cells as a monitor for marrow purging. In our initial clinical studies, however, we will make no clinical decisions solely on the basis of the outcome of the in vitro test. Only after demonstrating a correlation between the in vitro test and in vivo results will we use the test to determine clinical action.

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Long-Term Suspension Cultures of Bone Marrow Cells from Patients with Acute Myelogenous Leukemia

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INTRODUCTION

A possible presence of persisting leukemic cells in the remission bone marrow remains one of the major obstacles in the use of autologous bone marrow for the successful rescue of patients with acute leukemia after receiving high-dose intensification chemotherapy. Reliable methods for the detection of residual leukemic cells and their elimination in vitro are at present not available. In vitro growth of leukemic clones can only be achieved from bone marrow samples of patients with acute myelogenous leukemia (AML), whereas clonogenic growth of normal hematopoietic cells can be achieved from all normal marrows. This indicates a differential growth requirement for normal and leukemic clonogenic cells. In addition, the establishment of permanent cell lines of AML only indicates that preferential growth of the leukemic cell population may be obtained in a minority of cases. We have been interested in using long-term suspension cultures of human marrow as a model system for the comparative study of the growth and differentiation of normal and leukemic populations under defined conditions in vitro. Our working hypothesis was that under certain conditions the leukemic or normal population would grow preferentially. Such differential behavior of normal and leukemic cells could be exploited for either detection or separation of leukemic cells or both. The main purpose of this study was to evaluate the in vitro growth of leukemic bone marrows of previously untreated patients with AML.

MATERIALS AND METHODS

Bone marrow was obtained, during the initial work-up, from the sternum or posterior iliac crest of patients with previously untreated AML. After collection in preservative-free heparin (50 IU/ml), either the buffy-coat bone marrow (BCBM) cells were obtained or light density bone marrow (LDBM) (.2692 g/ml) cells were prepared using one-step Ficoll-Hypaque density gradient centrifugation (1).

Suspension Cultures of Bone Marrow

After preparing smears for morphology and plating for the assessment of the cells' initial cloning ability and growth pattern, bone marrow cells were seeded in Corning tissue culture flasks (25 cm^2 surface area). A total of 1 x 10^7 LDBM or 1.5 x 10^7 BCBM cells were resuspended in 10 ml of Iscove's modification of Dulbecco's minimal essential medium (IMDM) and supplemented with 10% fetal calf serum (FCS) and 20% horse serum. Hydrocortisone sodium succinate (HyC) was added to standard cultures at a final concentration of 1-5 x 10^{-6} M. The flasks were maintained at 37°C in an automatically regulated, fully humidified atmosphere of 5% CO_2 in air. Ninety percent of the medium was changed twice weekly. When BCBM was used, erythrocytes and mature granulocytes were removed on day 4 using a density gradient. If the number of non-adherent cells (NA) exceeded 1 x 10^6/ml at the time of medium change, the counts were adjusted to 1 x 10^6/ml. Once weekly the appearance of cultures was evaluated under an inverted-phase contrast microscope. The development of adherent stroma was assessed, and the number of NA cells was scored. Cytocentrifuge smears were then prepared for differential counts. A fraction of NA cells was assayed (usually 1 x 10^5/ml) in a double-layer semisolid agar-medium system for the presence of clonogenic cells.

Assay System for Clonogenic Cells

A modified (2) double-layer agar-medium system (3) was used throughout. The underlayer (1 ml) consisted of IMDM supplemented with 0.5% agar, 15% FCS, and 10% human placenta-conditioned medium (HPCM) as the source of colony-stimulating activity (CSA). At this dose and with the batch of HPCM used, the plateau growth of normal granulocyte-macrophage colony-forming cells (CFC-GM) was obtained. The cell population assayed for the presence of CFC was suspended in 1 ml of IMDM supplemented with 15% FCS and 0.3% agar and plated on top of underlayers. Quadruplicate cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air for 8 days.

Evaluation of Clonogenic Growth in Semisolid Cultures

On day 8 all cultures were scored under an inverted microscope. Clones of 8-49 cells were scored as clusters and clones of at least 50 cells as colonies. Overlayers were then transferred onto egg albumin-coated slides, dried, fixed in methanol, and stained with Giemsa's stain for morphologic examination of clones. Clones containing only immature cells with the morphology of blast cells (BL) or monocytoid cells were considered as being of leukemic origin (CFC-BL). Clones of cells containing myelocytes or more mature granulocytic cells, mature macrophages, or both were considered as being derived from normal CFC-GM.

Table 1. Maintenance in Flask Suspension Cultures of Clonogenic Cells from Leukemic Marrow of a Patient with AML-M2 (FAB Classification)

Days of Culture	Clones/1 x 10^5 Cells/Flask[a]		In Situ Morphology of Clones[b]
	Colonies	Clusters	
0	13 (1950)	3830 (5.75×10^5)	GM + Blast
4	9 (1800)	18 (3600)	GM + Blast
11	22 (3170)	26 (3740)	GM + Blast
21	13 (470)	13 (460)	GM
28	16 (144)	32 (288)	GM
34	46 (2024)	55 (2420)	GM
46	84 (336)	43 (172)	GM

[a] *Values are means from two flask cultures.*

[b] *In this patient, all colonies were granulocyte-macrophage colony-forming cells (CFC-GM) and contained mature granulocytes and macrophages, whereas clusters exhibited initially >90% blastic morphology.*

Table 2. Maintenance in Suspension Cultures of Clonogenic Cells from Marrow of a Patient with Acute Monocytic Leukemia

Day of Culture	Cells/Flask[a] 1 x 10^6	Clones/1 x 10^5 Cells[b] Colonies	Clusters	In Situ Morphology of Clones[c]
0	10	2700 ± 108	483 ± 108	Blasts 100%
5	15 → 10	540 ± 115	194 ± 11	Blasts 100%
12	18 → 10	27 ± 4	11 ± 3	Blasts 100%
20	1.2 → 0.7	0.7 ± 0.5	0	GM 100%
27	0.3 → 0	0	0	
35	0.3 → 0	0	0	
75	0.4 → 0	2.3 ± 1	0.3 ± 0.6	GM 100%
85	0.3 → 0	1.0 ± 2.0	2.0 ± 1.0	GM 100%

[a] *Because of extensive cellular proliferation during the first 2 weeks, the counts of nonadherent (NA) cells were reduced to 1 x 10⁶/ml on days 5 and 12. From day 27 on, all NA cells were removed.*

[b] *Mean ± SD of triplicate cultures.*

[c] *Granulocytes (G) and/or macrophages (M) containing clones.*

RESULTS

Bone marrow from 10 patients with various subtypes of previously untreated AML was studied in long-term cultures. The major emphasis was on the behavior of clonogenic (progenitor) cells. In all instances, the marrows were close to 100% cellular with more than 90% leukemic cell infiltration. In five cases, clonogenic growth was not detected at the time of initiation of cultures or during the subsequent culturing in vitro. Such cultures deteriorated within 3 weeks. In the remaining five bone marrow samples, either normal (CFC-GM), "leukemic" (CFC-BL), or both types of clones were detected in various numbers. This situation is characteristically demonstrated in Table 1. The most important observation was a short-term survival of progenitors of BL cells containing clones. In contrast, the presumably normal CFC-GM maintained their numbers throughout the 6-week observation period. From 3 weeks on, only clones containing mature cells were found. This observation was repeatedly made with marrow from acute monocytic leukemia (Table 2). In this case, both colonies and clusters were grown in semisolid marrow cultures initially. All of 200 scored clones were composed of easily identifiable large monoblastoid cells. The leukemic population rapidly lost its clonogenic potential in vitro. This was accompanied by extensive differentiation of monoblastic cells into macrophages. Albeit in very low numbers, CFC-GM could be found after 3 weeks of culture and were still detectable after 3 months in this system. They were readily recognized by the small size of the cells within the clones contrasting with large monoblastic cells in leukemic clones. The kinetics of the undifferentiated clone-producing BL cells and CFC-GM were further studied in another patient with acute monocytic leukemia (FAB-5A). The results are presented

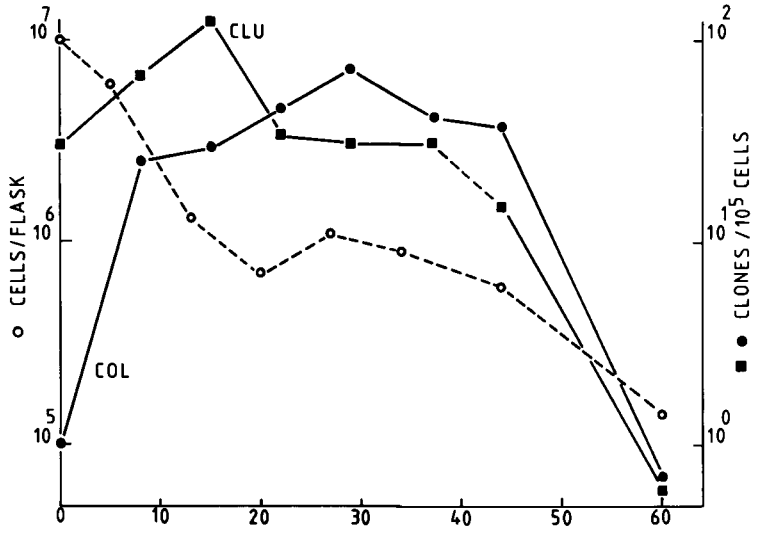

Figure 1. The total cellularity of the nonadherent fraction and the relative number of colonies and clusters in long-term (up to 60 days) suspension cultures of bone marrow from an untreated patient with acute monocytic leukemia.

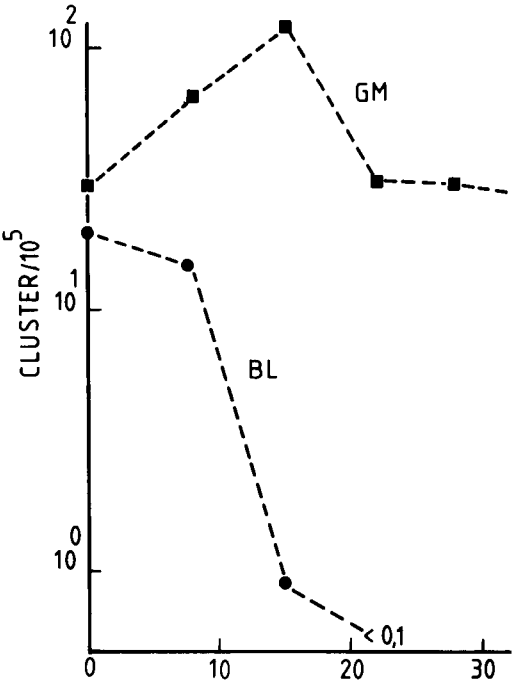

Figure 2. The relative distribution of leukemic (BL) and normal (GM) clusters as the function of time (over 30 days) in the nonadherent fraction of the long-term culture of bone marrow from an untreated patient with acute monocytic leukemia.

in Figs 1 and 2. In this instance, all of the 200 colonies scored were of granulocytic or macrophage morphology or both, whereas the clusters contained either undifferentiated BL or GM cells. Again, both types of

Table 3. Effect of Hydrocortisone on the Maintenance of Clonogenic Cells in Suspension Cultures of Bone Marrow from a Patient with Acute Monoblastic Leukemia

Day of Culture	5×10^{-6} M Hydrocortisone Sodium Succinate			No Hydrocortisone Sodium Succinate		
	Cells/Flask (1×10^6)	Clones/1×10^5 Cells[a]		Cells/Flask (1×10^6)	Clones/1×10^5 Cells[a]	
		Colonies	Clusters		Colonies	Clusters
0	10.0	0	200	10.0	0	200
3	4.2	ND	ND	7.4	ND	ND
10	1.2	10 ± 1	30 ± 1	2.0	2 ± 1	14 ± 1
28	1.6	ND	ND	1.2	ND	ND
35	1.1	30 ± 7	61 ± 9	1.4	0	0
42	0.8	ND	ND	2.6	0	0
49	0.8	5 ± 4	19 ± 5	3.6	0	0
56	0.5	9 ± 3	15 ± 5	12.2	0	0
63	0.4	4 ± 1	5 ± 1	29.0	0	0

[a] *Mean ± SD of triplicate cultures.*

Table 4. Effect of Hydrocortisone on the Differentiation Pattern of Leukemic Marrow in Suspension Cultures

Days of Culture	HyC[a] 5 x 10-6 M	Differential Count (%)						
		Blasts	Promyelocytes	Myelocytes	Metamyelocytes	Polycytes	Lymphocytes	Macrophages-Monocytes
0	-	90.0	2.0	1.5	1.0	1.0	2.0	2.5
10	+	4.5	45.0	3.0	0.5	1.0	5.0	40
	-	2.0	52.0	3.5	2.5	1.5	1.0	37
28	+	3.0	70.0	5.0	1.0	1.0	1.0	20
	-	65	6	1.0	0	1.0	0	26
35	+	3.0	32.0	20.5	11.5	30.0	0	2.5
	-	87.0	1.0	1.0	2.0	1.0	2.0	6.0
42	+	1.0	28.0	39.0	4.5	12.5	1.5	13.0
	-	94.0	0	0	0	4.0	0	2.0

Note: *Marrow from patient with acute monoblastic leukemia.*

[a]*Hydrocortisone sodium succinate.*

clones were readily distinguishable because of a much larger cell size as well as the morphology of BL clones. The kinetics of the different types of clonogenic cells in suspension culture show preferential growth of CFC-GM derived colonies (Fig 1) and clusters and disappearance of clusters of undifferentiated cells (Fig 2).

Since the standard culture system includes HyC supplementation, we evaluated the effect of its presence or absence in suspension cultures on the clonogenic growth and differentiation within the leukemic marrow (Tables 3 and 4). The rapid disappearance of the leukemic CFC growth was not affected by the drug. HyC was, however, critical for the development of a normal stromal cell layer and reappearance of CFC-GM progenitors, which were maintained for a period of more than 2 months. In the absence of HyC, a population of NA BL cells reemerged. These cells lacked the capacity to clone in semisolid media. The cells were negative for Epstein-Barr virus (EBV) genomic expression (EBV-NA staining) and were presumably of myeloid origin, as judged by their morphology (Table 4). Only in the presence of HyC was differentiation along the myeloid macrophage lineage found (Table 4).

DISCUSSION

The growth of the bone marrow from previously untreated patients with AML in long-term suspension culture revealed several distinctive findings: 1) the in vitro survival of leukemic clonogenic cells was invariably short in a culture system that included HyC, in spite of the development of a multicomponent stromal cell layer. 2) "Normal" CFC-GM had a selective growth advantage in the system and "reappeared" in the culture concomitantly with the decline in the leukemic cell population. When CFC-GM were present initially, they were maintained preferentially. In such situations, not only did the CFC-GM proliferate, but they also exhibited normal maturation.

The poor in vitro survival of mouse leukemic cells relative to normal hematopoiesis has been reported (7). More recently, it has also been found that in long-term cultures of bone marrow from patients with chronic myelogenous leukemia, the Philadelphia chromosome-positive clones are poorly maintained in such a culture system as compared to Philadelphia chromosome-negative progenitor cells (3). In the present report, the distinction between normal and leukemic cells has been based solely on the in vitro growth pattern and morphology. The BL-like morphology was considered as indicative of the leukemic origin of clones. Maturation of the clones along the GM pathway indicated origination from normal progenitor cells. In the system used, chromosomal markers are at present the only reliable markers for malignancy. Because of their absence in the initial leukemic specimen or for technical reasons, they could not be monitored in the present study.

This experiment, using bone marrow cultures either with or without HyC supplementation (Tables 3 and 4), indicates that conditions in long-term cultures may profoundly influence the growth of the leukemic marrow. The long-term suspension cultures in their standard form used in the present study favored normal hematopoiesis. In future studies, long-term cultures could be used to assess the size of the persisting normal stem cell pool in the leukemic marrow. The system appears unsuitable for the detection of persisting leukemia in remission bone marrow unless specific conditions for preferential growth of leukemic clones are better defined. The emergence of a leukemic cell line in the absence but not in the presence of HyC in culture media indicates that these goals can be achieved.

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Preclinical Studies on Detection of Minimal Residual Disease in Acute Leukemia

A. Hagenbeek and A. C. M. Martens

INTRODUCTION

Based on conventional methodology (light microscopy), the initial treatment of acute leukemia can only be monitored along a short part of the "log cell kill" scale. In the complete remission phase, an unknown number of leukemic cells, i.e., somewhere between 0 and 10^{10}, remain. Virtually no direct information is available on the number of residual leukemic cells or on their kinetics of proliferation. Because effective treatment of "minimal residual disease" (MRD) is today's most essential priority, methods to detect small numbers of leukemic cells are urgently needed. Use of autologous bone marrow transplantation (ABMT) will enable physicians to judge the quality of the graft and to evaluate the efficacy of various methods that are presently being applied to eliminate leukemic cells from the graft prior to reinfusion (e.g., monoclonal antibodies [MCAs], in vitro chemotherapy) (5). As discussed during a recent symposium, so far no means are available to provide this information in clinical practice (6). In other words, it is not clear whether a relapse that occurs after ABMT is due to residual leukemic cells in the host or to reinfused leukemic cells in the graft. Indirectly, in the near future this might be clarified to some extent by comparing the ultimate results of ABMT and isologous bone marrow transplantation in groups of patients with similar types of leukemias, remission-induction chemotherapy, phases of disease at the time of transplantation, and conditioning regimens.

Because of the many factors currently complicating these clinical studies, preclinical studies in a rat model for human acute myelocytic leukemia (AML) are performed, which focus on decreasing the detection level of MRD using MCAs and fluorescence-activated cell sorting (FACS). As described before (4), it has been possible to detect as low as 1 leukemic cell per 10,000 normal marrow cells. In this chapter, sequential studies on the quantification of MRD will be discussed, with emphasis on the growth kinetics of "invisible leukemia" that can be deduced subsequently.

MATERIALS AND METHODS

Rat Leukemia Model

The BN acute myelocytic leukemia (BNML), which was induced with 9,10-dimethyl-1,2-benzanthracene in a female Brown Norway rat, shows striking similarities to human AML (1,2). Some of the major characteristics of BNML are: (*a*) a slow growth rate, (*b*) a severe suppression of normal hematopoiesis owed to an absolute numerical decrease in the number of hematopoietic stem cells, (*c*) the presence of clonogenic leukemic cells, and (*d*) response to chemotherapy as in human AML. An additional advantage of this model is that normal stem cells and leukemic clonogenic cells can be selectively discriminated by modified spleen colony assays (3).

The BNML serves as a preclinical model in several cancer centers, such as The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas (Dr. K. A. Dicke), and The Johns Hopkins Oncology Center, Baltimore, Maryland (Dr. G. W. Santos, Dr. P. Burke).

Rm 124 MCA

This MCA IgM was produced and provided by Drs. H. Kaizer and R. J. Johnson of The Johns Hopkins University, Baltimore, Maryland. The speci-

ficity of this antibody for leukemic cells has been described previously (7).

Immunofluorescence Labeling of Cells

Bone marrow cell suspensions were prepared by flushing the femoral shaft with Hanks' HEPES buffered balanced saline solution (H.HBSS). The cell suspension was centrifuged and resuspended in H.HBSS supplemented with inactivated fetal calf serum (FCS) (5% v/v Flow Lab.) and iodine azide (0.01% v/v), which are used throughout the labeling procedure. For fluorescence labeling studies, 10^6 cells were pelleted and labeled with the MCA-Rm 124 (50 µl) at various dilutions at 0^oC for 45 min. After careful washing, the cells were incubated with a goat-anti-mouse-IgM (Fc) coupled to fluorescein isothiocyanate (GAM/IgM(Fc)/FITC 1/30) for 30 min at 0^oC. After washing, the cells were resuspended and processed on the FACS-II cell sorter.

Light-Activated Cell Sorter

After labeling, the cells were analyzed on a modified FACS II (Beckton Dickinson, Sunnyvale, CA) light-activated cell sorter with the laser beam at 488 nm (0.4 W). FITC fluorescence was measured through a combination of a broadband multicavity interference filter (520-550-nm transmission, Stamford, CT) and a 520-nm cut-off filter (Ditric) by an S-20 type photomultiplier. Perpendicular light scatter (PLS) intensity was measured by an A-11 type photomultiplier. PLS signals were linearly amplified. A logarithmic amplifier (T. Nozaki, Stanford, CA) was used for the fluorescence signals.

In case of kinetic studies, fluorescent leukemic cells were sorted into a DNA-staining solution containing propidium iodide, Triton X-100, and RNAse in phosphate-buffered saline. Thereafter, they were rerun through the FACS machine to determine a DNA histogram.

RESULTS AND DISCUSSION

After intravenous injection of 10^7 BNML cells into the rats, the number of leukemic cells present in various organs was determined at different times. This was done in a cell suspension of the given organ both by means of labeling with Rm 124 and FACS analysis and by subsequent in vivo bioassays. With the latter method, the absolute number of leukemic cells present in the suspension is derived from the survival time of recipient rats injected with a fixed number of cells (linear relationship between the survival time and the number of leukemic cells injected).

As an example, in the liver 3 days after intravenous leukemia transfer, leukemic cells already were detected with the MCA-Rm 124 (Fig 1). The number of leukemic cells was at that time already far above the minimal detection level, i.e., 10^7 leukemic cells were scored by the FACS machine in a total of 130 x 10^7 liver cells (1/130 cells). Serial determinations at various times after BNML inoculation yielded a growth curve of leukemia in the liver, characterized by a population doubling time (T_d) of 1.3 days (Fig 1, upper curve). The growth kinetics derived in this way are not different from those obtained by conventional bioassays (T_d = 1.2 days) (Fig 1, lower curve).

The observation that both curves differ in terms of time-tumor load relationship is most likely due to the fact that in the MCA experiments, only viable, nycodenz-enriched leukemic cells were injected, whereas in the bioassay "total cells" (including dead cells) were used.

These serial studies employing an MCA provide a relatively inexpensive method to study the kinetics of MRD. In Fig 2, data for the femoral bone marrow are added.

At present, studies are under way to study the kinetic characteristics of lower numbers of cells by serial determinations after inoculation with less than 10^7 BNML cells.

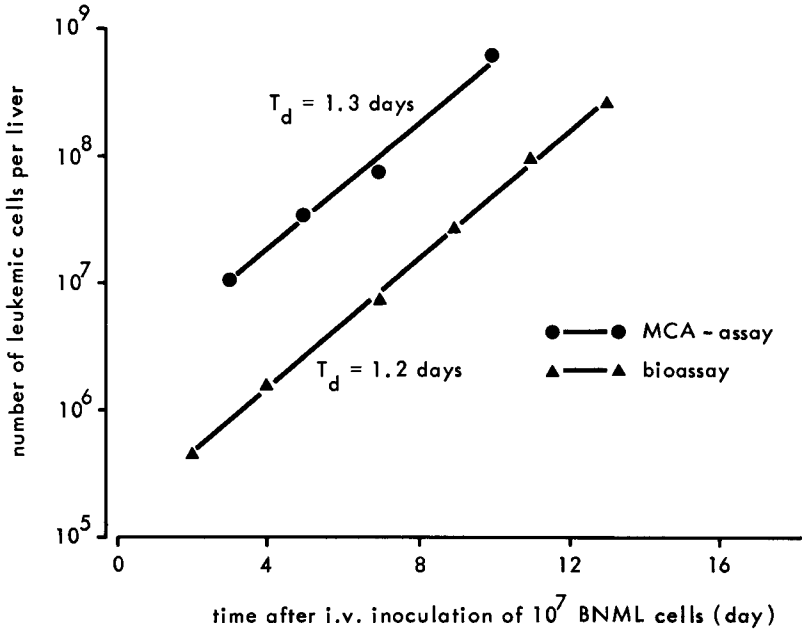

Figure 1. Growth kinetics of the BNML in the liver. Comparison of direct measurements with the MCA-Rm 124 and in vivo bioassays. T_d, doubling time of the leukemic cell population; MCA, monoclonal antibody.

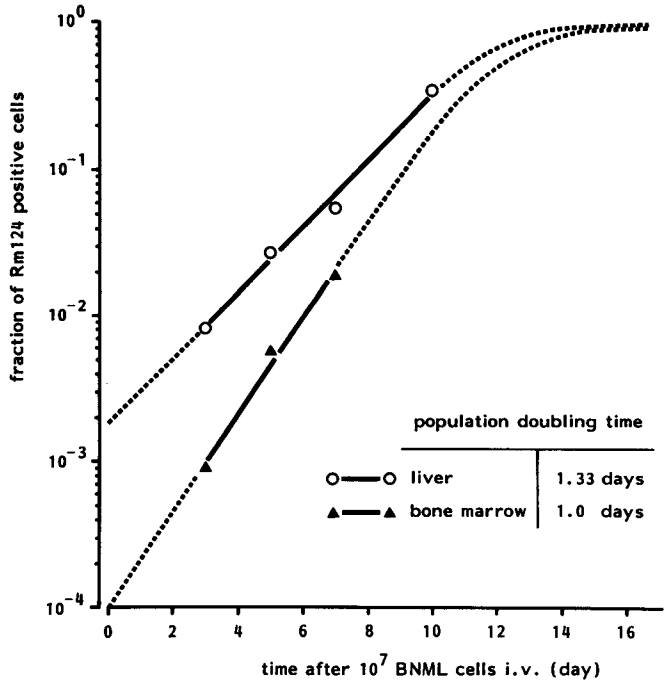

Figure 2. Growth kinetics of the BNML in the bone marrow and the liver as monitored with the MCA-Rm 124 (analyzed with FACS II).

Apart from the apparent feasibility of studying the kinetics of the leukemic cell population (T_d: doubling time), studies were initiated to analyze kinetics at the cellular level. Figure 3 shows how this was done

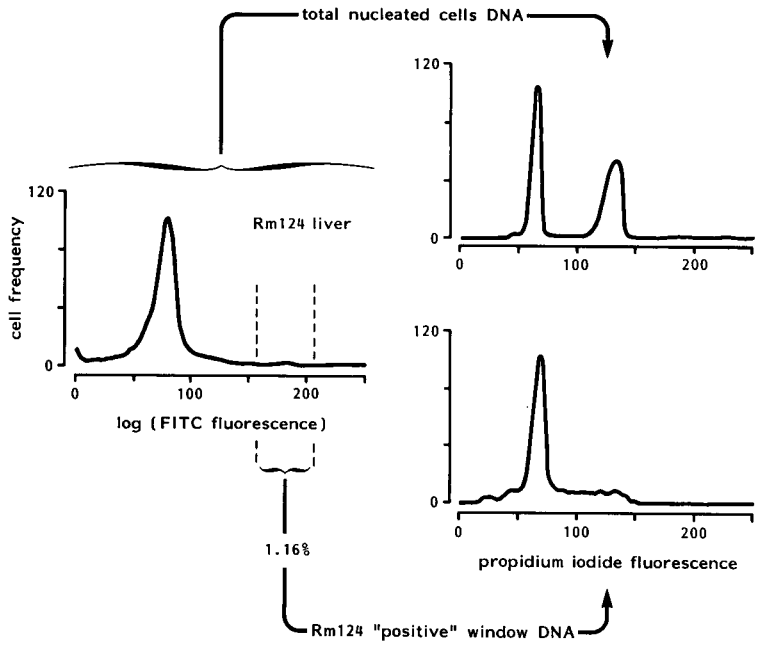

Figure 3. DNA histogram analysis of BNML cells present in a low frequency in the liver after fluorescence-activated cell sorting using the MCA-Rm 124. (This study was done on day 3 after intravenous inoculation with 10^7 BNML cells.)

in the leukemic liver. Three days after the injection of 10^7 BNML cells, a liver suspension was labeled with the MCA-Rm 124 (Fig 3, left). The fluorescent leukemic cells were sorted and subsequently subjected to a quantitative DNA analysis. The histogram obtained is given in the right lower panel of Fig 3. From this analysis an estimate can be made of the growth fraction of "residual leukemic cells" in the liver using a currently applied computer model. Obviously, this is impossible when the total liver is analyzed (Fig 3, right upper panel). The low number of leukemic cells is completely hidden in the DNA histogram of the diploid and tetraploid hepatocytes.

These preclinical animal model studies are meant to provide a base for clinical explorations. In this respect, it is recognized that the clinical conditions are more complex. First of all, the presently available MCAs against AML show quite a bit of cross-reactivity with normal marrow cells. Second, because only a relatively small fraction of the total marrow cellularity in man can be aspirated for diagnostic purposes and because cell-sorting technology is still rather "slow," the lower limit of detection of residual leukemia will be at most on the order of 10^6 cells.

However, from the rat model studies presented, several conclusions can be drawn.

- As described previously (4), the detection of 1 leukemic cell per 10,000 normal marrow cells is feasible with FACS analysis;
- Serial measurements using MCAs enable the study of population kinetics in minimal residual disease, showing a good correlation with conventional, expensive bioassays;
- Information on the distribution of leukemic cells over the cell cycle and hence, an estimate of the growth fraction can be obtained in minimal residual disease by establishing DNA histograms of MCA-labeled, FACS leukemic cells.

ACKNOWLEDGMENT

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Growth Factor Studies on Malignant B Lymphoid Cells:

Soft Agar Colony Formation in Human Lymphoma

R. J. Ford, Jr.

CONTROL OF HUMAN LYMPHOCYTE PROLIFERATION

The control of malignant lymphoid cell growth is presently an area of increasing interest in cancer research (1). Until the last decade, there was little information on what controlled the growth of normal T and B lymphocytes. The discovery of T-cell growth factor (2) (TCGF or interleukin-2 [IL-2]) changed this entire field drastically, and in the relatively few years since that discovery a great deal has been learned about growth factors controlling lymphocyte proliferation (3). During this time, we (4) and others (5,6) discovered that a similar lymphokine growth factor (also an interleukin), B-cell growth factor (BCGF), stimulated proliferation in normal human B cells. Further work on the interleukins indicated that the growth factor molecules share many characteristics (7). It is now apparent that distinct steps in human B-cell activation can be defined in which a resting G_0-phase B cell is triggered and transforms into an activated cycling B lymphoblast (8). These steps involve cross-linking of the cell surface immunoglobulin (SIg) receptor by antigen or an antigen simulator, such as anti-immunoglobulin M (anti-IgM), that activates the small resting G_0-phase B cell into a B lymphoblast (9). This activation step also causes the B cell to become responsive to its homologous growth factor (BCGF) presumably by expressing the cell surface receptor for this lymphokine, although this receptor has not as yet been identified or characterized. Activated B cells that bind BCGF are then stimulated into cell cycle progression and mitosis. The continued presence of BCGF in vitro can keep activated cycling B cells proliferating in vitro indefinitely (10).

This quantum jump in our understanding of normal lymphocyte activation and growth is now being extended into studies on neoplastic lymphoid cells. It has been shown, for instance, that a variety of malignant T-cell lines can make IL-2, or TCGF (11); other types of T-cell neoplasms have the receptor (Tac) for TCGF and can respond to exogenous TCGF but do not generate the growth factor in vitro or express the gene for TCGF (12,13). Human B-cell neoplasms, on the other hand, have not been as extensively studied as have the T-cell neoplasms, primarily because of the lack of purified BCGF, the assay systems needed, and growth factor-receptor definition. Our recent studies on a variety of B-cell non-Hodgkin's lymphomas (NHL) and chronic B-cell leukemias, such as chronic lymphocytic leukemia (CLL) and hairy cell leukemia (HCL), indicate that the malignant cells, as defined by SIg monoclonality (14) and the expression of the human malignancy-associated nucleolar antigen (HMNA) (15), can respond to human BCGF (16,17). The neoplastic B cells show both tritiated thymidine incorporation (Table 1) and cell growth in vitro when exposed to greatly purified human BCGF. These findings corroborate our hypothesis that since most malignant lymphoid cells retain the phenotype of their putative normal lymphocyte counterparts (18) they should also retain at least some corresponding functional capabilities as well, such as the ability to respond to homologous or lineage-specific growth factor(s). The finding that neoplastic B-lymphoid cells can respond to such growth factors implies that these tumors are not extremely aberrant from their normal lymphocyte counterparts in their basic cell structure (phenotype, morphology, etc.) and cell physiology but have probably undergone a subtle genetic change (e.g., nonrandom chromosomal translocation and oncogene expression) concomitant with or as a result of the neoplastic transformation process. It also implies that the tumor cells have retained some sensitivity to normal immunoregulatory signals, usually provided by lymphokines from accessory lymphoid cells, that control both cell growth and differentiation. The next logical question is, what is the

Table 1. Growth Factor Stimulation of Proliferation in Neoplastic Human Lymphoid Cells

Tumor Type[a]	Growth Factor[b]	^{3}H-Tdr Incorporated (CPM)[c]
Small cell NHL-B cell type[d]		
WDL	BCGF	10866[e]
WDL	BCGF	8010
N-PDL	BCGF	5017
N-PDL	BCGF	9460
Mixed cell	BCGF	8996
Small cell NHL-T cell type		
D-PDL	TCGF	11485
D-PDL	TCGF	8658
D-Mixed	TCGF	9876

Note: *^{3}H-Tdr, tritiated thymidine; NHL, non-Hodgkin's lymphoma; BCGF, B-cell growth factor; N-PDL, nodular poorly differentiated lymphoma; D-PDL, diffuse poorly differentiated lymphoma; TCGF, T-cell growth factor.*

[a]*Modified Rappaport classification, determined on hematoxylin- and eosin-stained paraffin sections.*

[b]*Greatly purified BCGF or TCGF preparations were obtained from diethylaminoethyl and gel filtration-purified lymphocyte-conditioned media.*

[c]*Microwell cultures containing 1 x 10^5 cells/well were stimulated with the growth factor preparation (10% vol/vol) for 72 h at 37° C. ^{3}H-Tdr (1 μCi/well) was added for the final 24 h of culture.*

[d]*Tumor cells were E_R-rosetted and adherent cell depleted and found to be >95% monoclonal by SIg light-chain typing. The tumor cells were also >95% human malignancy-associated nucleolar antigen positive.*

[e]*Counts per minute were calculated as experimental minus control (E - C) of triplicate cultures.*

source of the growth factor for malignant cells? By analogy to normal lymphocyte systems, one must consider the reactive normal accessory lymphoid cells ubiquitous in human lymphoid neoplasms. These lymphoid and monocyte/macrophage lineage cells appear to be immunocompetent, at least with respect to growth factor production, and probably represent some type of a cellular immune response to the neoplasm. While we have been able to demonstrate that such reactive T cells can make both BCGF and TCGF upon appropriate stimulation, it is unlikely that this is the primary mechanism driving the neoplastic lymphoid cell proliferation. A much more likely source is the neoplastic B cells themselves in an autocrine type of growth stimulation mechanism. Such a mechanism has recently been described for Epstein-Barr virus-transformed B-cell lines (19), and we have preliminary data that the same type of mechanism exists in a variety of human B-cell neoplasms (20). These findings suggest that we are on the verge of understanding many of the fundamental biologic processes involved in malignant lymphoid cell growth.

SOFT AGAR COLONY GROWTH IN HUMAN LYMPHOMA

The use of soft agar culture systems is widespread in experimental hematopoiesis (20). These systems have provided a great deal of basic

information about the nature of stem cells and their role as progenitors of the various hematopoietic cell lineages (i.e., myeloid, erythroid, etc.) present in the bone marrow (21). Soft agar systems have also contributed greatly to the discovery and definition of the colony-stimulating activities or factors (CSF) that control the growth and maturation processes in hematopoietic cells (22). A basic principle in virtually all of the systems relates to the requirement for a soluble factor, usually made by some type of accessory cell (e.g., granulocyte-macrophage [GM]-CSF made by a fibroblast) (23) that stimulates bone marrow progenitors or stem cells of a specific cell lineage (GM in the above example). These stem cells or cell renewal systems are typical of the myeloid system, where the concept of "stem cells" implies that cell renewal and maturation or differentiation are distinct alternatives; in addition, once a cell has been committed to a particular lineage, the renewal property of the putative stem cell is lost. Such constraints do not appear to be the case in the lymphoid system; however, where both T and B lymphocytes can be propagated indefinitely in the presence of the appropriate growth factor(s) (24,25). This finding has been utilized to develop soft agar cloning systems for both T cells (26) and B cells (27) based on the principles gleaned from the hematopoietic system, but with the use of specific interleukin growth factors usually in crude supernatant preparations. The lymphoid colonies or clones obtained can usually be picked from the agar and propagated further in vitro in the presence of the growth factor, a technique that is usually difficult if not impossible in hematopoietic stem cell systems.

This brings us to the subject of the soft agar colony or cloning assays for neoplastic human lymphoid cells. This is an area of considerable controversy, as the reproducibility of many of the techniques described has been questioned. One reason for the discrepancies noted may involve the different methodologies employed. Several types of double-layer soft agar assays have been reported that have yielded colonies of putative lymphoid tumor cells (28-31) derived from a variety of lymphoid neoplasms. These assay techniques have generally involved a variety of feeder layers of normal lymphoid cells plus or minus lectin, conditioned media from lectin-stimulated normal lymphoid cells, or various combinations of the above. Suffice it to say that it is difficult in most of the cases reported thus far to determine the exact nature of the cells plated in the assay, their viability at the beginning and the end of the culture period, and whether the "colonies" obtained were actually clonal progeny of single tumor cells or simply clumps or aggregates of tumor cells that formed before or after plating. Also, the subsequent characterization of the "tumor colonies" has been generally inadequate.

The above questions can, in general, be considered as the pitfalls of this type of methodology, which has sought to develop assay systems without a firm scientific data base. We have recently begun pilot studies in an attempt to apply what we have learned from our in vitro studies on the proliferative capabilities of human lymphoid cells to the development of a soft agar colony assay for neoplastic human lymphoid cells. Table 2 shows some preliminary results that we have obtained using greatly purified growth factors on representative human lymphoid tumors.

Our experience, utilizing a wide variety of human lymphoid tumors, indicates that for any measurable success in these techniques three general principles are necessary: 1) adequate cell surface phenotypic characterization, principally with monoclonal antibodies (MAb), and cell purification of the tumor cell population through cell separation procedures; 2) the use of defined if not purified growth factor stimulation; and 3) precise cell marking and definition of the putative tumor cells present in the obtained "colonies." Cellular phenotyping of lymphoid tumor cells is particularly difficult in the T-cell leukemias/lymphomas as clonal markers have not been described. We believe that the HMNA combined with a combination of the available T-cell antigens (e.g., -T_{11}, T_4, T_8) defined by MAb represents the best available methodology at this time. In the B-cell neoplasms, SIg light-chain typing in concert with the HMNA provides good phenotypic definition of neoplastic B cells (15). We have recently developed a double immunoenzyme technique for simultaneously identifying the presence of either SIg light chains or B-cell-specific

Table 2. Growth Factor-Mediated Colony Formation in Soft Agar

Tumor Type[a]	Growth Factor[b]	Colonies/ Plate[c]	Phenotype of Colonies[d]
D-WDL	BCGF	216	SIg+, B_1+, $^{\kappa}$+
D-WDL	--	2	N/D
HCL	BCGF	246	SIg+, $^{\kappa}$+, TRAP+
D-LCL-B type	--	0	N/D
D-LCL-B type	BCGF	360	SIg+, $^{\kappa}$+, B_1+
D-PDL-T type	TCGF	180	T_{11}, T_4+, SIg
D-PDL-T type	--	0	N/D

Note: *D-WDL, diffuse differentiated lymphoma; BCGF, B-cell growth factor; HCL, hairy-cell leukemia; D-LCL, diffuse large cell lymphoma; TCGF, T-cell growth factor; D-PDL, diffuse poorly differentiated lymphoma.*

[a]*Modified Rappaport classification.*

[b]*BCGF- or TCGF-containing preparations were added at 10% vol/vol.*

[c]*Double layer (0.3/0.5%) soft agar colony assays were set up with the growth factor incorporated into the bottom layer and the tumor cells present at 1 x 10⁵/ml final concentration in the upper layer. Colonies appeared 4-7 days after plating.*

[d]*Colonies were picked from agar and stained on slides by direct or indirect immunofluorescence.*

cell surface antigens (e.g., -B_1, B_2, B_4) together with the HMNA on individual tumor cells, which should be of great use in these assays.

The further development of agar colony techniques for neoplastic human lymphoid cells should provide a methodology for many future studies, not only for sensitivity testing of chemotherapeutic agents, but also as target cells for biologic response modifiers and other new treatment modalities.

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Detection of Burkitt's Cells in Remission Marrow by a Cell Culture Monitoring System: Implications for Autologous Bone Marrow Transplantation

I. Philip, M. C. Favrot, and T. Philip

INTRODUCTION

Between October 1980 and May 1984, 337 bone marrow (BM) aspirates from 66 patients were studied in a culture system that was derived from the technique of the first successfully cultured African Burkitt's lymphoma (BL) cell line by Epstein and Barr (1). The aim of this study was to detect early BM involvement in BL. The results of the cultures were compared with routine microscopic examination of BM aspirates. We concluded that purging procedures are necessary when BM is harvested for ABMT from patients in partial remission (PR), after relapse, or in first complete remission (CR) after initial central nervous system (CNS) disease.

PATIENTS AND METHODS

BM aspirates (337) from 66 patients were cultured at the same time regular monitoring or cytologic examination was done (i.e., protocol reported previously (2,3) and approved by the Ligue Regionale de Lutte Contre Le Cancer in Lyon, France).

Nineteen samples in 16 patients were clearly contaminated with tumor cells (>20% of BL cells). Of 318 samples from 50 patients, all were studied when results from cytologic examination of the marrow were normal (292 samples) or "suspect" (26 samples) (i.e., <5% putative BL cells).

In 26 patients, cells that were morphologically compatible with BL were observed in the marrow, but since the percentage was low (<5%) no conclusive diagnosis could be made. Their marrows were treated as potentially contaminated marrows or suspect marrows.

All aspirates were cytologically defined by a reference hematologist (Dr. Gentilhomme, Hopital Edouard Herriot, Lyon, France), who was independent of our team. Burkitt's cells were defined as monomorphic lymphoid cells of uniform maturity with a round nucleus and 3-4 nucleoli, a moderate amount of deeply basophilic cytoplasm, and clear vacuoles.

The in vitro liquid cell culture system has been previously described (4). Briefly, heparinized bone marrow samples (1-2 ml) were centrifuged on a Ficoll gradient (lymphoprep; 1.077 density, Nyegaard, Norway). After two washings, mononuclear cells were cultured in complete RPMI 1640 with 20% of heat-inactivated, irradiated, selected fetal calf serum, and L-glutamine and antibiotics (4). Cell suspensions were incubated in 25-cm^2 culture flasks at 37°C in 5% CO_2 atmosphere, in a vertical position. No feeder layer or other conditioned medium was used. Cultures were checked twice weekly with an inverted microscope, half of the medium was changed, and cytospin preparations were performed. The presence of Epstein-Barr virus-associated nuclear antigen (EBNA) was checked at the same time and at day 0 with indirect immunofluorescence technique (5). The cell lines in culture were characterized by cytologic, immunologic (6), or cytogenetic studies or both (7) (Fraisse, unpublished results).

Normal and malignant cell cultures have been observed. In normal cell cultures after a limited initial cell proliferation with the development of an adherent population, cells disappeared in 3-4 weeks, and only normal cells were observed in cytocentrifuged smears. In some cases, a haploid, Epstein-Barr virus (EBV)-positive lymphoblastoid cell line appeared after the third week, and the nonadherent myeloid cell population completely disappeared. In the malignant cell cultures, the first culture phase was similar to that of pattern I. During this period, malignant cells could readily be identified in cytocentrifuged, stained smears

between days 4 and 10 (4). If grown malignant cells were EBV-positive, they were easily defined by EBNA identification. The presence of malignant cells that grew in culture was easily defined before day 10, after culture by cytology and even if a cell line was not yet established (4). After establishment of the line, cytogenetics and immunologic analysis confirmed the growth of the same clone of malignant cells as the initial tumor (4).

RESULTS

Nineteen of the samples studied in 16 patients were clearly invaded by Burkitt's cells and identified by cytologic examination. In 14/19, we established a cell line (i.e., in 73.6% of the cases). Establishment of the cell lines was independent of the EBV status, the chromosomal translocation, or the clinical characteristics (relapse or diagnosis) at initiation of the culture.

Three hundred eighteen samples from the other 50 patients were studied: 292 samples were cytologically normal and 26 samples were suspect for tumor cells.

For 7/36 patients whose marrow was cytologically normal, we found Burkitt's cells in culture and in these cases, the in vitro monitoring assay was more sensitive than cytology (Table 1). Patient 4 had cerebrospinal fluid (CSF) relapse, and patient 2 had cytologically normal successive BM aspirations (23-day interval), from which Burkitt's cells grew in culture (BL 43 and BL 44, Table 1). A total of 8 cell lines were then established from these seven patients. For 2/14 patients whose marrow was cytologically suspect, Burkitt's cells were found in culture (Table 2).

Of these 50 patients, only 3 (two with normal cytologic bone marrow, one with suspect cytologic bone marrow) had subsequent relapses of the bone marrow after a negative result of the in vitro monitoring assay; however, in those patients, the primary tumor did not grow in culture. Hence, we conclude that we did not observe false-negative in vitro culture results in cases of BLs growing in culture. Details of this work have been published elsewhere (4).

We defined the sensitivity of the test using an artificial model in which the patients' marrow was contaminated with BL cells from its own cell line established at diagnosis. The in vitro culture system was able to detect $1/10^6$ cells in this experimental study. If we assumed that the limit of detection of cytology was 5%, these results showed a 3-4-log improvement of detection of minimal BM invasion.

Among these 50 patients, 234 marrow samples that were cytologically normal or were suspect were studied while the patients were in clinical CR. Burkitt's cells were found in 1/234 samples (patient 9, Table 2), i.e., 0.4% of the samples and 2% of the patients (1/50).

Of the 21 patients studied with cytologically normal or suspect marrow, three were in second CR, four were in PR after initial induction therapy, nine had CSF relapses, and five had other relapses. We found Burkitt's cells at culture in 8/21 patients (0/3 second CR, 2/4 PR, 5/9 CSF relapses, 1/5 other relapses).

DISCUSSION

The 4-year experience of monitoring minor BM involvement in BL allowed us to set up an in vitro cell culture monitoring system that can detect minimal contaminating BL cells in cytologically normal marrow. The level of detection is $1/10^6$ clonogenic BL cells in an artificial model of contaminated BM, either autologous (as presented in this chapter) or irradiated allogeneic BM (50 Gy) (see the chapter "Monoclonal Antibodies and Complement as Purging Procedure in Burkitt's Lymphoma").

As already indicated (see the chapter "Massive Chemotherapy with Autologous Bone Marrow Transplantation in 50 Cases of Non-Hodgkin's Lymphoma with Poor Prognosis"), ABMT and massive therapy are indicated for BL patients who are in relapse or PR after initial induction therapy. As

Table 1. Clinical Status and Culture Status of Patients 1-8[a]

Patient No./ Clinical Status at Culture	Status of BM at Culture	Results of Culture (Day of Positive Cytology)	Interval between Culture and Death (days)	Comments
1 CSF involvement	Normal	IARC/BL 8 (ND)	17	Death caused by interstitial pneumonitis. Suspect BM aspirate.
2 CSF relapse	Normal	IARC/BL 17 (10)	114	Died in Algeria. Inadequate reassessment.
3 CSF relapse and abdominal relapse	Normal	IARC/BL 35 (6)	20	Died of sepsis. Postmortem BM biopsy showed clumps of malignant cells.
4 CSF relapse	Normal Normal	IARC/BL 43 (9) IARC/BL 44 (7)	37 14	Died of progressive CSF disease. Postmortem BM aspiration showed 5% BL cells.
5 CSF relapse	Normal (TBM)	BL cells (7) EBV - cell line nonestablished	83	BM involvement (60%) at day 65: establishment of BL 2.
6 CSF involvement	Normal	BL cells (8)	20	Died of progressive CSF disease. Postmortem BM aspiration showed 5% BL cells.
46 Progressive disease Abdominal and cervical	Normal	IARC/BL 78 (9)	60?	CR obtained without evidence of BM involvement. Relapse and death with BM involvement at day 60.
7 CSF relapse	Normal (TBM)	No growth	45	BM involvement at day 35 (50% of BL cells) despite massive therapy and ABMT.
8 Abdominal and CSF relapse	Normal	Lymphoblastoid cell line (IARC/098)	113	BM involvement at day 88 (50% of BL cells) despite massive therapy and ABMT

Note: *BM, bone marrow; CSF, cerebrospinal fluid; IARC, International Agency for Research on Cancer; BL, Burkitt's lymphoma; CR, complete remission; ND, not determined; TBM, thawed bone marrow; EBV, Epstein-Barr virus; ABMT, autologous bone marrow transplantation.*

[a]*Patients 1-6 had normal bone marrow smears with the appearance of Burkitt's lymphoma cells in culture; patients 7 and 8 had negative results from bone marrow monitoring.*

Table 2. Clinical Status and Culture Status of Patients 9-11

Patient No./ Clinical Status at Culture[a]	BM Status at Culture	Results of Culture	Interval between Culture and Death (days)	Comments
9 Complete remission	Suspect	EBV-BL cells nonestablished cell line (contamination of the culture)	27	Death caused by Burkitt's interstitial pneumonitis (5% BL cells in BM at death).
10 CSF relapse	Suspect (<2%)	IARC/BL 28	70	Bone marrow graft cancelled (purge not available at this time). Died of CSF bleeding.
11 CSF relapse	Suspect	Normal	70	Failure of BM monitoring (a new sample was cultured at death, i.e., 100% involvement and IARC/BL 61 establishment).

Note: *BM, bone marrow; EBV-BL, Epstein-Barr virus - Burkitt's lymphoma; CSF, cerebrospinal fluid; IARC, International Agency for Research on Cancer.*

[a]*Patients 9 and 10 had positive in vitro cultures with suspect bone marrow. Patient 11 represents a failure of the bone marrow monitoring system.*

shown in the following data, BM harvested in a situation of clinical CR will be a safe procedure for BL patients (i.e., <2% of the patients will have marrow with Burkitt's cells at this time). However, the overall survival rate of patients in CR is approximately 80% if primary CNS disease is excluded (8), and it is not necessary nor is it too expensive to harvest 8/10 BMs without reinjection.

We decided to harvest the BM only at the time of BM transplantation indication (i.e., PRs or relapses). At that time, of the patients studied 8/21 patients (40%) with marrow that was cytologically normal showed BL cells at culture. Our conclusion was that BM would have to be purged prior to ABMT (see the chapter "Massive Chemotherapy with Autologous Bone Marrow Transplantation in 50 Cases of Non-Hodgkin's Lymphoma with Poor Prognosis" for the clinical data).

Eight of the 10 cell lines established from marrows that were cytologically normal or suspect were seen in patients with CNS disease. Despite three negative cultures of patients in CR of stage IV CNS, we decided to purge the marrow of these patients before ABMT because of the significant relationship between a positive culture and clinical CNS disease.

In conclusion, a purging procedure is necessary prior to ABMT in at least 40% of the patients with BL if the BM is harvested at the time of clinical indication of the BM transplantation.

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Malignant Markers in Colonies of Sheep Erythrocyte-Rosette-Positive Cells from Non-T Hematologic Malignancies

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INTRODUCTION

The growth of sheep erythrocyte-rosette-positive-forming colonies in culture (ERFC-C) in semisolid agar in response to phytohemagglutinin (PHA) stimulation has been extensively described (1-9). Clonal culture provides a system in which we can examine cell replication independent of cell-to-cell interaction and enables us to make a more definitive assessment of regulation at a defined cell level.

We, as well as others, have generated data suggesting that T lymphocytes cloned in agar with a mitogen or mitogens and conditioned medium are only a subset of T cells or, in some instances, may even be a pre-T cell (stem cell) (10-14). These cells do not grow preferentially in liquid culture.

We have also investigated the nature of ERFC-C derived from non-T hematologic malignancies cloned in semisolid agar. As a marker for malignancy we have used predominantly the human malignancy-associated nucleolar antigen (HMNA) assay and, in some instances, cytogenetic analysis.

Results of the HMNA assay have been consistently positive in malignant tumors of diverse nature and negative in benign tumors, inflammatory conditions, and normal tissue (15-20). Colonies of acute myeloid leukemia (AML) cloned in agar have also been positive on an HMNA assay, though their normal counterpart, granulocyte-macrophage colonies, has been negative (21-23).

In this chapter we report abnormalities of T cells cloned in agar from non-T hematologic malignancies (AML and B-cell neoplasms). Possibly, T-cell abnormality could affect the outcome of bone marrow purging with antibodies or drugs.

MATERIALS AND METHODS

A cell suspension of whole mononuclear cells (WMNC) was obtained from the peripheral blood, bone marrow, spleen, or lymph nodes for the experiments involving normal controls and from similar sources plus pleural effusions for the experiments involving hematologic malignancies.

The methods of cloning T cells in an agar bilayer system have been previously described (9,10). Sheep erythrocyte-rosette-positive (ER+) cells obtained from peripheral blood of normal donors were stimulated with PHA for 3 days. The medium was then harvested and used at a 50% concentration as the underlayer. The upper layer contained 5 x 10^5 WMNC, obtained from the sources mentioned above.

Cultures were analyzed after 10 days. To examine the percentages of HMNA-positive cells and ER+ cells, we rinsed the superficial colonies off the surface of the agar and then analyzed the cultures for these markers. If sufficient colonies were available and the tumor had a marker chromosome, cytogenetic analysis was attempted.

RESULTS

Cells in superficial colonies grown from normal persons and from patients with malignant disorders were routinely greater than 84% ER+. We initially examined the specificity of the HMNA assay by analyzing ERFC-C grown from bone marrow, peripheral blood, and spleen of normal donors, and from both normal and cancerous lymph nodes from patients with nonhematologic malignancies. The results were consistently less than 5% (Table 1).

Table 1. Analysis of assays on colonies of sheep erythrocyte-rosette-positive cells

Nature of Samples	Number of Samples Cultured	Number of Tests Yielding ERFC-C Growth (Cloning Efficiency Range)	HMNA[a] Assays No. Positive (Range % Positive Cells/No. Analyzed)	Cytogenetic Assays (No. Abnormal/No. Analyzed)
A. Normal controls Normal specimens	17[b]	17 (0.0018-0.348%)	0/15	0/1
Lymph nodes involved by nonhematologic malignancies	3	3 (0.0016-0.103%)	0/3	---
B. Hematologic malignancies				
AML	19[c]	17 (0.0028-0.342%)	15(31-98%)/17	1/1[d]
Lymphomas	21[e]	11 (0.0002-0.7444%)	7(5-94%)/9	1/1[f]
Hairy-cell leukemia (bone marrow)	1	1 (0.297%)	1(>99%)/1	---
C. AML in complete remission (bone marrow)	8	8 (0.0008-0.04%)	6(6-96%)/8	---

[a]Human malignancy-associated nucleolar antigen.

[b]Peripheral blood (8) and bone marrow (4) from normal donors; spleen (2) from accident victims; uninvolved lymph node (3) from patients with nonhematologic malignancies.

[c]Bone marrow (10), peripheral blood (8), involved lymph node (1).

[d]Lymph node (12), pleural effusion (5), bone marrow (3), spleen (1).

[e]Involved lymph node: 47XY+C chromosome aberration.

[f]Malignant pleural effusion: Dq+ chromosome aberration.

However, the great majority of specimens from patients with AML or B-cell neoplasms showed a high percentage of HMNA-positive cells. Cells from six of eight patients with AML in remission still showed persistent HMNA-positive ERFC-C.

We examined the cells of two patients whose cytogenetic analysis demonstrated abnormal results and detected the same chromosome aberrations in the cells from ERFC-C. In these two patients, the percentage of HMNA-positive cells in the ERFC-C was 88% and 98%.

DISCUSSION

These preliminary results suggest that abnormalities in cells from patients with non-T hematologic malignancies can give rise to colonies of cells with the ER+ T-cell marker. This possibility may be important in understanding the etiology of leukemia and lymphoma and in formulating new therapeutic approaches to these malignancies.

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Tumor Cell Detection in Autologous Bone Marrow Grafts

B. Barlogie

Infusion of autologous bone marrow has made possible the administration of substantially higher doses of cytotoxic drugs with side effects that are predominantly myelotoxic. The clinical success of this strategy is dependent upon two major premises: 1) the drugs employed have a dose-dependent antitumor effect, and 2) no contaminating tumor clonogenic cells are present in the marrow graft. The latter status can be achieved by using appropriate screening tests to carefully select patients or by purging in vitro those marrows that have either obvious tumor cell contamination or presumed involvement, as is the case with remission marrows from patients with leukemia.

In this chapter, I limit discussion to the problems of detecting tumor cells in the bone marrow of patients considered for autologous transplantation. Reflecting on the potentially successful use of this approach in patients with oat cell lung cancer, lymphoma, and neuroblastoma, one realizes that these generally chemotherapy-sensitive tumors display a considerable propensity for early dissemination and hence marrow involvement; this, of course, is also an intrinsic feature of the leukemias. The only clinical examination routinely applied to the marrow is the morphologic screening of marrow aspirate and biopsy material, which seems to be a fairly reliable test for detecting tumor cells of epithelial origin but less so for effectively screening for residual leukemia and lymphoma. Automated cytology in the form of flow cytometry appears to be an ideal tool for mass screening of the marrow, because it permits objective and quantitative cell analysis on many thousand cells per second (1). The question arises, however, as to which cellular property is most sensitive and specific for the purpose of tumor cell detection.

In an effort to distinguish tumor cells from normal, and specifically hematopoietic, cells, a number of physical, biochemical, and immunological cellular characteristics have been examined. In some circumstances, when direct microscopic inspection of the uncultured and unseparated marrow has been unsuccessful, clonogenic assays in vitro have preferentially expanded the tumor cells until tumor contamination could be detected.

DNA CONTENT ABNORMALITIES: ANEUPLOIDY

Tumor cell heterogeneity is schematically outlined in Fig 1. In general, an inverse relationship exists between the tumor's clonogenic potential and its degree of differentiation. The former appears to be most critical for the task under consideration. On this basis, genomic abnormalities commonly associated with tumors apparently represent an intrinsic tumor cell feature that is probably not subject to change with cell differentiation.

Instead of analyzing the mitotic karyotype, which requires in vitro DNA synthesis and progression of tumor cells into mitosis, it is more convenient to examine abnormalities of cellular DNA content in G_1. These abnormalities can be readily measured by flow cytometry after cells have been stained with a DNA-specific fluorochrome (Fig 2). Three abnormal DNA stem lines can be discerned in this example of acute lymphoblastic leukemia, but are no longer detectable during morphologic remission.

The collective experience in detecting aneuploidy with DNA flow cytometry is summarized by diagnosis in Table 1. The incidence of such abnormalities is high in the solid tumors and in myeloma (2), whereas only approximately 50% of all non-Hodgkin's lymphoma (3) and 25-35% of all leukemias (4) express such abnormality. For malignant lymphomas, the rate of aneuploidy is markedly higher in aggressive compared to indolent histologic types (Table 2).

An additional feature of great importance for detecting tumor cells in the bone marrow by DNA flow cytometry is their degree of DNA abnor-

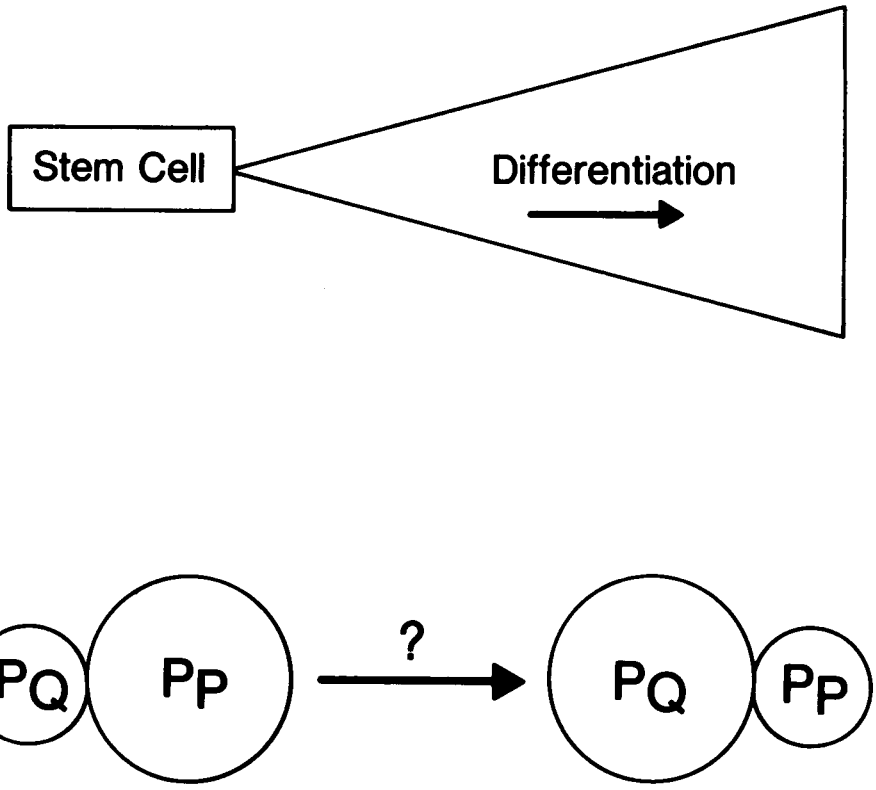

Figure 1. Cell kinetics of tumor stem cells and their progeny. P_Q, quiescent pool; P_P, proliferating pool.

mality, as schematically outlined in Fig 3. Sensitivity is particularly high when the aneuploidy level is either hypodiploid or hypertetraploid and thus does not interfere with the diploid marrow cells that, with their proliferative compartment, occupy the entire diploid-to-tetraploid range. We have conducted in vitro mixing experiments with normal marrow and aneuploid tumor cells in order to define how the degree of aneuploidy abnormality affects the sensitivity of tumor cell detection by DNA flow cytometry (Fig 4). We examined three different aneuploidy levels, a minor DNA excess of 10% (left panel), an intermediate near-triploid DNA excess (middle panel), and a marked hypertetraploid abnormality (right panel). The top histograms represent the initial unseparated cell populations. Below, we increasingly admixed normal marrow cells as indicated on the left margin, achieving dilutions down to 1:25. If the original sample contains 45% triploid cells, tumor cells can still be detected at a dilution of 1:15. If the original sample is a hypertetraploid DNA stem line, fewer than 1% of tumor cells remain readily identifiable at a 1:25 dilution.

The degree of DNA-content abnormalities in the hematologic malignancies and in solid tumors is delineated in Fig 5. Solid tissue neoplasms cover the entire spectrum from haploid to hyperoctaploid, but hematologic malignancies typically exhibit a low degree of hyperdiploid abnormality and a DNA excess of 10-20% (1). This, of course, markedly reduces the sensitivity of DNA flow cytometry for detecting residual leukemia, lymphoma, and myeloma on the basis of DNA content alone.

In a study comparing the results of morphologic analysis and DNA flow cytometry in oat cell lung cancer, we encountered rare instances in which either flow cytometry or microscopy were the sole indicators of tumor

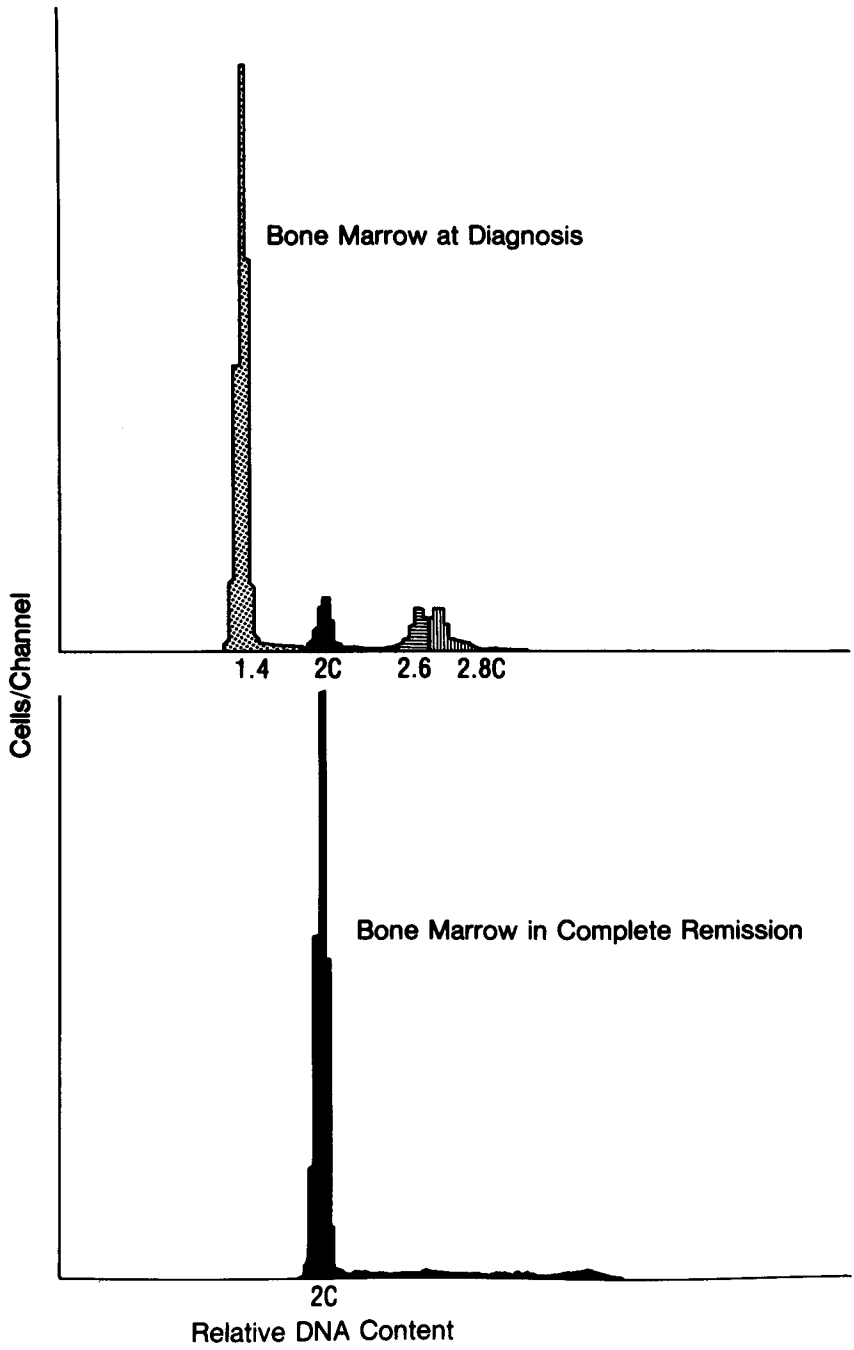

Figure 2. Abnormal DNA stem lines in acute lymphoblastic leukemia disappear when the disease is in complete remission.

cells in the marrow (Table 3). Earlier work had revealed that the degree of DNA abnormality was a unique feature of a patient's tumor, regardless of the site of tumor involvement (5). Hence, knowledge of the aneuploidy

Table 1. Aneuploidy in Human Malignancies

Diagnosis	No. of Patients	Percentage Aneuploid
Leukemia	595	22
Lymphoma	360	53
Myeloma	177	76
Colon cancer	135	62
Breast cancer	385	79
Lung cancer	353	85
Prostate cancer	147	62
Bladder cancer	459	82
Testis cancer	74	93
Melanoma	643	76
Sarcoma	41	98
All solid tumors	3,559	75
All malignancies	4,691	67

Table 2. Aneuploidy Rate in Malignant Lymphoma

Histologic Grade	No. of Patients	Percentage Aneuploid
Low	76	17
Intermediate	90	53
High	19	47

level of the primary tumor should facilitate a more directed search for such tumor cells in the bone marrow.

In order to distinguish between normal diploid cells in DNA synthesis and aneuploid G_1 cells, we can perform a bivariate analysis of DNA content and bromodeoxyuridine (BUdR) incorporation (6), as is diagrammed in Fig 6. Aneuploid tumor G_1 cells thus become clearly separated from normal hematopoietic cells engaged in DNA synthesis.

We have also evaluated cellular RNA and immunoglobulin light chain expression in conjunction with DNA to improve the sensitivity of tumor cell detection in the hematologic malignancies. Figure 7 illustrates the bone marrow from a patient with myeloma. This patient no longer had morphologic evidence of tumor involvement, and myeloma protein had disappeared from the serum and urine. Nevertheless, we were able to

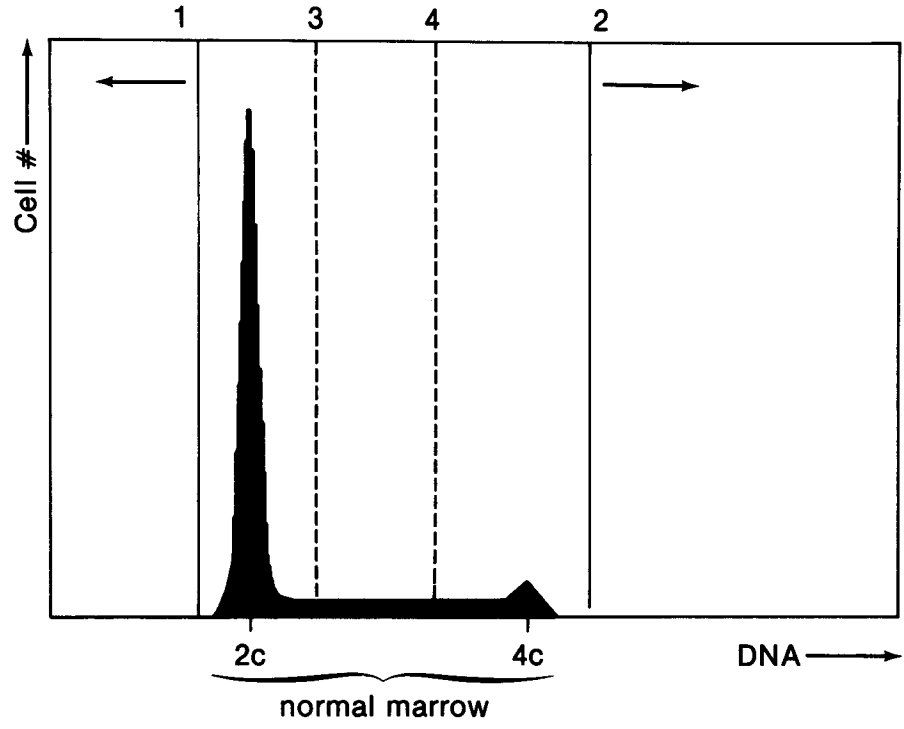

Figure 3. The degree of DNA abnormality (aneuploidy) in the bone marrow affects tumor cell detection by DNA flow cytometry. In the case of hypodiploid and hypertetraploid tumor DNA stem lines (examples 1 and 2), there is interference with normal bone marrow, resulting in a high sensitivity of detection; in examples 3 and 4, the hyperdiploid tumor $G_{1}/_{0}$ cells interfere with normal marrow S-phase cells, so that detection is limited to greater than 3-5% of the tumor cells.

identify, with a high degree of confidence, 3% of tumor cells in the marrow that displayed a distinct DNA and RNA abnormality and monoclonal lambda light chain expression. This information is very important to us, because we have begun to cryopreserve marrow from patients with myeloma who have achieved a marrow remission, in an effort to determine whether high-dose therapy and autologous transplantation may be a feasible way of extending patients' lives. If the marrow is clear of tumor, it can be obtained early and used to support high-dose salvage therapy several years later.

We have only rarely noted obvious DNA abnormalities persisting in patients with leukemia in remission. In Fig 8, an exceptional example, we noted the persistence of one of initially two aneuploid DNA stem lines during a brief period of morphologic remission.

ALTERNATIVE NEOPLASTIC MARKERS

Double-Stranded RNA

Since aneuploidy abnormalities have only limited use in detecting tumor cells in patients with hematologic malignancies, we have searched for potential substitute markers. Double-stranded RNA (ds-RNA) content, readily measured by the intercalating fluorochrome propidium iodide assay following DNA removal, is one interesting possibility (1,7). In acute myelogenous leukemia (AML), for example, the marrow typically expressed a

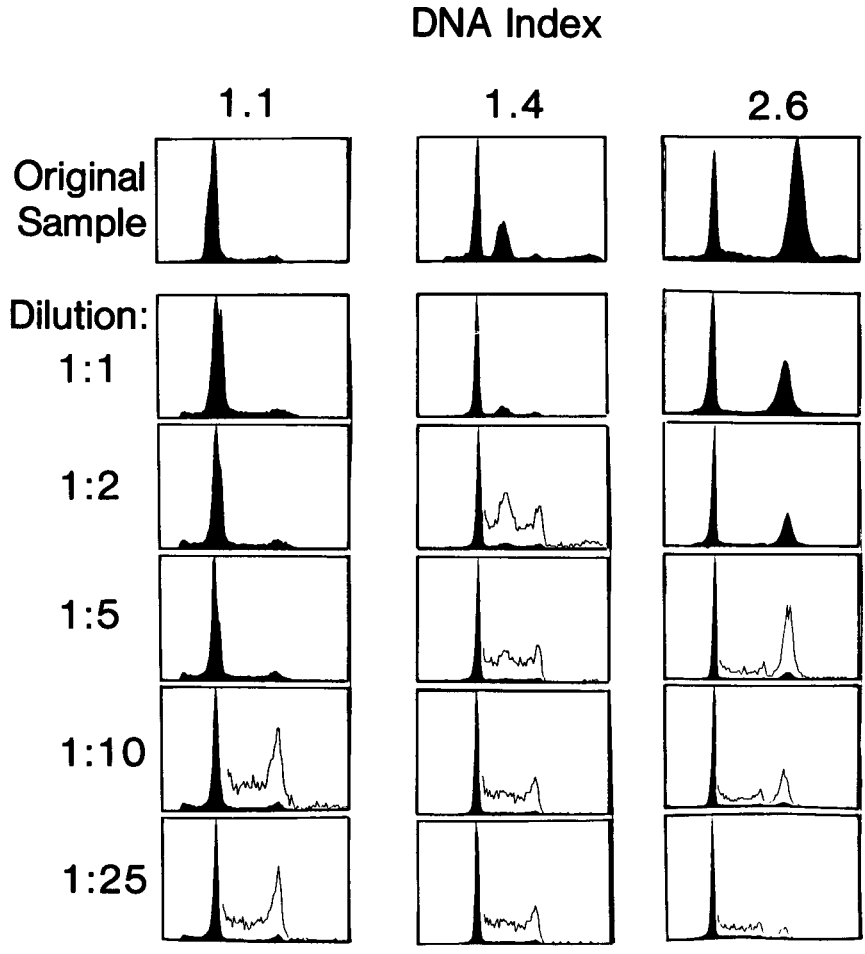

Figure 4. Sensitivity of tumor cell detection by DNA flow cytometry is a function of the degree of aneuploidy.

marked excess of ds-RNA, which can be readily quantitated by subtracting its distribution curve and the curve for normal marrow (7). In comparison to the very discrete expression of a G_1 DNA content, the ds-RNA feature is considerably more dispersed, which becomes a limitation whenever there is population overlap.

Our current experience with ds-RNA excess in patients with hematologic malignancies and solid tumors is summarized in Table 4. In general, we find a high incidence of ds-RNA excess expression in both AML and ALL, but in the malignant lymphomas, this feature appears to be dependent upon histologic grade (3).

Double-stranded RNA excess is expressed by a quarter of patients with leukemia in morphologic remission. These patients have a significantly shorter duration of disease control than the remaining three-quarters, whose ds-RNA excess falls within the normal marrow range (Fig 9) (7,8). However, we have not yet used this test to detect tumor cells in patients considered for autologous bone marrow transplantation.

Nucleolar Antigen

Another alternative neoplastic marker is the nucleolar antigen, initially identified by Busch and his colleagues, using antisera raised

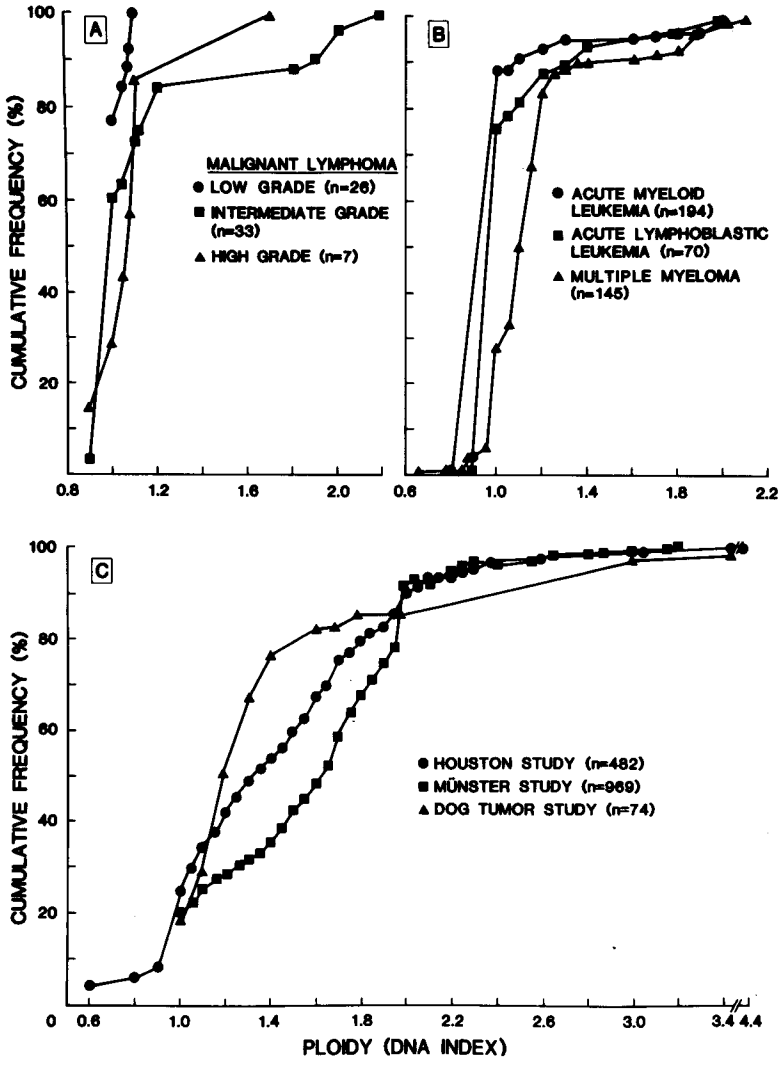

Figure 5. The frequency of DNA abnormalities is plotted for several hematologic malignancies and for solid tumors.

against HeLa-cell nucleoli. Several research groups have now confirmed the unique association between the nucleolar antigen and neoplastic cells, most recently Ford, Cramer, and Davis in a study on patients with malignant lymphoma (9). When this technique is used in conjunction with flow cytometry, however, it lacks the excellent discriminatory potential noted in the morphologic studies; furthermore, we noted an overlap between the nucleolar antigen-related fluorescence of aneuploid tumor cells and of normal hematopoietic cells (1). This may occur because conventional flow cytometry measures total cellular fluorescence rather than nucleolar staining.

Other Cell Markers

This chapter would be incomplete if it did not mention the use of heterologous or monoclonal antibodies to detect tumor cells in the bone

Table 3. Comparison of Two Methods of Tumor Cell Detection in the Bone Marrow of Patients with Oat Cell Lung Cancer: DNA Flow Cytometry and Morphologic Analysis

Morphologic Analysis	DNA Flow Cytometry		
	+	-	Total
+	7	3	10
-	5	64	69
Total	12	67	79

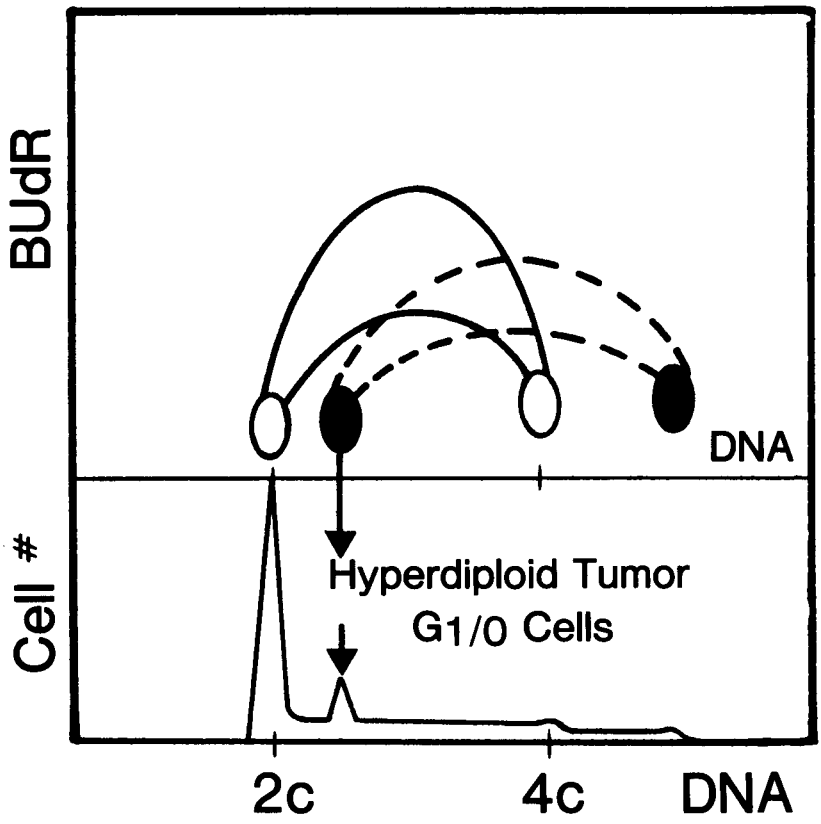

Figure 6. Diagram of bivariate DNA-bromodeoxyuridine analysis that distinguishes between normal cells in DNA synthesis and aneuploid tumor cells in G_1.

marrow. As already pointed out, cell surface antigen expression is subject to change with cell differentiation, and only a few monoclonal antibodies are available that presumably have a high degree of specificity for tumor cells. The lung cancer monoclonal antibodies developed by Minna's group are worth mentioning.

Finally, some cytoplasmic markers have recently been demonstrated by means of immunoperoxidase technology that distinguish epithelial and

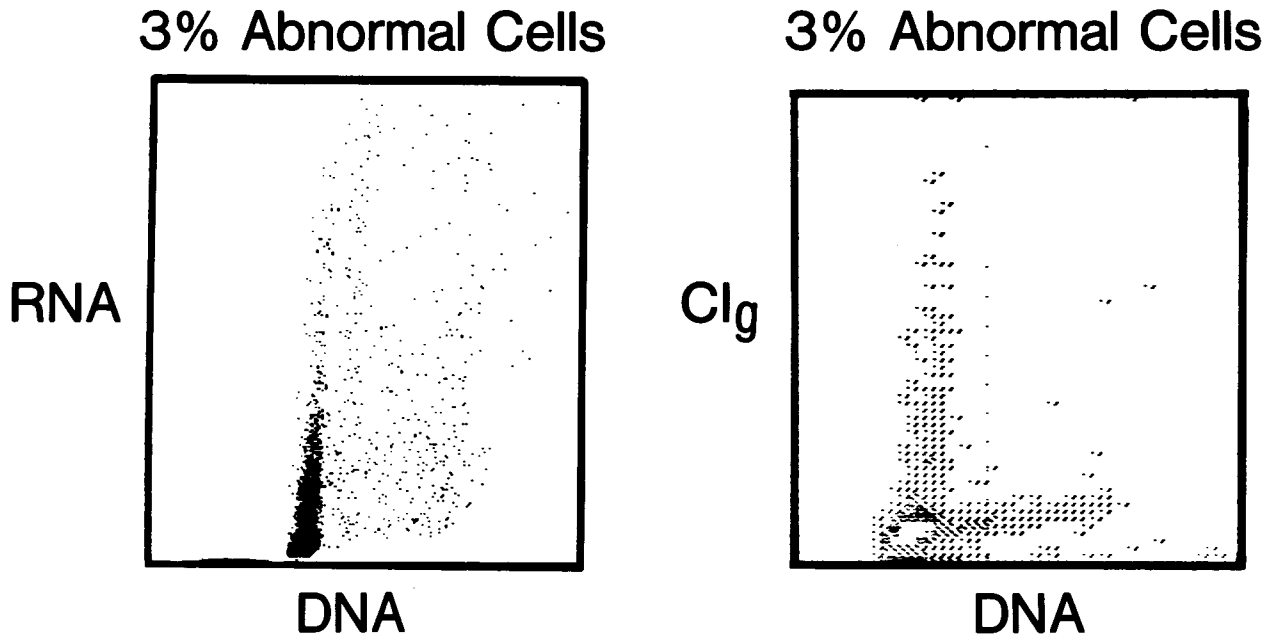

Figure 7. Evaluation of cellular RNA and immunoglobulin light chain expression increases the sensitivity of tumor cell detection in bone marrow cells from a patient with myeloma.

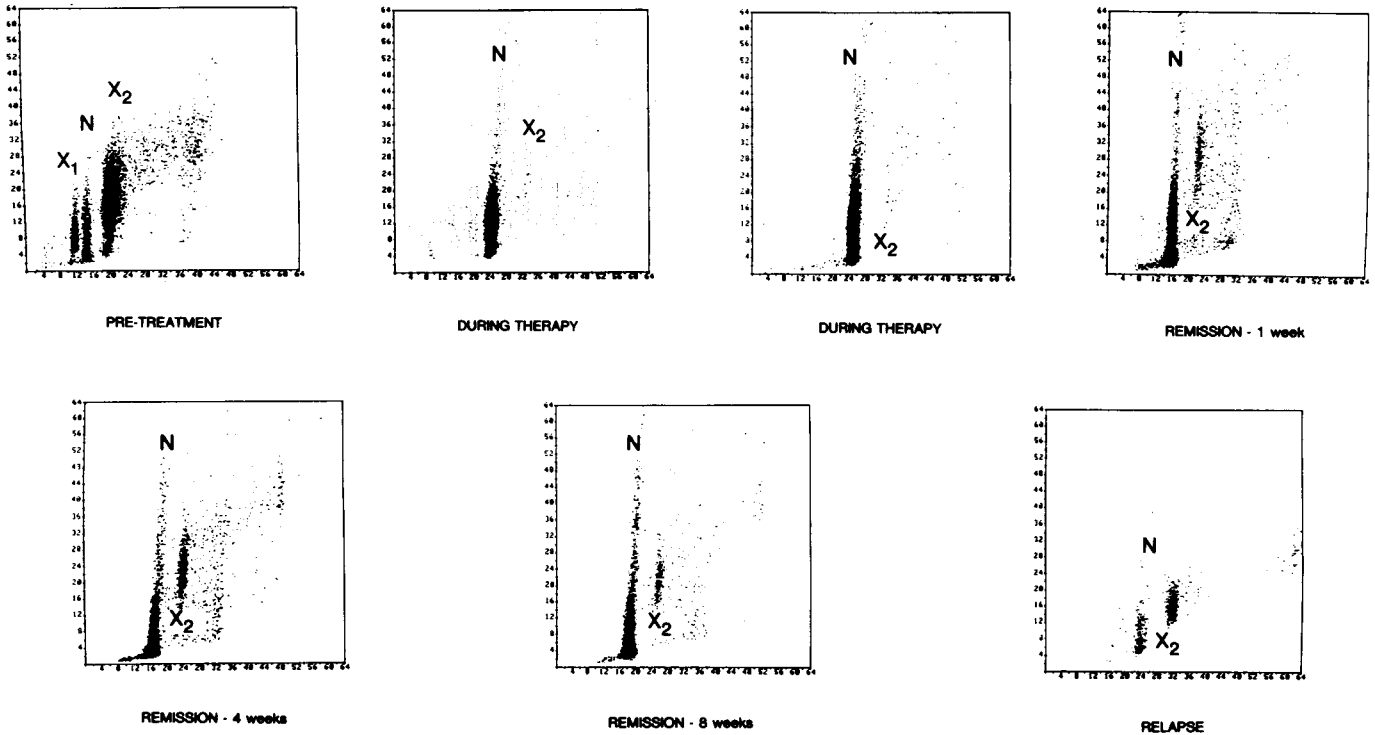

Figure 8. Example of a hyperdiploid DNA stem line that persisted, even though the patient was in remission from acute lymphocytic leukemia.

Table 4. Double-Stranded RNA in Human Malignant Tumors and Normal Tissues

Diagnosis	No. of Patients	ds-RNA Excess Median	ds-RNA Excess Range	Percentage with ds-RNA Excess >30%
Leukemia				
AML				
At diagnosis	32	44	2-92	78**
In remission (≤2 months in CR)	52	16	1-56	23**
In relapse	26	41	5-92	80**
ALL				
At diagnosis	8	32	5-54	62
In relapse	12	43	5-92	75
CML				
Benign phase	10	17	1-52	20**
Blast crisis	6	61	35-92	100**
Lymphoproliferative Conditions				
Lymphoma				
Intermediate-high grade	24	46	10-90	75**
Low grade	20	14	0-85	25**
Hodgkin's disease	9	17	13-41	17
AIDS	15	16	7-46	20
Multiple Myeloma				
>50% plasma cells	15	48	21-83	73**
≤50% plasma cells	27	26	1-66	48**
Solid Tumors				
Breast, seminoma, small-cell cancer of cervix, lymphoepithelioma, thyroid	5	56	29-78	80
Normal Tissues				
Bone marrow	23	15	0-33	9
Lymph nodes	3	17	1-29	0
Other tissues (lung, liver, pancreas, kidney)	8	5	0-24	0

Note: *ds-RNA, double-stranded RNA; AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; AIDS, acquired immune deficiency syndrome.*

**$P<.01$.

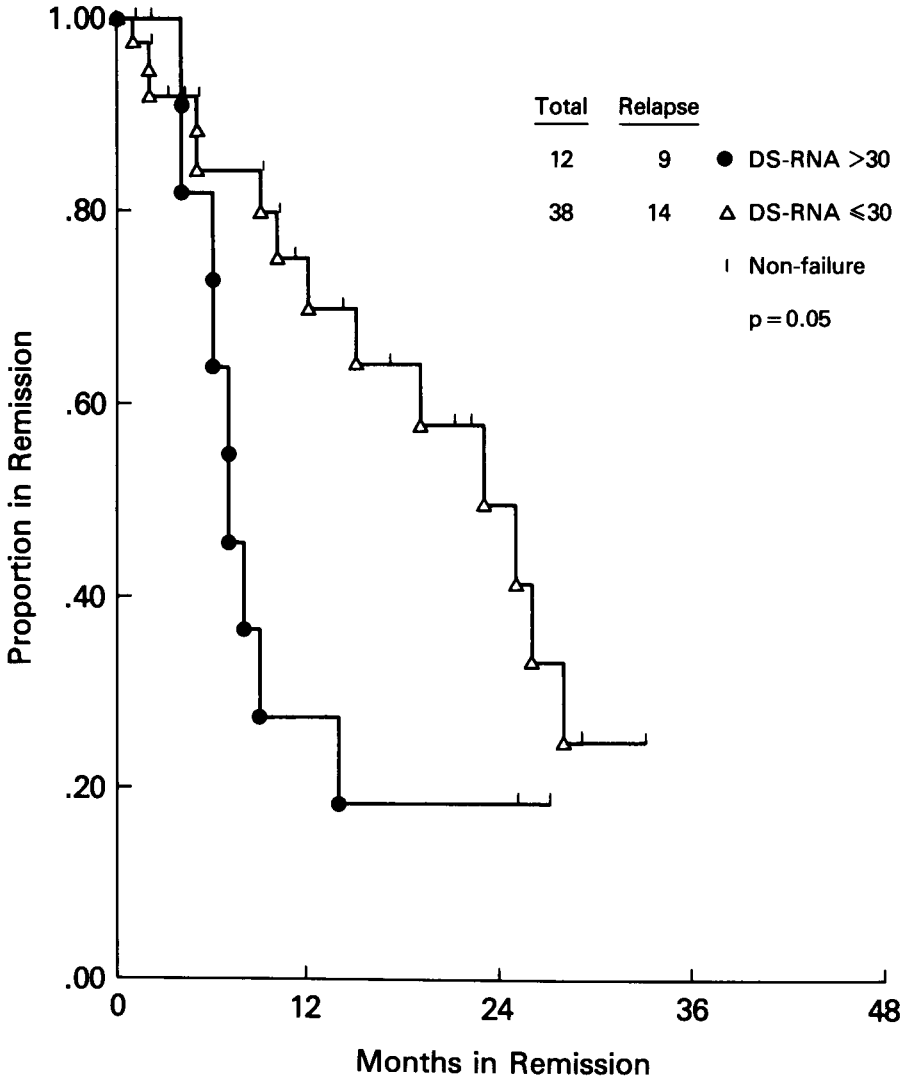

Figure 9. Length of remission in patients with acute myelogenous leukemia is related to the amount of double-stranded RNA excess expressed at the onset of the complete remission.

mesenchymal tumors. Specifically, keratin and vimentin reagents should be useful for detecting rare and possibly immature nonhematopoietic tumor cells in the bone marrow.

CONCLUSION

I have limited discussion in this chapter to some of the cellular probes amenable to flow cytometric investigation as an expedient tool for mass screening of marrow cells. Although the presence of DNA-aneuploid stem lines is an unequivocal stigma of neoplasia expressed in a most discrete and uniform fashion, its value for detecting tumor cells among normal hematopoietic cells is compromised when the aneuploidy level is in the diploid-to-tetraploid range. Detecting tumor cells immunologically,

on the basis of either cell surface or cytoplasmic characteristics, may provide a better discrimination when reagents do not cross-react with hematopoietic tissue, as would be expected in the case of keratin and vimentin markers. Relying on cell-differentiative characteristics may prevent our detecting poorly differentiated or undifferentiated tumor clonogenic cells. However, both histologic and cytopathologic experiences indicate the presence of differentiated progeny in the bone marrow.

ACKNOWLEDGMENT

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Use of Hoechst 33342 for Sensitive Detection of Viable Cells Seeded into Bone Marrow

C. P. Reynolds, A. T. Black, and J. N. Woody

INTRODUCTION

Sensitive detection of small numbers of cells in bone marrow is essential for developing effective marrow-purging methods. This is true whether one is purging tumor cells from autologous marrow (1-4), or T lymphocytes from allogeneic marrow (in an effort to prevent graft-versus-host disease [GVHD]) (5). Detecting rare cells in bone marrow with flow cytometry is difficult and is limited to a sensitivity of 1/100 to 1/1000 (6,7). In certain select systems, culture techniques can detect as low as 1 target cell per 100,000 marrow cells (6,8), and a method for immunostaining using immunoperoxidase capable of detecting 1 neuroblastoma cell per 100,000 marrow cells has been reported (9). However, these techniques are not applicable to a wide variety of cell types.

We describe here a method for marking viable cells with the supravital DNA stain Hoechst 33342 (H342) (3,10) prior to seeding the stained cells into bone marrow. The stain does not significantly affect cell viability, yet allows rapid detection of one target cell per million marrow cells, owing to the bright nuclear fluorescence of the H342. Moreover, we have found that the vital dye trypan blue (11) quenches the H342 fluorescence, limiting detection to only viable cells.

This technique is applicable to model systems with a variety of cell types; target cells (such as a cell line) are prestained and then seeded into the marrow. It should prove useful in optimizing methods for purging marrow because of the high sensitivity and rapid nature of the assay and its ability selectively to detect viable target cells in marrow.

MATERIALS AND METHODS

MOLT-3, a T-cell leukemia line (12), was obtained from the American Type Culture Collection, Rockville, MD; the human neuroblastoma cell line LA-N-5 was a gift of Dr. R. C. Seeger, UCLA School of Medicine, Los Angeles, CA (4); and the SMS-SB line, a pre-B leukemia cell line, was a gift of Dr. R. G. Smith, The University of Texas Southwestern Medical School, Dallas, TX (13). Cells were cultured in RPMI-1640 with 15% fetal calf serum (RPMI-1640/FCS) in 5% CO_2 at 37°C. Human bone marrow was obtained from kidney-donor vertebral bodies by modification of a previously described procedure (14), and bone marrow mononuclear cells were separated by equilibrium sedimentation over Ficoll-Hypaque (15).

Hoechst 33342 (H342) is a DNA-specific stain that can penetrate viable cells and brightly stain DNA. H342 has minimal or no effect on cell viability (3,10,16). Target cells (1 to 1.5 million/ml) were incubated in 1 μg/ml of H342 in RPMI-1640/FCS for 1 h at 37°C; they were then cultured for 2-3 h in RPMI-1640/FCS without stain to allow H342 that is not tightly bound to DNA to diffuse out of the cells. Failure to do this results in the non-DNA-bound dye diffusing from the target cells and staining adjacent marrow cells in the mixture. After the staining and poststain incubation periods, the H342-marked cells were seeded into bone marrow at various concentrations, counterstained with 0.15% trypan blue, and examined on slides under a coverslip using a Leitz fluorescence microscope. Alternatively, for target cell concentrations less than 0.1%, 100,000 to 1,000,000 cells of the mixture per well were placed into 96-well flat-bottom microtiter plates and examined with a Leitz inverted fluorescence microscope. Microscopes were equipped with ultraviolet (UV)-excitation (350 nm) blue-fluorescence-emmission (460 nm) "D" filter blocks.

Complement lysis of the cells was carried out by treating fresh or H342-stained MOLT-3 cells with a mixture of monoclonal antibodies (MAbs)

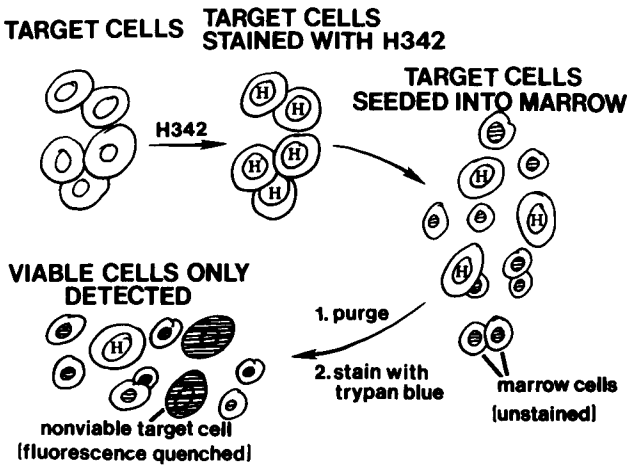

Figure 1. The Hoechst 33342 (H342) assay.

W6/32 (17) and BBM.1 (18) in the presence of various concentrations of fresh rabbit complement (Pelfreeze Biologicals, Rogers, AR) for 1 h at 37°C. Lysis of LA-N-5 was carried out in the same manner, using the MAb 459 (19) provided by R. C. Seeger. Viability of the treated cells was determined by staining with 0.7% trypan blue and enumerating the percentage of viable cells in a hemocytometer (11).

RESULTS

This assay provides a method for highly sensitive detection of viable tumor cells in model systems. The technique involves the premarking of a population of tumor cells (such as a cell line) followed by seeding of the premarked cells into marrow specimens (Fig 1).

Staining cells for 1 h followed by poststain incubation for 2 h results in 100% of the cells demonstrating the bright H342 nuclear fluorescence. It is important to wash out unbound dye before mixing target cells with the marrow, to prevent the marrow cells' staining with H342 that has diffused from stained target cells. As shown in Fig 2, there is no significant difference in the viability of the H342-stained cells and fresh, unstained cells (as assessed by trypan blue exclusion).

We have found that H342 fluorescence is quenched in nonviable cells that are permeable to trypan blue. The ability of trypan blue to quench the H342 fluorescence suggested that this phenomenon could be used to discriminate viable from nonviable cells. To further assess this, we treated H342-stained cells with a complement-fixing MAb and fresh rabbit complement. Such treatment did not affect the H342 fluorescence. Subsequent counterstaining of the cells with trypan blue quenched the H342 fluorescence in the nonviable cells, so that only the cells that remained viable after complement/antibody treatment exhibited nuclear fluorescence.

To test the effect of H342 staining on the susceptibility of cells to complement-mediated lysis, MOLT-3 leukemia cells were stained with a mixture of complement-fixing MAbs and then treated with fresh rabbit complement. As shown in Fig 2, there was no statistically significant difference in the viability of antibody/complement-treated H342-stained cells and fresh, unstained cells ($P > .8$ for all points by Student's t-test).

The sensitivity of the assay for detecting cells seeded into bone marrow was evaluated using a series of seeding experiments. Mixtures of bone marrow containing various concentrations of H342-marked cells from the SMS-SB leukemia cell line were made. The mixtures were then examined

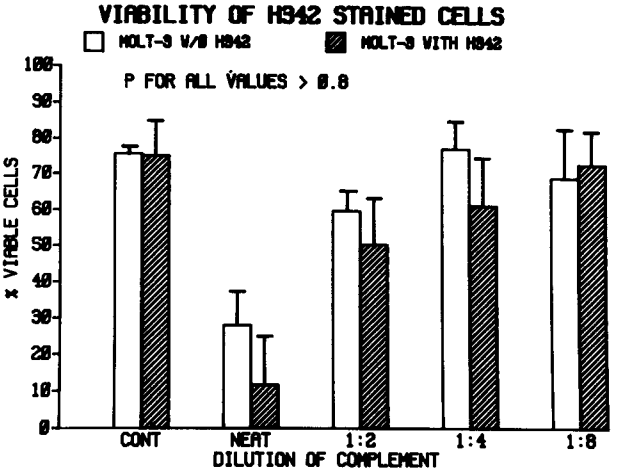

Figure 2. Lack of significant difference between viability of H342-stained MOLT-3 cells and that of fresh, unstained MOLT-3 cells. Both fresh and H342-stained cells were treated with monoclonal antibodies and various dilutions of complement, as described in text (NEAT, 1:2, 1:4, 1:8), or with complement and no antibodies (CONT). Values represent average of three separate experiments, and error bars represent standard deviation. Significance: P >.8 by Student's t-test for all pairs.

by fluorescence microscopy and the number of H342-stained cells counted. As shown in Fig 3, detection of H342-stained cells was accurate for seeded cell concentrations down to 0.0001% (one per million). The same level of detection was achieved for various cell lines from human T and B leukemias, neuroblastoma, rhabdomyosarcoma, and for normal lymphocytes. Variance in the number of cells detected was higher for solid tumors than for hematopoietic cells. This is probably a result of the greater difficulty in making dilutions with solid tumor cells, which have a high degree of cell clumping.

DISCUSSION

The development of purging procedures requires highly sensitive methods for detecting target cells remaining after treatment of the marrow. Infusing viable tumor cells into patients during autologous marrow transplantation could result in disease recurrence (1,4,8,20,21). Similarly, alloreactive cells seeded into the patient during allogeneic marrow transplant may cause a graft-versus-host reaction (GVHR) (5). Failure to effectively purge such alloreactive cells can be detected rather soon after transplantation by the emergence of GVHR. Although clinical relapse may also appear rapidly after autologous transplantation with purged marrow, it is also possible that small amounts of tumor infused with the marrow could produce recurrent disease months or years after marrow transplantation. The possibility of such late relapses mandates caution in the early evaluation of clinical trials using autologous marrow transplantation.

To minimize the chances of such problems, it is essential to develop methods of purging the marrow that are highly efficient. The assay described here using H342 and trypan blue will facilitate the development of effective marrow-purging procedures. It allows testing of the effects of antibody/complement treatment of target cells in the presence of marrow. The assay is ideally suited to measuring the effect of physical depletion methods, such as immunomagnetic depletion (1,4,21,22) or centrifugal elutriation (20,23). Optimization of such methods is possible because of the sensitivity and speed of the assay (4,22).

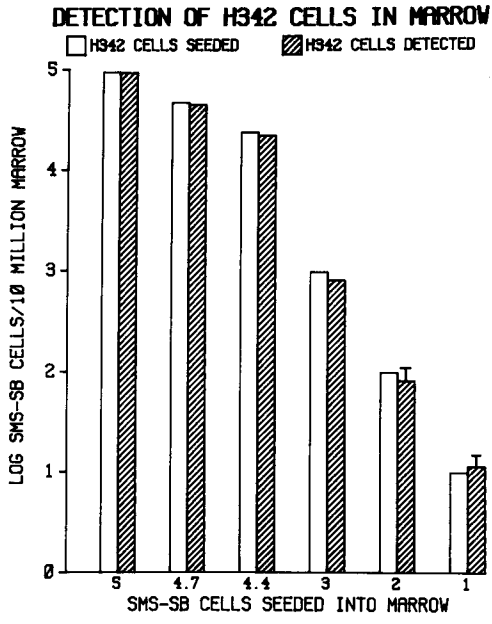

Figure 3. Comparison between number seeded and number detected for various seeded concentrations of H342 SMS-SB leukemia cells. Values for H342 cells detected are the log of the average number of H342-stained cells detected per 10,000,000 marrow cells. Error bars represent standard deviations (SD), not shown for SD <.02.

The H342 assay is a rapid and highly sensitive method for monitoring target cell contamination in model systems, but is of little value in evaluating actual clinical marrow samples. However, use of the assay with well-designed preclinical model systems will increase the probability of success when the purging method is used in clinical trials.

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IX. Marrow Purging Methods

Removal of Malignant Cells from Human Bone Marrow Using Monoclonal Antibodies and Complement

R. C. Bast, Jr., P. DeFabritiis, M. Bregni, V. Raso, C. Reynolds, J. M. Lipton, J. S. Greenberger, L. M. Nadler, S. E. Sallan, and J. Ritz

INTRODUCTION

Selective and complete removal of malignant cells from human bone marrow in vitro should facilitate autologous bone marrow transplantation for leukemias and lymphomas. Monoclonal antibodies might prove useful for selective deletion of malignant cells provided that these reagents bind to tumor-associated antigens and not to the pluripotent stem cell(s) required for bone marrow reconstitution after ablative chemoradiotherapy. Binding of antibody to tumor cells does not, of course, assure destruction of malignant cells. One mechanism by which antibody can destroy tumor cells is through the activation of complement (C') components.

In animal studies, treatment of bone marrow with rabbit C' and polyclonal antisera eliminated 2-3 logs of tumor cells (1-4). In the case of a rat leukemia that grew progressively after intravenous injection of 10-100 cells, a single treatment with antiserum and C' could destroy 1 x 10^4 leukemic cells that had been mixed with 1 x 10^8 syngeneic bone marrow cells (3). Repeated treatment with antibody and C' eliminated 1 x 10^5 leukemic cells, provided that mature granulocytes and erythrocytes had been removed on gradients of Ficoll-diatrizoate.

SELECTIVE REMOVAL OF ^{51}Cr-LABELED CALLA-POSITIVE CELLS FROM HUMAN BONE MARROW WITH ANTIBODY AND C'

Development of monoclonal reagents that react with human leukemia and lymphoma cells has permitted the elimination of tumor cells from human bone marrow using complement-dependent lysis. The J5 monoclonal antibody, for example, is an IgG2 immunoglobulin that will bind to the common acute lymphoblastic leukemia antigen (CALLA) and will lyse CALLA-positive cells in the presence of rabbit C'. The J5 antibody will react with cells from approximately 70% of patients with acute lymphoblastic leukemia (5). In preclinical studies we had found that optimal lysis of ^{51}Cr-labeled CALLA-positive leukemic cells was obtained by treatment with the J5 antibody and absorbed rabbit C' for a total of 90 min (6). Three treatments for 30 min were more effective than two treatments for 45 min or a single treatment for 90 min. Separation of marrow mononuclear cells on Ficoll-diatrizoate was not essential but did reduce the number of nucleated cells requiring treatment. This was of value in that high concentrations of bone marrow (>2 x 10^7 cells/ml) inhibited elimination of leukemia. Importantly, repeated treatment with J5 and C', which eliminated greater than 99% of leukemic cells, produced a 50% loss of nucleated cells but no selective loss of colony-forming units in culture (CFU-C), erythroid burst-forming units (BFU-E), or bipotent granuloerythropoietic colonies.

SELECTIVE REMOVAL OF CLONOGENIC BURKITT'S LYMPHOMA CELLS FROM HUMAN BONE MARROW WITH ANTIBODY AND C'

Use of ^{51}Cr release to determine tumor cell lysis can detect at best 2 logs (99%) of tumor cell elimination. Moreover, ^{51}Cr-release assays cannot distinguish between destruction of clonogenic and nonclonogenic tumor cells. Consequently, a more sensitive assay has been developed that depends upon the high clonogenic efficiency of Burkitt's lymphoma cells (8). These tumor cells bear a number of well-characterized cell surface markers including CALLA, gp26, B1, B2, IgM, and B2 microglobulin, as well as class I and class II major histocompatibility antigens. Monoclonal

antibodies that react with each of these determinants and fix C' are available. Much of our work has been done with the Namalwa cell line, which bears CALLA, gp26, and B1. Namalwa cells have been mixed with a 20-fold excess of irradiated human bone marrow before treatment with one or more monoclonal antibodies and C'. Clonogenic tumor cells remaining in the mixture are enumerated by limiting dilution on a feeder layer of irradiated human bone marrow cells.

For the J5 anti-CALLA, J2 anti-gp26, and the anti-B1 antibodies, a 1:100-1:200 dilution of ascites provided optimal elimination of clonogenic units (8). Using optimal antibody concentrations, three treatments with the J5 antibody and C' eliminated almost 2 logs of clonogenic cells. Three treatments with J2 anti-gp26 eliminated 3 logs of Burkitt's cells, but multiple treatments with anti-B1 removed less than a log of malignant clonogenic cells. Treatment with a combination of J2+J5 or J2+B1 was more effective than treatment with any single reagent (8). Treatment with all three antibodies was not more effective than the optimal combination of two reagents. Using individual monoclonals, the concentration of normal bone marrow cells limited the efficacy of the antibody and C'. Inhibition of C'-dependent lysis was observed with marrow concentrations greater than 5×10^6/ml. When two or three antibodies were used in combination in the presence of an optimal concentration of bone marrow, three treatments for 30 min still proved superior to a single treatment for 90 min. Provided that multiple reagents and multiple treatments were used, elimination of clonogenic Burkitt's cells proceeded as efficiently in the presence of 2×10^7 bone marrow cells/ml as it did in the presence of an optimal marrow concentration of 5×10^6 cells/ml. Addition of 5% human serum to the incubation mixture strongly inhibited tumor cell lysis. This might relate to the presence of an inhibitor of C'_1 in human donors with angioedema as in 5% fetal calf serum.

CHARACTERIZATION OF BURKITT'S CLONES THAT RESISTED TREATMENT WITH ANTIBODY AND C'

To gain some insight into the mechanism by which malignant cells resist treatment, clones have been isolated that grew at limiting dilution following treatment of Namalwa cells with antibody and C'. Substantial heterogeneity was observed in the expression of CALLA, gp26, and B1 by different clones. After treatment of Namalwa cells with the J5 anti-CALLA antibody and C', clones exhibited less CALLA on the average, judged by the binding of J5 measured by indirect immunofluorescence and flow cytometry (P DeFabritiis, M Bregni, J Lipton et al., unpublished observations). The phenotype of clones remained stable for at least 2 weeks in cell culture. Clones could be isolated that differed in sensitivity to lysis with J5 and C', but there was no correlation between antigen expression and susceptibility to C'-dependent lysis, judged either by ^{51}Cr release from labeled clones or by the clonogenic assay. Clones isolated after treatment with J2 anti-gp26 or anti-B1 and C' failed to exhibit consistent abnormalities in phenotype or susceptibility to C'-dependent lysis. Interestingly, however, the expression of CALLA and of gp26 was strongly correlated after treatment with C' and J2 or anti-B1, but not after treatment with C' and J5 or a control antibody (JO). The mechanism underlying this correlation is obscure, but the empirical observation suggests that after treatment with J2 or anti-B1, those cells that are relatively deficient in CALLA will also be relatively deficient in gp26.

SELECTIVE REMOVAL OF CLONOGENIC BURKITT'S LYMPHOMA CELLS WITH 4-HYDROPEROXYCYCLOPHOSPHAMIDE

Several other techniques have been proposed for the removal of malignant cells from bone marrow, including treatment with cytotoxic drugs (9) and immunotoxin conjugates (10,11). Consequently, we have undertaken studies with the cyclophosphamide metabolite 4-hydroperoxycyclophosphamide (4HC) (in the form of Asta Z 7557) and with ricin A-chain monoclonal antibody conjugates to compare the efficacy of these

other approaches to that of treatment with monoclonal antibodies and C'. Incubation with 60-100 μg/ml of 4HC for 30 min proved optimal (12). Using each of three different Burkitt's cell lines, from 2-3 logs of malignant cells, mononuclear cells did not inhibit the elimination of clonogenic Burkitt's cells using 4HC. When fresh marrow was treated with 4HC under these same conditions, there was a quantitative loss of granulocyte-macrophage (GM) CFU-C. Within 1 week during continuous bone marrow culture, however, production of CFU-C-GM returned, and the cumulative production of cells in long-term culture was inhibited less than 50%.

SELECTIVE REMOVAL OF BURKITT'S LYMPHOMA CELLS WITH RICIN A-CHAIN MONOCLONAL ANTIBODY CONJUGATES

Different conjugates have been prepared with monoclonal antibodies and ricin A chain (13). In contrast to results with complement-dependent lysis, ricin A-chain conjugates reactive with CALLA, gp26, and B1 were relatively ineffective in eliminating clonogenic Burkitt's lymphoma cells (M Bregni, P DeFabritiis, V Raso et al., unpublished observations). Variable results were obtained against five different Burkitt's lymphoma cell lines with conjugates directed against components of cell surface immunoglobulin. The most consistent results were obtained using conjugates directed against the nonpolymorphic region of the alpha chain of Ia. A 12-h incubation in the presence of 10-mM NH_4Cl and 1 x 10^{-7} M ricin A chain conjugated with anti-Ia or anti-μ chain removed 4 logs of malignant cells. Addition of an excess of irradiated bone marrow inhibited elimination of clonogenic Burkitt's cells, related perhaps to a limited amount of antibody and the presence of Ia-positive precursors in normal marrow. Incubation of fresh human bone marrow with ricin A-chain-anti-Ia conjugates eliminated CFU-C-GM. As in the case of 4HC treatment, however, production of CFU-C-GM was observed within 1 week during continuous bone marrow culture.

CONCLUSION

In summary, there appear to be several methods currently available that permit the selective removal of malignant cells from human bone marrow. Comparison of three different methods suggests that multiple treatments with multiple monoclonal antibodies and C', a single treatment for 30 min with 4HC, or a single 12-h incubation with ricin A-chain monoclonal antibody conjugate can each be effective in removing clonogenic Burkitt's lymphoma cells. The high clonogenic efficiency of the Burkitt's lymphoma cells required for this assay may have biased results in favor of 4HC, and it remains to be seen whether this agent would be as effective against tumor cells exhibiting more indolent growth. Treatment with antibody and C' failed to affect growth of CFU-C, BFU-E, or bipotent granulo-erythropoietic precursors, consistent with the successful engraftment of bone marrow in patients who have received marrow treated with J5, J2, and C' following ablative chemoradiotherapy (SE Sallan, JM Lipton, J Ritz et al., unpublished observations). Treatment either with 4HC or with ricin A-chain monoclonal antibody conjugates eliminated CFU-C-GM, but these precursors could be found within 7 days in continuous culture. Reappearance of marrow progenitors is consistent with the experience of the transplant group at The Johns Hopkins University, who have observed engraftment after treatment of marrow with similar concentrations of 4HC (14). Whether or not the temporary elimination of normal precursors by immunotoxins reactive with Ia would limit the utility of this particular reagent remains to be explored in future trials.

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Autologous Bone Marrow Transplantation for Pediatric Non-Hodgkin's Lymphoma: In Vitro Purging of the Graft with Anti-Y 29/55 Monoclonal Antibody and Complement

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INTRODUCTION

High-dose cytoreductive regimens followed by autologous bone marrow transplantation (ABMT) have been successfully used to induce long-term remissions in patients with advanced or relapsed non-Hodgkin's lymphoma (NHL) of high malignancy, particularly of Burkitt's type (1-3). However, there is a substantial risk that the reinfused bone marrow may contain undetected clonogenic tumor cells that may cause treatment failure. Accordingly, we have developed an in vitro system for purging lymphoma cells from bone marrow prior to ABMT. It involves the B cell neoplasia-associated monoclonal antibody anti-Y 29/55 and complement. We present our results with seven pediatric patients and compare their status to that of an earlier patient group treated with unpurged marrow.

MATERIALS, METHODS, AND PATIENTS

Monoclonal Antibody and Bone Marrow Processing

This complement-binding monoclonal antibody (IgG 2A, kappa) of murine origin has been described previously (4). It recognizes a large spectrum of malignant lymphoid cells derived from human plasma cell precursors. It also binds to nonmalignant B lymphocytes normally confined to secondary lymphoid organs. It does not react with cells of acute lymphocytic leukemia of null, common, pre-B, and T types nor with normal hematopoietic elements, including granulocyte-macrophage precursors (5,6). We have tested tumor cells of 11 different pediatric patients who had Burkitt's or other B-cell NHL for their anti-Y 29/55 reactivity. All of them were strongly positive at a high percentage, usually above 95% (unpublished data).

Bone marrow of patients was harvested after they went into remission, usually after one or two courses of VAC (vincristine [2 mg/m^2], Adriamycin [60 mg/m^2], and cyclophosphamide [45 mg/kg]). At this time the percentage of malignant cells in the bone marrow was minimal (below 1%). The mononuclear cell fraction was obtained by density gradient centrifugation (sodium metrizoate/Ficoll, specific gravity 1.077). Cells were washed and resuspended in plasma protein solution (1-3 x 10^7/ml). The antibody (30-10 µl ascites per milliliter cell suspension) and complement (adult rabbit serum, final concentration 20%) were added. The mixture was incubated for 90 min at 20°C. Finally, the cells were washed twice and cryopreserved as usual. Indirect immunofluorescence detected no residual tumor cells after this procedure. Growth of colony-forming units in culture (CFU-C) was not significantly altered after the purging process. Experimental data (unpublished) indicate that this procedure is able to reduce the tumor-cell load by approximately 2 logs.

Cytoreductive Therapy and Patients

We induced remission in our patients by administering VAC courses every 3-4 weeks, prednisone 60 mg/m^2 daily, vincristine 2 mg/m^2 weekly, and intrathecal triple therapy every 2 weeks. Cranial irradiation (20-24

Table 1. Characteristics of Patients

No.	Age (yr)	Sex	Murphy Stage	Involvement of BM	Involvement of CNS	No. VAC Courses	Radiotherapy	Remission Status at ABMT	Interval Diagnosis to ABMT (wk)	Survival after ABMT (mo)
1	7.0	M	IV	Yes	Yes	4	CNS+sacrum	1. CR	14	34+ NED
2	10.5	M	IV	Yes	No	3	None	1. CR	12	29+ NED
3	14.0	M	IV	Yes	Yes	2	CNS	1. CR	10	27+ NED
4	5.5	M	III	No	No	2	None	1. CR	10	18+ NED
5	9.5	M	III	No	No[a]	2	Sacrum	1. CR	10	8+ NED
6	15.0	M	IV	Yes	Yes	2	CNS	1. CR	12	2+ R
7	9.5	M	IV	Yes	No	1	Tumor bulk	2. PR	30	1.5 R

Note: *BM, bone marrow; CNS, central nervous system; VAC, vincristine (2 mg/m²), Adriamycin (60 mg/m²), cyclophosphamide (45 mg/kg); ABMT, autologous bone marrow transplantation; CR, complete remission; NED, no evidence of disease; R, relapse; PR, partial remission.*

[a]*Presence of a lumbosacral epidural mass, nerve root compression, and negative CSF cytologic study.*

Gy) was added if signs of central nervous system (CNS) involvement were present. Local lumbosacral irradiation was given for epidural lesions to two patients (nos. 1 and 5 in Table 1). High-dose consolidation therapy with autologous bone marrow rescue, as shown in Table 2, was initiated as soon as remission was achieved (2-3 months after diagnosis). Systemic chemotherapy was discontinued thereafter except for intrathecal medication.

Seven patients have been treated with this protocol. Their characteristics are shown in Table 1. They all had histologically and usually also immunologically proven NHL of Burkitt's or other B-cell type. The primary location was in the abdomen in five patients, in the cervical region in one patient (no. 5), and generalized in the other patient (no. 1). Reactivity of anti-Y 29/55 was demonstrated in five patients. In two patients (nos. 2 and 4) no appropriate material was available for immunologic studies. Based on our previous experience with this type of tumor, however, we can assume positive anti-Y 29/55 reactivity for these patients also.

RESULTS

Hematopoietic reconstitution was observed in all patients, providing evidence of marrow engraftment. No delay was recorded in patients with purged marrow as compared to 16 patients with unmanipulated marrow (Table 3).

Five out of six patients transplanted in first remission are presently free of disease and in good general condition. In one patient of the same group the tumor has recurred 6 weeks after ABMT in the bone marrow, which was massively involved initially. The only patient transplanted in second (partial) remission has relapsed in the abdomen 6 weeks after ABMT.

Acute complications included severe but reversible liver dysfunction with massive ascites (veno-occlusive disease?) in two patients (nos. 2 and 5), severe polyneuropathy and prolonged food intolerance in patient 3, and *Candida* sepsis in patient 7. Long-term problems included a mild psychoorganic syndrome in patient 1 and bilateral cataracts in patient 3.

DISCUSSION

This study demonstrates that ABMT plus in vitro purging of the graft, using the monoclonal antibody anti-Y 29/55 and complement, is feasible for pediatric patients with NHL of the B-cell type.

Table 2. Pretransplant Regimen

Therapy	Protocol Day							
	-7	-6	-5	-4	-3	-2	-1	0
Vincristine (mg/m^2)	2							
Adriamycin (mg/m^2)	60							
Cyclophosphamide (mg/m^2)		45	45	45	45			
Total body irradiation (Gy)							6	
ABMT[a]								Δ

[a] *ABMT, autologous bone marrow transplantation.*

Table 3. Hematologic Reconstitution after ABMT

Component	Blood Level (x 10^9/l)	Duration after ABMT[a] (days) Anti-Y 29/55 Purged Marrow (*N*=7)	Duration after ABMT[a] (days) Unpurged Marrow (*N*=16)
WBC[b]	1.0	13 (9-27)	15 (9-50)
Neutrophils	0.5	14 (10-36)	19 (12-49)
Platelets	50	27 (8-117)	30 (15-48)[c]
Reticulocytes	1%	17 (11-33)	20 (15-49)

[a] *Median and range.*

[b] *WBC, white blood cells.*

[c] *No recovery in one patient.*

We have used a similar protocol, but without bone marrow purging, in an earlier group of patients who had various tumors that included 10 with NHL of the B-cell type (2). A comparison of the two groups with respect to hematologic (Table 3) and immunologic (data not shown) recovery does not reveal any significant influence from the purging procedure. However, the clinical significance of the purging procedure for tumor control cannot be finally evaluated in this study, because a randomized control group is lacking.

We can compare six of our patients with purged marrow to a historical group of eight patients with unpurged marrow (2). When we consider those six patients who underwent transplantation in first remission, we observe the following facts:

1. In the "unpurged" group, three of eight patients have relapsed. One was lost because of complications. Four of eight patients (50%) have been in continuous remission for 31-62 months (median, 48 months).
2. In the "purged" group one of six patients has relapsed. The remaining five patients (83%) are alive and have been in continuous remission for 8-34 months (median, 27 months).
3. The "purged" group contained more "unfavorable" cases, i.e., patients with CNS disease and/or epidural lesions, than the "unpurged" group (four and one, respectively).

Concerning tumor control, these observations are in favor of the purging procedure as described here.

Complications appeared to be more frequent in patients receiving purged marrow. This tendency, however, did not influence the overall survival. None of the observed problems could be attributed to the in vitro manipulation of the marrow.

The role of ABMT in the treatment of children with NHL of B-cell type has still to be defined. Indications for ABMT have become more restrictive as treatment results with conventional regimens have markedly improved in the last few years (7,8). Patients with CNS disease, however, still represent a poor-risk group. We believe that early ABMT with purged marrow might improve their prognosis.

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Realistic Study Design to Prove the Biologic Effect of Purging

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INTRODUCTION

One of the most burning questions in the treatment of acute leukemia with high-dose cytoreductive therapy is whether autologous bone marrow can be used to restore hematopoiesis in the recipient (1). We all know the hazards of autologous marrow: the high chance that leukemic cells are present in the marrow cell suspension used for transplantation. If present, these leukemic cells may induce leukemia after transplantation.

At remission, the total number of leukemic cells in the marrow may vary from $100\text{-}10^{10}$; we consider 10^8 cells realistic, although of course the actual number depends on the quality of remission. Assuming there is a homogeneous distribution of leukemic cells in the marrow, the number of leukemic cells in the marrow cell suspension to be transplanted is 10^6, since 1% of the entire bone marrow reserve is aspirated during marrow harvest (2). Concisely stated: At remission, approximately 10^8 leukemic cells are present in the marrow. The number of marrow cells aspirated is $1\text{-}2 \times 10^{10}$, which is 1% of the total marrow pool. Therefore, the number of leukemic cells in the graft is approximately 10^6. As yet, the fraction of clonogenic leukemic cells cannot be identified, so every leukemic cell must be removed.

In this volume various methods of eliminating leukemic cells, or purging, are described. In this chapter, therefore, we propose a realistic study design with which to determine the biologic role of marrow purging. In order to design a realistic study, several questions need to be addressed: the time of transplantation, the natural history of the various acute leukemias, the type of cytoreductive regimens used as conditioning for transplantation, data analysis, the number of patients needed, and the time of the marrow harvest.

TIME OF TRANSPLANTATION

We consider the optimal time of transplantation to be when the disease has reached a phase in which conventional doses of chemotherapy no longer induce a long-term remission, and yet the tumor still responds to high-dose cytoreductive therapy in conjunction with infusion of leukemia-cell-free marrow (e.g., from allogeneic identical-twin donors). When such treatment induces long-term disease-free survival in a substantial proportion of patients (30-50%), it indicates that high-dose cytoreduction is still effectively eradicating residual leukemic cells. We believe that patients in a second remission from acute myelogenous leukemia (AML) and second and subsequent remissions from acute lymphocytic leukemia (ALL) fulfill these criteria.

NATURAL HISTORY OF THE LEUKEMIAS

The most important facts about adult AML can be summarized as follows (3-5):

1. About 30% of AML patients are long-term disease-free survivors.
2. A continuous complete remission (CCR) can be predicted on the basis of prognostic factors.
3. Patients who relapse on chemotherapy have a 29% chance of remission reinduction.
4. Patients who relapse off chemotherapy have a 60% chance of remission reinduction.

5. Patients who fail first salvage therapy have a 9% chance of remission induction with a second salvage program.
6. The median duration of a second complete remission (CR2) is 4.5 months.

Approximately 70% of the patients achieve complete remission (CR) (3) and, according to the predictive model developed by Keating et al (4), it is possible to predict an individual patient's chance of staying in remission for 1 year.

The natural history of childhood ALL presents different parameters for marrow transplantation. About 90% of children achieve a first complete remission (CR1), and about 60% maintain the remission; the exact proportion depends on the immunologic cell-surface markers on the leukemia cells and the age of the patient (4). A CR2 can be induced in 85%. However, its medium duration is only 6 months. The duration is shorter when the patient has relapsed during the first year of CR1. The chances of cure are high after an extramedullary relapse without a marrow relapse (5,6).

We think that performing a transplantation during relapse does not offer the best opportunity to assess the role of purging, since the remission duration after transplantation is short. Of 95 relapsed patients with acute leukemia who had undergone autologous bone marrow transplantation, 60 achieved CRs. However, the CR lasted for a median of only 6 months (range, 1+-38 months), and only four patients were in CCR for 2 years. Recurrence of leukemia in patients transplanted during relapse is probably due to residual disease after high-dose cytoreductive therapy. The data reported above has been compiled from several teams; note that only four long-term disease-free survivors have been reported, or less than 5% of the entire population transplanted in relapse.

Transplantation performed during CR1 may also present difficulties to a study of the effect of purging, since the prognosis of a patient after bone marrow transplantation depends on the length of the CR prior to transplantation. A group of patients 6 months into CR1 can expect 25% to have long-term disease-free survivals, whereas more than 75% of the patients who remain in CR1 for more than 2 years achieve a long-term disease-free survival with conventional chemotherapy (Fig 1). Careful analysis of the timing of transplantation is necessary, therefore, to assess the contribution of transplantation to long-term survival. Table 1 lists the results of transplantation in CR1 obtained by three different groups. As expected from the arguments above, the effect of transplantation cannot be assessed in this patient population. Of 13 patients, five have survived longer than 2 years, but the time of transplantation in remission has varied from 6 months to more than 15 months after the start of CR1.

REGIMEN

Another important factor to consider is which high-dose cytoreductive regimen to use. We believe that the regimen CBV (6 mg/m^2 cyclophosphamide, 300 mg/m^2 BCNU, and 750 mg/m^2 VP-16-213) is an effective antileukemic regimen: two out of five patients transplanted during CR2 with allogeneic marrow are long-term survivors. Table 2 indicates the CRs achieved when CBV was used as a conditioning regimen and patients were transplanted in relapse. Note in Table 3 that seven of 11 patients (63%) treated with CBV as a second salvage therapy achieved CR, which is much better than the 9% CR rate achieved with conventional regimens.

STUDY PROPOSAL

Based on the reasons mentioned above, we propose a two-arm study to be conducted during second and third remissions. One arm will comprise a patient group treated with "crude" marrow; the population in the second arm will be treated with "purged" marrow. Following are the end points for evaluation:

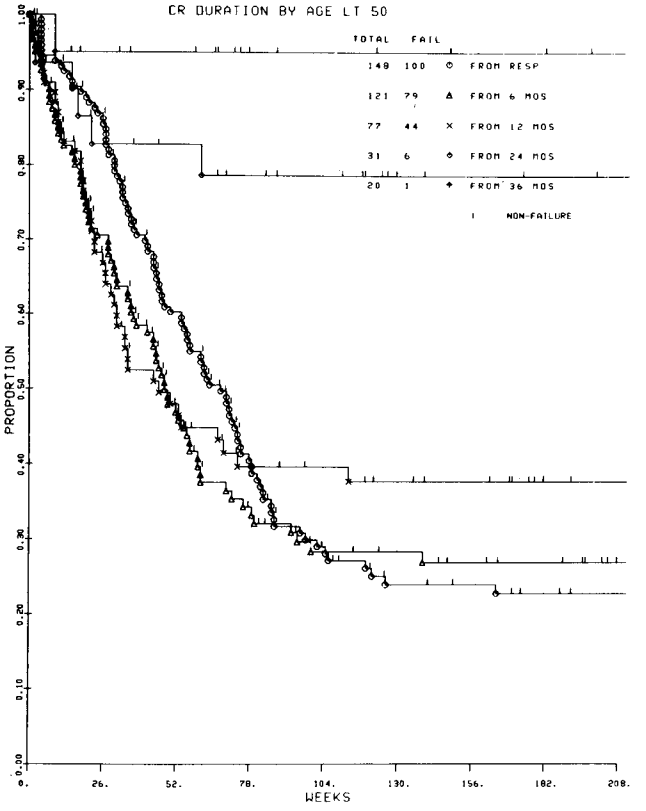

Figure 1. Influence of duration of complete remission (CR) on chance of continuous long-term disease-free survival. The horizontal axis indicates the time in weeks, and the vertical axis the percentage of patients surviving disease free. Twenty-six percent of the patients who are in CR for 6 months (triangles) *and 38% of those who are 1 year in CR (X) are long-term survivors; the percentage increases to 78% when the patients remain in CR for 24 months* (diamonds). *When patients are in CR for 36 months or longer* (top line, arrow), *the figure increases to 95%.*

Table 1. Length of Long-Term[a] Disease-Free Survival after ABMT without Marrow Purging during Remission of Acute Myelogenous Leukemia (Preliminary Results)

Study	No. of Patients Undergoing Transplantation	CR	1-Year Remission
Besancon	3	1, 2	2
St. Louis	7	1	1
Rotterdam	3	1	2
Total	13		5

[a] *1 year.*

Note: *ABMT, autologous bone marrow transplantation; CR, complete remission.*

Table 2. Results of CBV[a] Salvage Therapy for Relapsed Patients with Adult Acute Leukemia

Disease	No. of Patients	Complete Remissions[b]
Acute myelogenous leukemia	12	6
Acute lymphoblastic leukemia	9	1
Total	21	12 (57%)

[a]*CBV, cyclophosphamide, BCNU, VP-16-213.*

[b]*Median duration of complete remission was 3.5 months.*

Table 3. Relationship between Time of Bone Marrow Salvage and Length of Complete Remission in Patients with Adult Acute Leukemia

Salvage Attempt	No. of Patients	CR[a] Duration (mo)
First	1	1
Second	11	7
Third	8	4
Fourth	1	0

[a]*CR, complete remission.*

1. Is the median duration of the CR in which transplantation occurs longer than the median duration of CR obtained with conventional doses of chemotherapy?
2. Is the median CR duration in the "purged arm" longer than in the "nonpurged arm"?
3. Can remissions be achieved that are longer than the preceding remission? If so, is the proportion of patients who sustain longer transplantation-induced remissions greater in the "purged arm" than in the "nonpurged arm"?

We will assess not only the duration of the CR after transplantation, but also the total length of the remission in which transplantation occurs. We will then compare the duration of this remission with the length of the preceding remission. We consider the effect of transplantation to be positive when the "transplantation" remission is longer than its preceding remission, and we will study the patients in each group whose transplantation remission is longer than the preceding remission.

Part of the preliminary results--the data for the patient group treated with CBV and nonpurged marrow--are listed in Table 4. Transplantation occurred in CR2 or CR3. In only one patient was the transplantation remission longer than the remission preceding transplant. On rare occasions this phenomenon can also be achieved with conventional-dose chemotherapy, as in patient 4. In this patient CR1 lasted 24 months but CR2 lasted 93 months.

In the 11 patients transplanted, the median duration of the transplantation remission was 7.5 months, whereas the median duration of the preceding remission was 14 months. The number of patients needed to

Table 4. Preliminary Results of CBV and Nonpurged Marrow in Adult AML in Second or Subsequent Remission

Patient No.	Length of CR Preceding Transplant (mo)	Length of CR2 or CR3 in Which Transplant Occurs (mo)		
		Pre-TR	Post-TR	Total
AML				
1	9 6	31+	37+	
2	10 1	4	5	
3	8, 5	3.5	1	4.5
4	24, 93	3	7	10
5	14	(1 week)	(Early death)	
6	29 2	4	6	
7	18 3	5	8	
8	18 2	6	8	
ALL				
9	11, 11	2	5	7
10	17 1.5	6	7.5	
11	19, 14	1.5	8	9.5
Median duration (mo)	14	7.5		

Note: *CBV, cyclophosphamide, BCNU, VP-16-213; AML, acute myelogenous leukemia; CR, complete remission; TR, transplant; ALL, acute lymphocytic leukemia.*

prove a difference between these two remissions is dependent on the results obtained. For example, of the 11 patients, the transplantation remission exceeded the CR1 in one patient (9.9%). Assuming that marrow purging induces longer transplantation remissions in 20% of the patients, 60 patients would be needed; or in 30% of the patients, 25 patients would be needed; or in 40% of the patients, 13 patients would be needed to conclude that the rate exceeds 9.9% (significance level <.05, power >.8).

MARROW HARVEST

We do not know if the time of marrow harvest is a major point to consider. We prefer to perform a transplantation with CR2 marrow, although we consider CR1 a possible time. Our reasons are:

1. We know that a patient in CR2 is eligible for the program.
2. The quality of the marrow is still sufficient to induce hematopoietic restoration after high-dose cytoreduction.
3. High-dose cytoreductive treatment with CR2 marrow can lead to unmaintained remission.
4. Monoclonal antibody (MAb) screening can be done during the first relapse. If MAbs are available that react with 95% of the leukemic cells, as determined by immunofluorescence, we can see these MAbs for marrow purging before performing the CR2 transplantation.

In conclusion, the biologic role of purging has not yet been evaluated, but it can be assessed by a two-arm study in CR2. Transplantations

performed in CR1 will probably not provide a definite answer. Moreover, purging may have negative side effects, which have not been fully explored.

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Monoclonal Antibodies and Complement as Purging Procedure in Burkitt's Lymphoma

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INTRODUCTION

Massive therapy and autologous bone marrow transplantation (ABMT) is, in 1984, the best available therapy for Burkitt's lymphoma (BL) patients who have relapsed or patients who do not achieve complete remission (CR) after 2 months of conventional therapy. Its efficiency is still controversial for consolidation of first CR in initial central nervous system (CNS) disease (1-3). Based on our retrospective clinical analysis of cases of nonpurged ABMT, the most important obstacle in using this procedure is the frequency after ABMT of relapse in bone marrow (BM), an infrequent relapse site after conventional therapy. The BM relapse after ABMT may be attributed to tumor cells present in the infused marrow cell suspension (1,4,5). In the patient group eligible for our ABMT program, we found contamination of BL in remission marrow (defined by cytology) in 40% of the patients. Tumor cells were detected by a liquid-culture system--the details of which have been published elsewhere (5).

Various techniques are available to purge in vitro malignant cells prior to ABMT without affecting pluripotent stem cells to the same extent (6-8). Our team currently uses an immunomagnetic depletion procedure to purge neuroblastoma cells (9,10) and a chemical procedure with Asta Z in various solid tumors (unpublished data). Considering the preliminary published results on the efficiency of an immunodepletion by monoclonal antibodies (MAbs) and complement (11-15), we have initiated the use of this procedure to clean BM in BL, keeping in mind that we would probably have to combine various techniques (16).

Different membrane phenotypes of the BL cells have previously been described (17). According to this analysis, we selected three MAbs to use for purging remission marrow. Y29/55 and B_1 are two pan-B MAbs, AL_2 is an anti-CALLA (common acute lymphoblastic leukemia antigen). All three were previously used for such procedures in B-cell lymphomas or acute lymphocytic leukemia (ALL) (13,16,18). We determined the optimal conditions of purging with those three MAbs and cytotoxic rabbit complement by testing those antibiodies in BL cell lines, as well as staining malignant cells with Hoechst vital dye 33258, which allowed a viable quantitation of up to $1/10^4$ of the remaining BL cells (19) confirmed by a more sensitive cell culture monitoring system (20). This procedure was not toxic on normal progenitors' BM cells. The possibility of extending the results to fresh tumor samples is discussed.

MATERIALS AND METHODS

Blood and Bone Marrow

Peripheral blood lymphocytes (PBL) and normal BM mononuclear cells were obtained from healthy donors, collected in tubes with phosphate-buffered saline (PBS) without calcium and magnesium (Gibco) and a preservative-free heparin. The cells were separated on a Ficoll gradient (1.077 density; Niegaard, Norway), washed twice in PBS, and checked on viability by the 0.1% trypan blue exclusion test.

BL Cells

Fresh tumor samples were minced and suspended in PBS, separated on Ficoll gradient, and washed twice in PBS.

BL cell lines (Raji, Daudi, IARC BL_{17}, Ly_{67}, IARC BL_{63}) were kindly provided by Gilbert Lenoir (International Agency for Research on Cancer, Lyon, France). Their characteristics, as well as their reactivity with various B-cell MAbs, were previously described (17,21). Cells were cultured at 37°C in a 5% CO_2 atmosphere in air in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (Gibco). Cells were generally harvested for experiments 24 h after change of medium. Cell viability (trypan blue exclusion) was checked before and after 2 h of incubation with complement; the assay was performed only when the viability was 80% or higher. The expression of the three markers used for the cytotoxic assay was also checked before the procedure.

Monoclonal Antibodies

G. Janossy (London) kindly provided RFT8, a MAb cytotoxic with the complement and expressed on the suppressor or cytotoxic subpopulation of PBL, which is 20% of the mononuclear cells. It was used at 1:5 final dilution in cytotoxicity and indirect immunofluorescence assays (14).

Y29/55 was kindly given by Dr. K. Forster (Hoffmann-La Roche, Basel), AL_2 was provided by A. M. LeBacq (Bruxelles), and B_1 was provided by Coultronics (France). Expression of the three MAbs on BL lines and normal B cells was previously described (17). In indirect immunofluorescence assays, Y29/55 was used at 1:10, AL_2 at 1:500, and B_1 at 1:20 final dilution, with goat antimouse Ig Fab'2 fluorescein conjugates (Coultronics) as the second layer at appropriate dilutions (17).

Complement

We collaborated with the Institut Pasteur (Lyon, France) in preparing the complement. White baby rabbits (4-5 weeks old) were first tested for the absence of human anti-A and anti-B allohemagglutinins in their sera so that further complement absorption on human RBCs could be avoided. The rabbits were then bled aseptically; blood was allowed to clot for 2 h at 4°C; the serum was then separated by centrifugation and ultracentrifuged (150,000 g) for 90 min, as sterilized through 0.22 filters, and stored at -70°C. Bacteriologic and virologic controls were all negative.

Treatment of the Cells and Detection of Residual Contaminating Cells

Optimal conditions of treatment were successively studied in two models.

T8+ Cell Lysis in PBL

All preliminary experiments were done using a well-known model to set up the condition for using the complement (Tables 1-3). In PBL, the 20% T8+ cells (suppressor cytotoxic) were lysed with RFT8 MAb (14,22). All conditions were standardized at a temperature of 24°C. PBL (2×10^7 cells/ml) were incubated with RFT8 (1:5 final dilution) for 30 min and the complement was added. One or two treatments, with one wash between treatments, were carried out. The optimum medium appeared to be Hanks' or Earle's, or RPMI or PBS (Gibco) with increased $CaCl_2$ concentration (pH 7.4) and supplemented with 10 nM HEPES. The dilution curve of the complement and the duration of incubation were determined. Residual T8+ cells were detected by indirect immunofluorescence and dead cells were excluded by ethidium bromide (EB) staining (1:500 dilution) (22) on a Leitz Ortholux II fluorescence microscope (490-nm excitation peak) (Table 4).

Clonogenic BL Cell Lysis

Before the lysis procedure was begun, BL cells were stained with vital Hoechst dye fluorochrome 33258: 1.5×10^6 cells/ml were incubated in

Table 1. Percentage of Residual RFT8+ Cells after Lysing with RFT8 and Complement (One Treatment)

Medium	Percentage of Residual RFT8+ Cells by $CaCl^2$ Concentration (mg/l)				
	0	44	132	185	Control
Hanks' solution	8	7	5.5	3	30%
Phosphate-buffered saline	8.5	8.5	7	5	30%

Table 2. Percentage of Residual RFT8+ Cells in Peripheral Blood Lymphocytes after Lysing with RFT8 and Complement (One or Two Treatments)

Treatments	Percentage of Residual RFT8+ Cells by Complement Dilution (vol)			
	1:2 (200 µl)	1:3 (100 µl)	1:4 (75 µl)	Control
One	1.5	2.5	3	30
Two	0.2	0.3	0.7	30

Table 3. Percentage of Residual RFT8 Cells in Peripheral Blood Lymphocytes after Lysing with RFT8 and Complement Treatment: Variation of Incubation Duration

Treatments	Percentage of Residual RFT8+ Cells by Duration of Incubation (min)			
	30	45	60	Control
One	7.5	4	2.5	30
Two	4	2.5	0.6	30

RPMI with 10 µg/ml of fluorochrome for 60 min and washed for an additional hour in RPMI (37°C). Hoechst dye staining was observed using a fluorescence microscope (excitation peak, 360-365 nm; emission peak, 470 nm) (19,23).

All experiments were done at 24°C in Hanks' solution (185 mg/l $CaCl_2$) for half an hour incubation with MAbs, one or two complement treatments of 1 h each (one wash between the two treatments), and two final washes.

In the first group of experiments, BL-stained cells were mixed with normal PBL. Tables 5-7 show BL cells and 4 x 10^6 normal PBL in 200 µl medium (2 x 10^7 cells/ml) were treated under various conditions--variable dilutions of each MAb with one and two complement treatments (dilution 1:3, as set up on T8+ cell lysis experiments) (Table 5); variable dilutions of the complement with one or two treatments of cells preincubated with MAb cocktail at optimal dilution (Table 6); incubation of samples with each MAb and the cocktail, followed by complement treatments (1:3

Table 4. Expression of B-Cell Markers on 28 BL Cell Lines: Indirect Immunofluorescence Detection

Cell Lines	No.	Surface Immunoglobulins	No. of Expressing Cell Lines: Y29/55	B_1	BA_1	CALLA	38.13	TU$_1$ and/or BL$_{13}$
Group I								
African	5							
N. African	4	8	12	12	1	0	11	12
Caucasian	3							
Total	12							
Group II								
N. African	5							
Reunion Isl.	1	7	7	7	5	7	7	7
Caucasian	1							
Total	7							
Group III								
Caucasian	9	7	6	8	9	9	4	0

dilution)--to compare their efficiency (Table 7). In two sets of experiments (Tables 8 and 9), the effect of cell concentrations on complement activity was tested. First, 4 x 10^5 BL cells and 4 x 10^6 PBL (200-µl suspension) were incubated at an optimal dilution of MAb cocktail, washed, and resuspended in either a 200-µl suspension (2 x 10^7 cells/ml) or a 50-µl suspension (8 x 10^7 cells/ml) with complement; second, 4 x 10^5 malignant cells were mixed with increased numbers of normal PBL (4 x 10^6 cells, 8 x 10^6 cells, and 12 x 10^6 cells) and resuspended in 200 µl medium, incubated with optimal dilution of MAbs, and two complement treatments (1:2 dilution: 200 µl) (Table 9). In all groups of experiments, control samples were incubated with complement alone. After the purging procedure, residual stained BL cells were counted in a hemocytometer (count of 2 x 10^4 total cells), with the exclusion of dead cells by 0.1% trypan blue (detection of $1/10^4$ viable residual cells). In Tables 5-9, the results were expressed as total number of residual BL cells in the sample.

In a second set of experiments (Table 10), BL cells were mixed with irradiated allogeneic BM mononuclear cells at various concentrations from 1 to 20% (4 x 10^6 total cells/sample, 2 x 10^7 cells/ml) and treated with the MAb cocktail and two complement treatments (1:2 dilution). The entire procedure was done under sterile conditions. Detection of residual malignant cells was done either by counting the Hoechst viable stained cells or by a cell culture monitoring assay (20). After the purging procedure, 4 x 10^6 cells were poured in 5 ml of RPMI medium into a 25-cm^2 plastic culture flask (Falcon) with MRC5 feeder layer and incubated at 37°C in a 5% CO_2 atmosphere. After 8 days of culture, the percentage of growing BL cells was evaluated by cytologic examination of a cytocentrifuged smear, as described previously (20). If no BL cells were seen, the culture was kept 1 more week to conclude that BL cells did not grow. The quality of the detection is described in the Results section.

BL cells were mixed with 50% autologous BM mononuclear cells and treated in optimal conditions described in the aforementioned set of experiments. The toxicity of the procedure was evaluated by the total

Table 5. Elimination of 4 x 10^5 Malignant Cells Mixed with 4 x 10^6 Normal Cells (2 x 10^7 cells/ml) with Variable Dilution of Complement (One or Two Treatments)[a]

Treatments	No. of Residual Malignant Cells by Complement Dilution (vol)			
	1:2 (200 µl)	1:3 (100 µl)	1:4 (75 µl)	Control
One	7 x 10^3	1 x 10^4	ND[b]	4 x 10^5
Two	5 x 10^2	1 x 10^3	4 x 10^3	

[a]*Cells were preincubated with the cocktail of MAbs.*

[b]*ND, not determined.*

Table 6. Efficiency of the Lysis of 4 x 10^5 Malignant Cells Mixed with 4 x 10^6 Normal PBLs (2 x 10^7 cells/ml) with each MAb and Cocktail[a]

Cell Lines	No. of Residual Malignant Cells by MAb or Cocktail				
	B_1	29	AL_2	Cocktail	Control
Ly_{67}	8 x 10^4	2 x 10^5	ND	4 x 10^4	4 x 10^5
IARC BL_{17}	8 x 10^3	4 x 10^4	8 x 10^3	3 x 10^3	4 x 10^5
Raji	4 x 10^2	2 x 10^4	2 x 10^4	4 x 10^2	4 x 10^5
IARC BL_{63}	4 x 10^2	1.5 x 10^4	2 x 10^4	4 x 10^2	4 x 10^5
Daudi[b]	6 x 10^3	5.5 x 10^3	6 x 10^3	5 x 10^3	4 x 10^5
	4 x 10^2	8 x 10^2	5 x 10^3	2 x 10^2	4 x 10^5
	8 x 10^3	8 x 10^3	4 x 10^3	2.5 x 10^3	4 x 10^5

[a]*Two complement treatments at 1:3 dilution.*

[b]*Each cell line result is the mean of at least three experiments. The three experiments are detailed for Daudi to show the variation from one experiment to another in the same cell line.*

Note: *PBLs, peripheral blood lymphocytes; MAb, monoclonal antibody; ND, not determined.*

granulocyte-macrophage colony-forming unit (CFU-GM) recovery in agar of 2 x 10^6 BM-treated cells (24).

RESULTS

As shown in Table 1, we obtained a better complement activity when a medium with rather high concentrations of $CaCl_2$ (185 mg/l) was used. In

Table 7. Expression of the Three Markers on the Selected Cell Lines at Time of the Assays[a]

MAbs	Percentage of Positive Cells by Cell Line (Fluorescence Intensity)				
	Ly_{67}	BL_{17}	Raji	BL_{63}	Daudi
B_1	100 (++)	100 (++)	100 (++)	100 (++)	100 (++)
Y29/55	80 (+)	100 (++)	80 (+)	80 (+)	70 (+)
AL_2	(-)	100 (+)	30 (+/-)	100 (+)	40 (+/-)

[a]*The fluorescence intensity was evaluated as strong (++) or weak (+/-).*

Table 8. Elimination of 4 x 10^5 Malignant Cells Mixed with 4 x 10^6 Normal Cells with Variable Concentration of the Cell Suspension and Variable Volume of Complement[a]

Volume of Complement	No. of Residual Malignant Cells by Volume of the Suspension (Cell Concentration)		
	200 µl (2 x 10^7/ml)	50 µl (8 x 10^7/ml)	Control
100 µl	8 x 10^2	9 x 10^2	4 x 10^5
75 µl	1 x 10^3	1 x 10^3	

[a]*Cell suspensions were preincubated with optimal concentration of MAbs cocktail, in 200-µl medium, centrifuged, and resuspended in appropriate volume of medium.*

Table 9. Efficiency of the Lysis of 4 x 10^5 Malignant Cells Mixed with Various Concentrations of Normal Cells[a]

Volume of Complement	No. of Residual Malignant Cells by No. of Normal Cells (Concentration)			
	4 x 10^6 (2 x 10^7/ml)	8 x 10^6 (4 x 10^7/ml)	12 x 10^6 (6 x 10^7/ml)	Control
200 µl	4.5 x 10^2	1.6 x 10^3	2.4 x 10^3	4 x 10^5

[a]*All samples were in 200-µl medium and pretreated with MAbs cocktail (optimal concentration for 200-µl suspension).*

addition to a constant $CaCl_2$ concentration, the complement was less efficient in a PBS than in a $NaHCO_2$-buffered solution such as Hanks' medium (or Earle's or RPMI), which probably was due to the inhibition of the enzymatic reaction by the high molecular weight of the phosphate ion. We obtained a 0.5-log increase of more kill using Hanks' medium (185 mg/g $CaCl_2$) than with any medium without $CaCl_2$.

Table 10. Efficiency of the Purging Procedure on Clonogenic BL Cells at Various Concentrations in Normal Irradiated Bone Marrow Mononuclear Cells

Cell Lines	Residual Malignant Cells in Purged Samples by BL Cell Concentration in Samples before Purging					BL Lysis (Log)
	20%	10%	5%	1%	0.1%	
BL_{63}						
Hoechst	$7/10^5$	$1/10^4$	0	0	0	$\simeq 3$
D8 culture			0	0	0	$\simeq$
Raji						
Hoechst	$1/10^4$	$1/10^4$	0	0	0	$\simeq 3$
D8 culture	32%		0	0	0	$\simeq 3$
Daudi						
Hoechst	$1/10^3$	$7/10^4$	$5/10^4$	$1/10^4$		$\simeq 2$
D8 culture				0	0	> 4
BL_{17}						
Hoechst	$1/10^3$	$4/10^4$	$2/10^4$			$\simeq 2$
D8 culture				0	0	> 3
Ly_{67}						
Hoechst	$5/10^3$					$\simeq 1$

[a] *By the Hoechst technique, the residual viable malignant cells are quantified immediately after the purging procedure by their concentration in the total cell population. In the culture system, cells were examined cytologically on cytocentrifuged smears at D8 and quantified by the percentage in the total population of cells on the smears; these results have to be compared to the limit of detection by the system (Table 11). In addition, for all assays the control (sample with 1% malignant cells treated by complement alone) gave growth to 100% BL cells at D8 in culture.*

As shown in Table 2, two treatments with complement were more efficient than one. In addition and of financial interest, the optimal dilution of complement was higher when two treatments were applied instead of one; consequently, the same final quantity of complement was more efficient when half/half was used in two treatments than in-full in one treatment.

As shown in Table 3, incubation at 24°C, with either one or two treatments with complement, produced a better lysis if the duration of incubation was extended from 30 to 60 min. We did not achieve an increase in killing with two 30-min treatments than we did with one 60-min treatment--probably because the complement we used maintained an equal activity for at least 1 h at 24°C and needed 2 h to achieve complete lysis.

We have published the immunophenotype of 28 Burkitt cell lines analyzed with seven distinct MAbs (17,21) (Table 4). B_1 and Y29/55 were expressed on almost all cell lines; cell lines established from Caucasian BL patients strongly expressed pre-B cell markers as CALLA or BA_1. Hence, we selected Y29/55, B_1, and AL_2 as relevant MAbs to purge BL cells.

We obtained a plateau of optimal activity at 1:80 final dilution for B_1, 1:50 for Y29/55, and 1:1000 for AL_2. However, in individual assays, we obtained a slight improvement in killing cells with increased amounts of MAb, probably because of some variations in sensitivity of BL cell

Table 11. Efficiency of the Cell Culture Monitoring System to Detect Contaminating BL Cells from Each Cell Line Mixed with Various Dilutions in Irradiated Human Bone Marrow[a]

Cell Lines	Percentage of BL Cells in the Culture at Day 8 by Dilution of BL Cells in Initial Poured Sample					Limit of Detection
	1/10²	1/10³	1/10⁴	1/10⁵	1/10⁶	
BL_{63}	100		30	0		1/10⁴
Daudi	100		70	50	45	>1/10⁶
BL_{17}	100		90	90		>1/10⁵
Raji	100	61		6		>1/10⁵

[a]*Lecture at day 8 of the culture.*

lines. We then selected the dilution shown to be optimal for all individual assays: 1:50 final dilution for B_1, 1:25 final dilution for Y29/55, and 1:500 final dilution for AL_2. These dilutions were optimal for cell samples containing 10-50% BL cells. In the cocktail, MAbs were used at the same final dilution as when used alone.

We obtained a better lysis with two complement treatments at half/half volume than with one treatment at the full quantity (Table 5). The badge of complement used in those experiments was slightly more active at 1:2 than 1:3 final dilution.

The cocktail of three MAbs was usually more efficient than each individual MAb. However, in two cell lines, BL_{63} and Raji, B_1 alone was as active as the cocktail to lyse BL cells. Using the cell culture monitoring system to detect residual malignant cells, we obtained an equal killing with B_1 or the cocktail with samples contaminated with 1% BL cells, which were from Daudi, BL_{17}, or BL_{63}. In Daudi samples, Y28/55 + AL_2 was also as efficient as B_1. Note that the activity of each MAb and the cocktail varied from one cell line to another and even in the same cell line between different assays (see Table 6 for results of Daudi lines). The variation in cytolytic activity was not strictly correlated to the expression of markers on the cells (indirect immunofluorescence staining), as shown in Table 7. To determine whether those variations could be explained by antigen modulation during the assay, BL cells incubated with MAb alone or with complement alone for 180 min were stained by indirect immunofluorescence with each MAb and a significant decrease of fluorescence was detected.

As shown in Table 8, the activity of the complement was not influenced by the varying cell concentration (i.e., from 2 x 10^7 to 8 x 10^7) provided the same final volume of complement was used for an equal total number of cells. This implied that the dilution curve of the complement depended on the cell concentration. In addition, as shown in Table 9, the activity of the complement on the same number of malignant cells decreased when the concentration or the total number of normal cells increased. In complementary assays (data not shown) we did not observe significant improvement of the killing by increasing the final volume of complement in samples containing 30-50% malignant cells (2 x 10^7 cells/ml). It was concluded that for purging samples contaminated 10-50% with BL cells, the final optimal volume of complement depended on the total number of malignant or normal cells. Note that these results were obtained by mixing BL cells with PBL. Similar results were obtained when BL cells were mixed with normal irradiated bone marrow. However, when BL cells were mixed with the erythroleukemic cell line K562, we found a better killing for the same final quantity of complement, probably because of the absence of any reactivity of these cells with complement.

The efficiency of the procedure varied between the lines (Tables 10 and 11). It was possible to eliminate 3 logs of malignant cells from BL_{63} or Raji and BL_{17} cell lines and 4 logs from the Daudi cell line, whereas we did not destroy more than 1 log from Ly_{67}. It is noteworthy that a good correlation was found in the evaluation of the killing up to 3 logs with either the Hoechst technique or the cell monitoring system. However, for Daudi and BL_{17} lines the culture system allowed us to define a higher level of killing. The explanation could be that the assays, followed by culture detection, were done with 1% BL cell-contaminated samples and the purging procedure could be more efficient if 3 or 4 logs of malignant cells could be destroyed from 1% contaminated samples rather than from 10% contaminated cell suspensions, as monitored by the Hoechst technique. Otherwise, the cell still viable just after the purging procedure and detected by the Hoechst technique might not be able to grow further in culture.

Toxicity of the Entire Procedure on BM Progenitor Cells

Under the conditions described above, we tested the complement and MAb toxicity on hematopoietic progenitor cells by the CFU-GM technique. As shown in Fig 1, the cloning efficiency of 2×10^5 mononuclear cells was 72% of the control when cells were tested with complement alone and 50% when cells were treated with the MAbs cocktail and complement. A major point to consider is the loss of mononuclear cells during the procedure; this loss is especially caused by the agglutination variable from one marrow sample to another and it is independent of the complement toxicity on CFU-GM. The total recovery of CFU-GM from 2×10^6 BM pretreated cells was 62% of the control with complement treatments alone and 42% with MAbs plus complement treatments. This total recovery was not significantly improved when the manipulations were performed in PBS without calcium. This indicated that calcium probably did not play a major role in the agglutination phenomenon, which was probably due to the lysis of granulocytes. Therefore, recently we added pancreatic DNAase (nonpyrogenic, sterilized, and tested for viral and bacterial contamination) (Sigma) at 5 U/ml in all samples 15 min before the end of each complement procedure.

Preliminary Results on Fresh BL Tumor Samples

We have performed three experiments on fresh tumor BL cells mixed with autologous BM. None of these BL cells were able to grow in culture; hence, the efficiency of the killing was evaluated by the combination of the IF staining of the remaining malignant cells and the EB exclusion of the dead cells. Under the conditions described above and as shown in Fig 2, the BM cell treatment with one MAb and one complement incubation allowed us to kill 1 log of malignant cells; the killing increased to 1.5 logs with the cocktail of MAbs and to 2 logs (maximum limit of a viable detection with IF + EB analysis) with the cocktail and two complement treatments.

CONCLUSIONS

We described a procedure using three MAbs--Y29/55, B_1, and AL_2--with baby rabbit cytolytic complement to kill Burkitt's cells. A 1- to 3-log kill of clonogenic cells that varied from one line to another was obtained. We confirmed previously published data obtained with a similar procedure meant (*a*) to eliminate contaminating T-cells in BM, (*b*) to avoid graft-versus-host disease in allogeneic BM transplantation (14), or (*c*) to eliminate CALLA-positive cells or B-malignant cells from marrow cell suspensions before ABMT in CALLA or B-cell lymphoma (11, 13,15,16). The procedure was set up to purge BM in BL cases, but it could probably be rapidly extended to B lymphomas using the same cocktail for centroblastic centrocytic lymphomas or Y29/55 and B_1 only in CALLA or B-cell lymphomas.

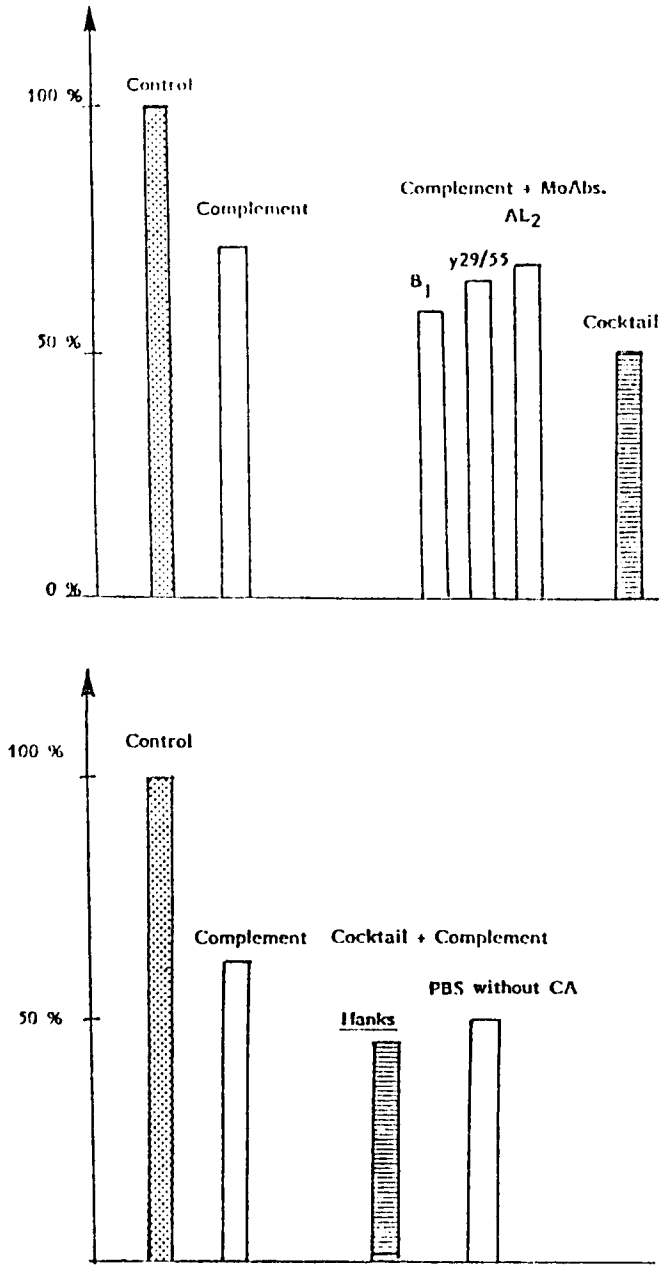

Figure 1. Toxicity of the bone marrow treatment on CFU-GM culture. Top, *Cloning efficiency of 2 x 10^5 mononuclear cells (mean of 10 experiments).* Bottom, *Total recovery of CFU-GM from 2 x 10^6 bone marrow mononuclear cells treated with complement and monoclonal antibodies (mean of 7 experiments).*

However, it is still questionable if any results obtained with cell lines might be extended to fresh tumor samples.

Our study with five different BL cell lines showed considerable variations in the efficacy of the cocktail under the same experimental conditions; those variations were not strictly correlated to the expres-

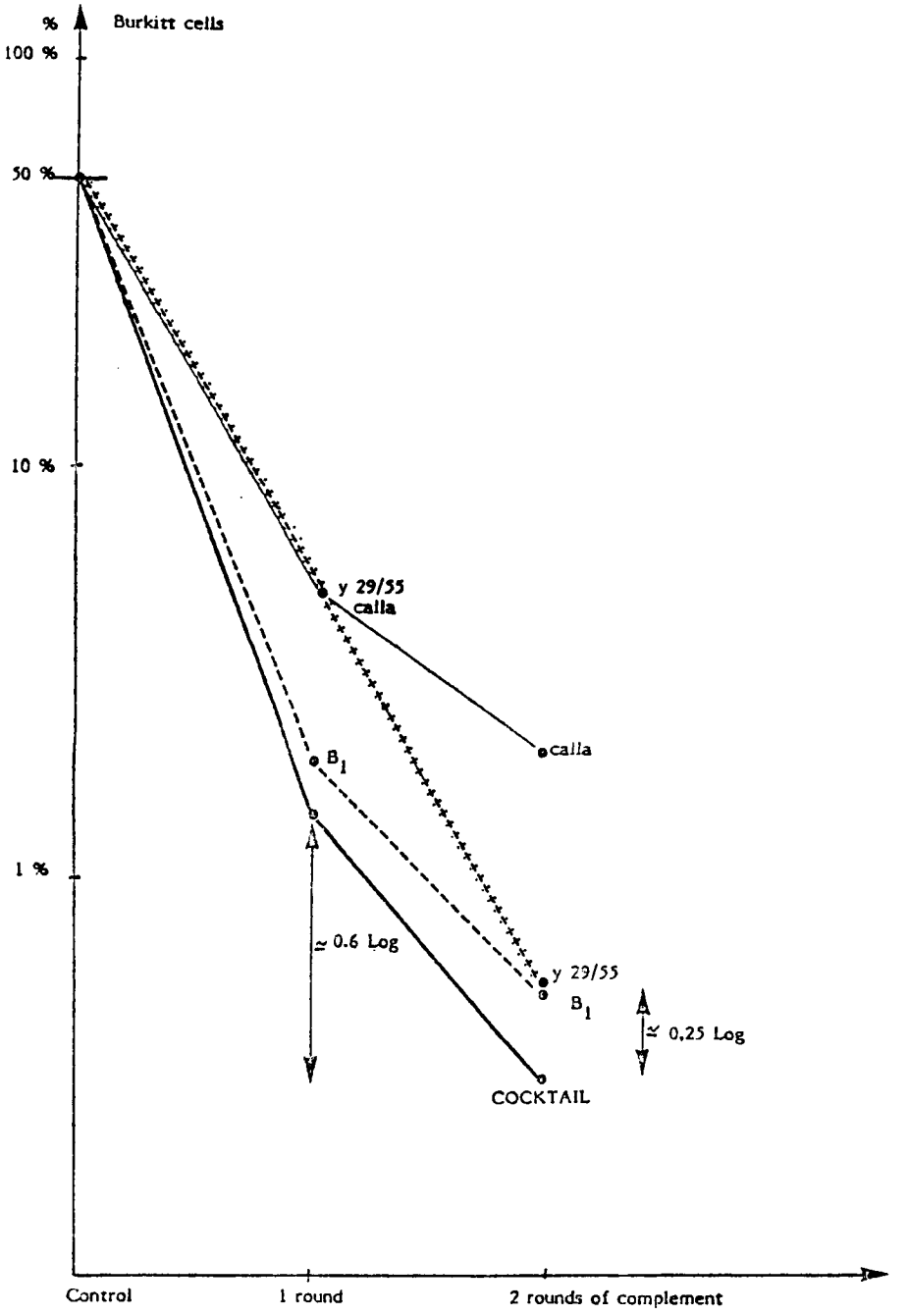

Figure 2. Efficiency of the procedure on fresh tumor samples (IF + EB detection of residual malignant cells).

sion of markers on the membrane and suggested a varying resistance or sensitivity of the cells to the complement lysis. If those variations are present in fresh tumor samples, the efficiency of the purging procedure will differ from patient to patient.

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Marrow Purging with Immunotoxins in Allogeneic and Autologous Bone Marrow Transplantation: Pilot Clinical Studies

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INTRODUCTION

Immunotoxins, toxins that are covalently linked to monoclonal antibodies (MAbs), offer an efficient option for ex vivo marrow purging. We have previously reported that a single 2-h incubation of donor bone marrow with a cocktail of anti-T cell immunotoxins yields the following: 1) good cell recovery (approximately 35% of total nucleated cells harvested), 2) effective T depletion, and 3) encouraging results with respect to the prevention of acute graft-versus-host disease (GVHD) (1). We have extended our experience in allogeneic transplantation to 14 patients by using immunotoxins that consist of well-characterized anti-T cell MAbs that are covalently linked to whole ricin (2) and we have also treated three autologous grafts with a combination of two of these immunotoxins: TA-1 ricin (anti-p95/170) and T101 ricin (anti-p65) prior to cryopreservation. We have elected to use whole ricin immunotoxins since preclinical studies demonstrated that the presence of ricin B chain in the conjugates facilitates entry of the toxic A chain into antibody-targeted cells, even though direct (nonspecific) B-chain binding to cell membranes is blocked by a saturating concentration of the inhibiting sugar and lactose in the incubation medium (3). Protein synthesis is inhibited in T cells poisoned by immunotoxin (4) with the consequence that the T cells in donor marrow are no longer able to proliferate in response to alloantigenic stimuli and thus die within 1-7 days of treatment.

Potent immunotoxins can be generated with MAbs that do not bind complement. Large batches of immunotoxins can be prepared in advance and stored for prolonged periods of time which eliminates the variability encountered with other biologic reagents, such as sheep erythrocytes or complement. Toxicity studies with rodents indicate that the LD50 of immunotoxins used in our pilot studies is more than 100-fold greater than the maximum amount of immunotoxins that might be infused after marrow treatment (Vallera DA, unpublished data).

PATIENTS AND METHODS

Patients who had high-risk leukemia and histocompatible sibling donors were selected for the pilot study of immunotoxin marrow treatment for prophylaxis of acute GVHD. Three patients who lacked human leukocyte antigen/mixed leukocyte culture (HLA/MLC)-identical donors, one each with high-risk leukemia, Wiskott-Aldrich syndrome, and Wolman's disease consented to the pilot study of immunotoxin treatment for prophylaxis of acute GVHD in mismatched related transplantation. Three patients who had T-cell acute lymphocytic leukemia (ALL) in first or second remission and lacked HLA/MLC-identical sibling donors were selected for autologous transplantation with immunotoxin purging when their leukemia cells were found to have greater than 50% binding to the MAbs TA-1 or T101 or both (see antibody description below). Permission to participate in these pilot studies was obtained under the guidelines of the Committee on the Use of Human Subjects at the University of Minnesota.

All patients were hospitalized in private high-efficiency particulate air-filtered rooms in the bone marrow transplantation ward at the University of Minnesota Hospital from the onset of pretransplant cytoreductive therapy until satisfactory hematologic and systemic recovery was achieved. All leukemia patients in remission were conditioned with intravenous cyclophosphamide intravenously (60 mg/kg/day x 2) followed by fractionated total body irradiation (TBI) (total of 1320 cGy split into 8

doses over 4 days). Two patients (unique patient numbers [UPNs] 373 and 382) with refractory acute lymphoblastic leukemia were conditioned with cytarabine intravenously (3 g/kg x 12) followed by single-dose TBI (850 cGy). The two mismatched patients with nonmalignant genetic diseases (Wolman's disease and Wiskott-Aldrich syndrome) received pretransplant regimens of intravenous cyclophosphamide (intravenously) and single-dose TBI, with or without pretransplant antithymocyte globulin.

Immunotoxins

Anti-T cell MAbs were covalently bound to intact ricin by means of a thioether linkage (5). The three MAbs were: TA-1 (6), which recognized a p90/175 dimeric glycoprotein on most peripheral T cells, monocytes, and some myelomonocytic bone marrow precursors; T101 (7), which recognized a p65 antigen on most peripheral T cells; and UCHT-1 (8), which recognized a p19 structure on immunocompetent late thymic and postthymic T cells (recognized by OKT3). Preclinical studies showed that the mixture of these three immunotoxins was more efficient in depleting proliferative T-cell responses than equivalent amounts of any individual immunotoxin alone. Human pluripotent hematopoietic stem cells (CFU-GEMM) were not significantly reduced in number at the immunotoxin concentrations used (2).

Ex Vivo Marrow Treatment with Immunotoxin "Cocktail"

Bone marrow nucleated cells (4 to 6 x 10^8/kg) recipient body weight were collected under sterile conditions and added to heparinized tissue culture medium. Subsequent bone marrow treatment was carried out in laminar air-flow hoods with reusable glassware prerinsed with phosphate-buffered saline (PBS) containing 1% human serum albumin (Cutter Biologicals). Bone marrow cells were mixed with 0.66% Hetastarch (American McGaw), and the RBCs were permitted to settle over 1-2 h in 50-ml graduated glass cylinders until the buffy coats reached 50% of the original volume. Buffy-coat cells were aspirated, washed, and adjusted to 25 x 10^6/ml RPMI medium with 25 mmol/l of HEPES, 300 mg/l of glutamine (Gibco), and 1% of human serum albumin, and centrifuged on isolymph gradients (Gallard-Schlesinger). Interface mononuclear cells were resuspended at 10^7 cells/ml and incubated for 2 h in RPMI 1640 with 100 mmol/l of lactose and the immunotoxin mixture at 37°C in 5% CO_2 in 500-2000-ml flasks with continuous stirring.

For allogeneic transplantation (GVHD prophylaxis) 300 or 600 ng of immunotoxins (equal amounts each of TA-1 ricin, UCHT-1 ricin, and T101-ricin) were added to 10^7 mononuclear cells/ml. For autologous purging, 600 ng (equal amounts each of TA-1 ricin and T101 ricin) were added to 10^7 cells/ml. After incubation the bone marrow was washed in lactose-containing medium and then incubated in RPMI 1640. Allogeneic marrow was infused in normal saline (approximately 50 cc intravenously over 15 min). Autologous marrow was cryopreserved in dimethyl sulfoxide (DMSO).

In Vitro Assays

Untreated bone marrow samples and immunotoxin-treated samples were studied for residual T cells through in vitro stimulation with the mitogenic lectin HA-17, in the mixed leukocyte reaction (MLR), and in a cytotoxicity assay as described previously (1). Bone marrow cultures were plated in methylcellulose and scored at 14 days for the presence of colony-forming units in culture (CFU-C), erythroid burst-forming units (BFU-E), and mixed colonies CFU-GEMM (9). Autologous marrows harvested in remission were studied prior to treatment for double staining with anti-TdT (terminal deoxynucleotidyl transferase) and T-cell MAbs (TA-1 and T101 together), as described previously (10), to quantitate residual malignant cells immunologically. Preclinical studies of immunotoxin killing of T-cell lines in the presence of excess marrow have been published elsewhere (2,11).

Table 1. Allogeneic Bone Marrow Transplantation: GVHD Prophylaxis by Marrow Purging with Three Anti-T Cell Immunotoxins

UPN	Age/Sex/Disease	GVHD	Vital Status
Histocompatible Donors			
289	6/F/ALL (3 REM)	None	Alive > 500 days; relapsed day 221
300	13/F/ALL (3 REM)	None	Alive > 450 days
338	21/M/ANLL (2 REM)	Skin II	Died day 232; relapsed day 166
344	15/M/ALL (3 REM)	None	Alive > 300 days; relapsed day 73 with autologous recovery
366	31/M/ANLL (2 REM)	None	Died day 185; relapsed
373	7/M/ALL (REL)	None	Alive > 150 days
381	38/M/ANLL (1 REM)	Skin II	Alive > 130 days; relapsed day 83
382	15/M/ALL (REL)	None	Alive > 130 days; relapsed day 75
394	18/F/ANLL (REL)	None	Alive > 70 days
399	29/M/ANLL (1 REM)	None	Alive > 50 days
403	54/M/CML (accelerated)	--	Alive > 30 days
Related Partially Matched Donors			
332	8/M/WAS	None	Alive > 300 days; autologous recovery with partial donor RBC chimerism
375	4/M/Wolman's Disease	--	Died day 18; gram-sepsis
389	4/M/ANLL (1 REM)	None	Alive > 100 days; autologous recovery with partial donor PBL chimerism

Note: *UPN, unique patient number; GVHD, graft-versus-host disease; ALL, acute lymphoblastic leukemia; REM, remission; ANLL, acute nonlymphocytic leukemia; REL, relapse; WAS, Wiskott-Aldrich syndrome; RBC, red blood cell; PBL, peripheral blood lymphocytes.*

RESULTS

Clinical Trials

Descriptions of the patient characteristics and clinical outcome of allogeneic and autologous bone marrow transplantation involving marrow purging with anti-T cell immunotoxins are presented in Tables 1 and 2.

Table 2. Autologous Bone Marrow Transplantation for T-Cell Leukemia/Lymphoma in Remission: Purging with TA-1 Ricin and T101 Ricin[a]

UPN	Age/Sex	Disease[b]	Engraftment Day[c]	Vital Status
379	23/F	T-cell ALL (1 REM)	20	Alive > 130 days, skin relapse day 64
388	17/M	T-cell ALL (2 REM)	50	Alive > 100 days, bone marrow relapse day 53
395	17/M	T-cell ALL (2 REM)	13	Alive > 79 days

[a]*Treated marrows contained less than 0.5% cells with double staining for TdT and T-cell leukemia monoclonal antibody cocktail.*

[b]*All patients had TA-1+, T101+ phenotype.*

[c]*Third consecutive day with WBC > 1000/mm³ posttransplant.*

Note: *UPN, unique patient number; ALL, acute lymphocytic leukemia; REM, remission.*

The clinical results of the three patients who received immunotoxin-purged autologous marrow are shown in Table 2. Although all three patients are alive at this time, two relapsed within 2 months postgrafting. The first patient (UPN 375) had evidence of leukemia in the skin prior to bone marrow transplantation and had a relapse (skin) 64 days posttransplant without marrow relapse. The second patient (UPN 388) achieved second clinical remission pretransplant only after multiple chemotherapeutic agents and investigational drugs were used. Unfortunately, marrow relapse was diagnosed 3 days after this patient met the criteria for hematologic engraftment posttransplant. Relapse was suspected because of the rapid rise in peripheral blood lymphocyte counts at day 45 posttransplant.

In general, engraftment of immunotoxin-treated marrow in both allogeneic and autologous marrow transplant patients was prompt. None of the patients had unusual requirements for blood product support and mucositis was only a minor problem.

CONCLUSIONS AND DISCUSSION

Following extensive preclinical studies with identified potent combinations of immunotoxins for the specific inactivation of normal and malignant T cells, we began phase 1 clinical trials with these reagents for ex vivo marrow purging in allogeneic and autologous transplantation. Precedence for the use of immunotoxins for T-cell depletion in bone marrow transplantation in the mouse was established (12). To date, these clinical trials confirm the safety of ex vivo purging with immunotoxins. We plan to proceed to comparative trials to investigate the clinical efficacy of these techniques. In allogeneic transplantation, histocompatible patients will be randomized to receive either immunotoxin pretreatment or standard (Minnesota) in vivo GVHD prophylaxis (13). This approach will permit comparison of time to engraftment, posttransplant complications, GVHD rates, survival, and impact on immunoreconstitution. With respect to autologous marrow transplantation, patients with acute T-cell leukemia who are in remission and lack histocompatible donors will receive pretransplant conditioning therapy (prior to reinfusion of immunotoxin-purged

marrow) that is identical to that used in allogeneic transplantation of T-cell leukemia for patients with histocompatible donors. Thus, time to engraftment, rate of relapse, and survival can be evaluated to assess the relative success of this approach.

ACKNOWLEDGMENTS

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Monoclonal Antibodies and Magnetic Microspheres Used for the Depletion of Malignant Cells from Bone Marrow

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INTRODUCTION

Magnets can be employed as cell separators when one population of cells contains iron that can be rendered magnetic. For example, malaria-infected erythrocytes (RBC) containing breakdown products of hemoglobin that are paramagnetic can be enriched from normal RBC in which iron is complexed with oxygen and thus is nonmagnetic (9). As few cells are intrinsically magnetic, several techniques have been devised to artifically render them so. Sheep RBC in which the hemoglobin has been converted to Met-Hb (magnetic) have been used to rosette and subsequently remove T cells from human peripheral blood (8). Antibodies coupled to ferritin and cobalt have also been used to render cells magnetic (7,10). These methods rely on the use of relatively high field strength magnets to affect a separation.

To facilitate both ease of separation and removal of the magnetic material from cell suspensions, several particulate preparations of magnetic compounds have been investigated as targeting agents. These include Magnogel 44 (hydrophilic agarose-polyacrylamide spheres) (4), albumin microspheres (17), methacrylate microspheres (11), and polystyrene microspheres (15,16). Polyacrolein beads have been used to separate fowl erythrocytes from sheep RBC to a purity of 99% (6). While this is encouraging, the yield of cells recovered from the separation was too low for the methodology to be directly applied to the purging of tumor cells from bone marrow. Unquestionably, the most monodisperse and uniform preparation of microspheres mentioned above are those made from the polymerization of styrene divinylbenzene (15,16). This chapter describes the methods developed to use these beads for the removal of tumor cells from bone marrow harvested for autologous transplantation.

METHODS

Preparation of Microspheres

Three-micrometer polystyrene beads containing magnetite, made by the polymerization of styrene divinylbenzyne and modified to render them hydrophilic and magnetic, are uniform in size with a pitted surface, giving them a large surface area (approximately 100-150 m^2/g). To ensure monodispersity in solution, microspheres were sonicated (MSE 100 W ultrasonic disintegrator, wavelength 12 μm) for 10 sec at 4°C and were sterilized by washing in alcohol. Following transfer to sterile phosphate buffered saline (PBS), antibodies were added to the microspheres for 18 h at 4°C (ratio beads/protein 10:1 wt/vol). Antibody bound to the microspheres as polystyrene nonspecifically adsorbs protein. Microspheres were washed free of unbound antibody directly before use. Trace label studies indicated 100 mg of beads bind approximately 7.0 mg of protein under these conditions (14).

Collection of Bone Marrow and Preparation of Buffy-Coat Fraction

For development of the methodology, single bone marrow aspirates were harvested from the iliac crest of normal volunteers. Bone marrows (BMs) for autologous transplantation were harvested under general anesthetic from the iliac crest or sternum or both into tissue culture medium con-

taining preservative-free heparin. Buffy-coat preparations were made by centrifugation of bone marrow at 220 x g for 20 min. Approximately 80% of the original nucleated cell fraction was retained, with a 75% depletion of RBCs.

Monoclonal Antibodies

Five monoclonal antibodies of the IgG class (UJ 13A, UJ223.8, UJ127.11, UJ181.4, αThy-1) were individually purified by means of protein A affinity chromatography. The sixth, 5.1.H11, was supplied by Dr. F. Walsh (5). These reagents bind to neuroblastoma cells but not to normal bone marrow progenitors as determined by indirect immunofluorescence assays. Each antibody is stored at a concentration of 1.0 mg/ml and is added to tumor cells in bone marrow under conditions calculated to saturate antigen-binding sites.

Either contaminated BM or BMs into which the human neuroblastoma cell line CHP100 was titrated 1-50% infiltration were incubated with monoclonal antibodies for 30 min at 4°C. Following centrifugation at 220 x g for 10 min and washing with PBS containing 5% purified plasma protein fraction (PPF), microspheres coated with anti-mouse Ig were added to the BM. For purging marrows for transplantation, 100 mg of beads were added to between 5 x 10^9 and 5 x 10^{10} nucleated cells for 2 h at 4°C before passage through a series of chambers surrounded by magnets.

Magnetic Cell Separation

Test experiments were performed using LP3 tubes (Luckham's) and a horseshoe magnet (two poles, 2 cm x 1 cm). The initial magnetic apparatus designed to separate tumor cells from large volumes of BM is an enclosed sterile system of polycarbonate chambers linked by silicone rubber tubing (Fig 1). Ten samarium-cobalt permanent magnets (1 cm x 1 cm x 0.3 mm) are placed in the base of the main polycarbonate chamber so that they are separated from the interior by 2.0 mm of polymer (chamber size 19 cm, internal volume 22.0 ml). In order to ensure maximum efficiency of separation, a second smaller chamber with an internal volume of 3.0 ml is linked to the first. This contains two samarium-cobalt magnets in its base. A third chamber is positioned between the poles of a water-cooled electromagnet, field strength 1.0 tesla. Free microspheres or tumor cells escaping capture in the first two chambers are removed by this magnet. The apparatus is assembled under sterile conditions and is primed with normal saline containing 20% PPF. Pretreated BM is pumped through the system at 1.5 ml/min, and the chambers are flushed free of hematopoietic cells with saline containing 5% PPF. This permits 5 x 10^9 BM cells to be purged of tumor cells in approximately 3 h. Eighty-five percent of the nucleated cells entering the system can be recovered to be either cryopreserved or returned to the patient directly after high-dose chemotherapy.

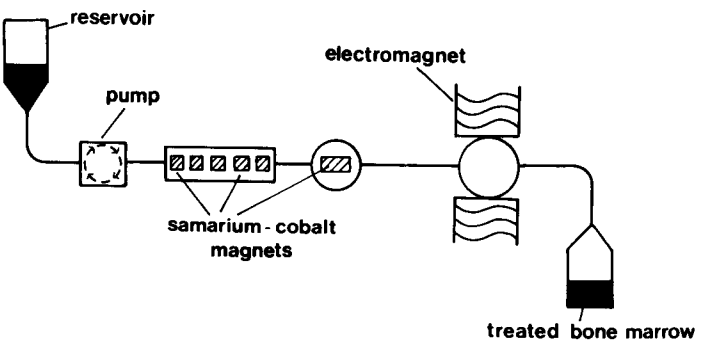

Figure 1. Flow system for the removal of tumor cells from bone marrow.

Tumor Cell Removal and Hematopoietic Cell Recovery

In model systems, estimates of tumor cell lines removed from BM were made by cloning studies in soft agar. Tumor cell removal from marrow to be used for transplantation was assessed by conventional histological techniques as well as by indirect immunofluorescence studies using up to 12 different monoclonal antibodies and fluorescein-conjugated sheep anti-mouse Ig.

Colony assays on fractions taken throughout the procedure were undertaken according to the method of Fausner and Messner (1,2). Erythroid colony-forming units (CFU-E) (8-50 hemoglobinized cells in a single cluster) were counted on day 7. Granulocyte-monocyte colony units (CFU-C), megakaryocytes (CFU-MEGA), and mixed colonies of erythroid and granulocyte-monocyte elements (CFU-GEMM) were counted on day 14. Samples were checked at each stage of the procedure for bacterial and fungal contamination.

RESULTS

Tumor Cell Removal

Using cloning assays (efficiency 7.5-12%) to assess tumor cell removal, it has been possible to deplete 99.9% of neuroblasts from an equal population (5×10^7) of hematopoietic progenitor cells. This was achieved using an indirect system, attaching anti-mouse Ig to 3-μm beads and mixtures of monoclonal antibodies to neuroblastoma cells. In this indirect system, the use of 2- or 3-μm beads achieved 99.2-99.6% removal of tumor cells from a 50% infiltrate of CHP100 cells in bone marrow, whereas only 60% of neuroblasts were removed using 10-μm microspheres. In addition, 3-μm beads containing different amounts of magnetite (10, 15, and 20% wt/vol) were tested for their efficiency at tumor cell removal. A horseshoe magnet would not efficiently remove beads containing 10% magnetite from suspension, whereas effective bead and tumor cell removal was achieved with microspheres containing 20% magnetite.

Panels of monoclonal antibodies need to be employed to efficiently target microspheres to tumor cells. Direct coupling of individual antibodies to separate batches of microspheres or direct coupling of six reagents to one batch of beads did not give efficient tumor cell removal. Seventy percent of CHP100 cells was removed from a mixture of 5×10^7 neuroblastoma cells mixed with 5×10^7 nucleated BM cells. This contrasts with the 99% removal of CHP100 cells that was achieved in comparable experiments using anti-mouse Ig-coated beads. Attempts to "orientate" reagents on the microspheres using protein-A spacers did not improve the efficiency of depletion, although this was difficult to assess since the original methodology resulted in 99.5-99.9% cell removal.

In an indirect system, six different anti-neuroblastoma monoclonal antibodies mixed with artificially contaminated BM were more efficient at removing tumor cells than was a single reagent (UJ13A). Only 85% of CHP100 cells could be removed from equal mixtures of neuroblastoma and nucleated bone marrow cells (5×10^7 each) using a single monoclonal antibody and anti-mouse Ig-coated beads, whereas a panel of 6 reagents repeatedly gave depletions of over 99% of tumor cells. All estimates of tumor cell removal from BM have to be made on the "magnetic negative" fraction because no method has yet been found to successfully remove beads from the surface of coated cells.

Using the magnetic separation device described previously for handling large volumes of bone marrow and equal numbers of tumor and BM cells, a 99.9-99.95% depletion of tumor cells was achieved. Decreasing the ratio of tumor cells to bone marrow resulted in a decrease in cloning efficiency of the cell line, making accurate estimates of tumor removal impossible. However, in artificial mixing experiments and 6 of 7 bone marrows purged of fresh tumor cells (3-30% contamination), no neuroblasts were found following indirect immunofluorescence studies on the treated marrow. This was ascertained using a panel of up to 12 antibodies and

Table 1. Illustration of Colony Assay before and after Purging Marrow with Monoclonal Antibodies and Magnetic Microspheres

Time	Nucleated Cells (x 10)		
	CFU-C	CFU-E	CFU-GEMM
Prepurging	178	119	55
Postpurging	194	94	46

Note: *CFU-C, granulocyte-monocyte colony units; CFU-E, erythroid colony-forming units; CFU-GEMM, mixed colonies of erythroid and granulocyte-monocyte elements.*

counting at least 250 fields that contained approximately 80 nucleated cells per field.

In a series of 34 purging procedures, all beads were removed from the treated BM by the samarium-cobalt and electromagnets (assessed by light microscopy). All marrows were shown to be free from bacterial and fungal contamination. Overall recovery of nucleated cells was 50% of the original, with a mean of 1.7 x 10^8 cells/kg body weight cryopreserved for reinfusion. As 32% (means of 34 procedures) of nucleated cells were lost during the preparation of the buffy-coat fraction, losses in the actual "magnetic depletion stages" on the procedures were minimal. Viability of cells passing through the separation chambers was in excess of 98% (trypan blue exclusion).

Colony assays for CFU-C, CFU-MEGA, CFU-GEMM, and CFU-E showed no significant fall after separation when compared with colonies grown from the buffy-coat fraction (Table 1).

Of 30 purged bone marrows from neuroblastoma patients, three await reinfusion. Seven were not reinfused because the patients relapsed during induction therapy prior to grafting. Two patients died of fungal infection prior to reengraftment. In the remainder, engraftment was rapid unless patients had been exposed to high and prolonged doses of chemotherapy (12-21 days needed to attain 500 neutrophils in the peripheral blood), particularly alkylating agents. In this latter group, the time to attainment of 500 neutrophils in the peripheral blood was up to 60 days.

Because the first patient to receive high-dose therapy with fractionated TBI (12 Gy twice a day for 3 days; vincristine, day 0: 1.5 mg/m; days 1-4: 0.5 mg/m intravenously; and melphalan, 180 mg/m) and cleaned marrow using the magnetic beads was treated 14 months ago, it is too early to assess the effectiveness of therapy. However, preliminary data suggest that patients treated in relapse with overt marrow contamination prior to purging have a poorer prognosis than do those given megatherapy and consolidation therapy and treated in complete remission.

DISCUSSION

The use of monoclonal antibodies and magnetic microspheres to remove tumor cells from bone marrow has several advantages over other techniques. Testing and absorption of complement is not required (12), and unlike other immunological methods relying on either opsonization or toxin killing, this method allows rapid estimation of tumor cell removal from the "magnetic negative" fraction (13).

The indirect approach has several advantages over a direct system in which microspheres are coated with monoclonal antibodies. Apart from being more efficient, the indirect approach of attaching microspheres to cells is more economical on reagents and can be simply adapted for use

with other tumors by changing the panel of monoclonal antibodies. Currently, we have used it to purge marrows from two patients with B-cell lymphoma and from four with common acute lymphoblastic leukemia. The use of the technique for T-cell depletions from bone marrow to be used for allogeneic transplantation also is being explored (3).

Covalent linkage of antibody to beads to reduce protein leakage from the surface also is being assessed, with other polymers being grafted onto the exterior of the microspheres. Initial experiments have shown that beads of this type are as good as or better than those currently in use and that they have the advantage of requiring less anti-mouse Ig to coat their surface (J Ugelstad, unpublished observation). All of these beads can be used in conjunction with inexpensive permanent magnets. Currently, we are attempting to design a disposable chamber for the magnetic separation, thus eliminating the worry of viral or bacterial contamination of marrow occurring through the repeated use of the polycarbonate chamber.

The separation technique described depletes tumor cells from bone marrow to a level of at least 1:1000-1:10,000 nucleated cells in test systems using human neuroblastoma cell lines. However, extrapolation of results obtained on small-scale experiments and the use of cell lines may not reflect the true efficiency of any technique when it is used to remove fresh tumor cells from the large volumes of bone marrow used for autologous transplantation. In the latter situation, the indirect immunofluorescence technique which uses panels of monoclonal antibodies is the most sensitive approach available for detecting tumor cells. Here however, we feel that it is not possible to detect tumor cells at levels lower than 0.1%. Therefore, only long-term follow-up studies of patients receiving high-dose therapy with or without purged bone marrow will show the effectiveness of this approach to therapy.

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Optimal Elimination of Leukemia T Cells from Human Bone Marrow with T101 Ricin A-Chain Immunotoxin

G. Laurent, P. Casellas, A. Fauser, and F. Jansen

We investigated the elimination of malignant cells from bone marrow cell suspensions before autologous bone marrow transplantation (ABMT) by the use of immunotoxins (IT). An anti-T cell IT was prepared by coupling ricin A chain to the monoclonal antibody (MAb) T101 (T101 IT); the antibody binds to the T1 differentiation antigen expressed by normal T lymphocytes and most T cell-derived malignant disorders. The clinical value of the T101 (IT) for purging human bone marrow was evaluated with regard to (*a*) efficiency in killing leukemia cells, (*b*) optimal conditions for clinical use, and (*c*) toxicity to bone marrow stem cells.

EFFICIENCY OF REACTIVITY AGAINST LEUKEMIA CELLS

Appropriate experiments have enabled us to analyze the different phases in the mechanism of T101 IT's action on target cells. Specific fixation of T101 IT is identical to that of T101 MAb, whose affinity is very high (Ka = 10^{10}/M). Cytofluorometric analysis of binding to leukemic cell lines and to numerous fresh samples has confirmed this specific fixation. Cells negative for T101 fix neither the antibody nor T101 IT (1).

We studied inhibition of protein synthesis by measuring [^{14}C] leucine incorporation (1). A 50% reduction of CEM cells was obtained by exposing cells to 10^{-11} M of ricin A chain conjugated to T101 (Fig 1). In contrast, a 10^{-7} M A-chain concentration was necessary to obtain the same cell reduction. Higher concentrations of a nonspecific IT were also necessary to obtain this 50% reduction (Fig 1). With 10 mM NH_4Cl, the specific activity of T101 IT increased significantly so that to obtain a 50% inhibition, only 10^{-13} M was needed. This increased activity was the result of a considerable increase in the rate of protein synthesis inhibition (with NH_4Cl, 90% was inhibited in 5 h, whereas without NH_4Cl 60 h were necessary). According to a sensitive clonogenic assay for CEM cells, a log reduction in CEM colony-forming cells was obtained if cells were incubated with T101 conjugated with 10^{-8} M ricin and incubated with 10^{-2} M NH_4Cl.

OPTIMAL CONDITIONS FOR CLINICAL USE

Optimal conditions for in vitro treatment with T101 IT were defined using clonogenic assays for normal hematopoietic progenitor cells (2; NC Gorin et al., unpublished data).

The study of nonspecific target cells such as hematopoietic progenitor cells demonstrated a nonspecific cytotoxicity at concentrations of conjugated A chain greater than 10^{-8} M (Fig 2). This is in contrast to the effect of conjugated A chains on specific target cells (see Fig 1).

Cytoreduction of CEM cells was determined after different times of incubation with T101 conjugated A ricin. The optimum incubation time was 20 h (Fig 3). The role of cytoreduction of CEM cells incubated with T101 IT was determined at 8°C, 25°C, and 37°C. The optimum temperature appeared to be 37°C (data not shown).

Hematocrit and plasma concentration had no influence on the efficacy of T101 IT. Moreover, studies of fresh unseparated human bone marrow contaminated by CEM showed no modification in the cell death rate. Thus, with strict regard to the biological effect of T101 IT, bone marrow cell separation seems to be unnecessary. Furthermore, because of the very low toxicity of A chain no washing procedures are needed; this will greatly simplify bone marrow manipulation (NC Gorin et al., unpublished data).

Nucleated cell concentrations greater than 2×10^7/ml limit the effect of T101 IT.

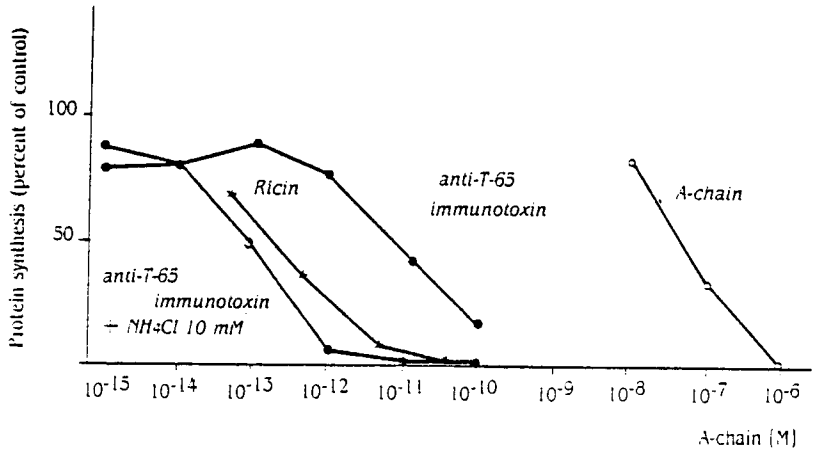

Figure 1. Effect of various immunological agents on protein synthesis in CEM cells. Anti-T-65 immunotoxin, T101 conjugated with ricin A-chain.

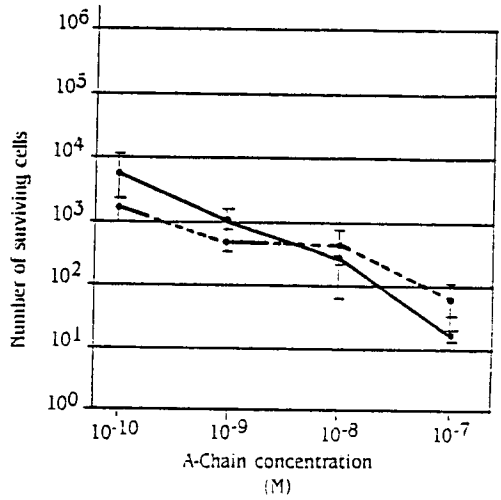

Figure 2. Effect of conjugated T101 ricin A chain on nonspecific, hematopoietic progenitor cells.

TOXICITY ON BONE MARROW STEM CELLS

Granulocyte-macrophage colony-forming units, erythroid burst-forming units (data not shown), and pluripotent hematopoietic stem cells (CFU-GEMM) assays were performed after 24-h incubation at 37°C in the presence of NH_4Cl (10^{-2} M) (P Casellas et al., unpublished data). These studies were carried out by Dr. Axel Fauser (Freibourg, Federal Republic of Germany). No toxicity was observed up to the concentration of 10^{-8} M (Figs 4 and 5) (2). The nonspecific toxicity observed of concentrations higher than 10^{-8} M appears to be linked to A-chain toxicity. Furthermore, it was shown that nonspecific cytotoxicity was not influenced by shortening the incubation time or modifying the incubation temperature to 25°C.

CONCLUSION

The data confirm that the use of T101 IT is a simple, highly efficient (4 logs of cytoreduction as assessed on clonogenic cell lines) and

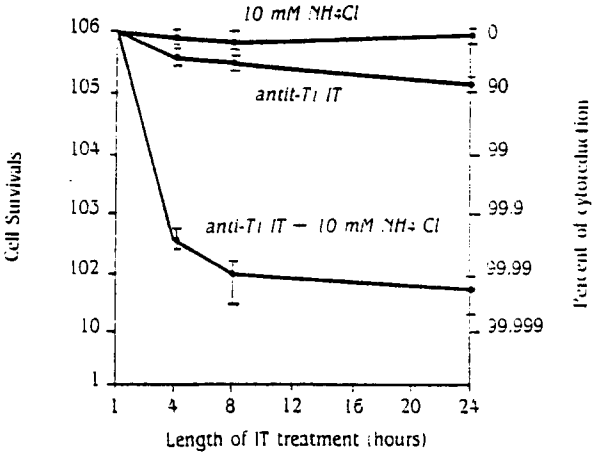

Figure 3. On the horizontal axis, the time of incubation with NH_4Cl, with anti-T_1 IT, or with anti-T_1 IT, and 10 mM NH_4Cl has been plotted; on the vertical axis, the cytoreduction (CEM cells). Note that incubation of CEM cells with anti-T_1 IT in the presence of NH_4Cl kills more than 99.99% of the cells, whereas only a one log reduction can be achieved by incubation with anti-T_1 IT alone. NH_4Cl incubation without antibody had no effect on CEM survival.

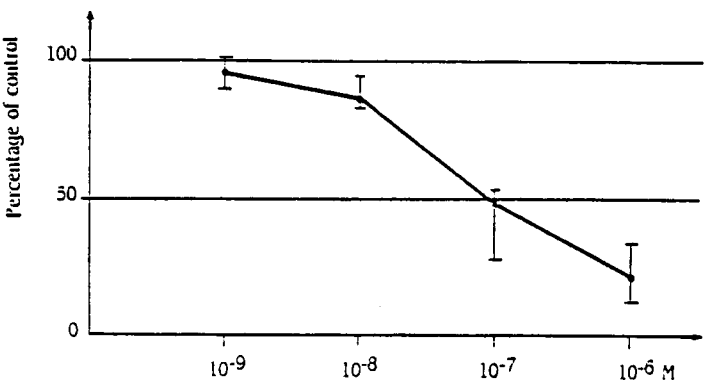

Figure 4. Influence of conjugated T101 ricin A chain on granulocyte-macrophage colony-forming units. The immunotoxin was incubated with CEM cells and 10^{-2} M NH_4Cl for 24 h at 37°C.

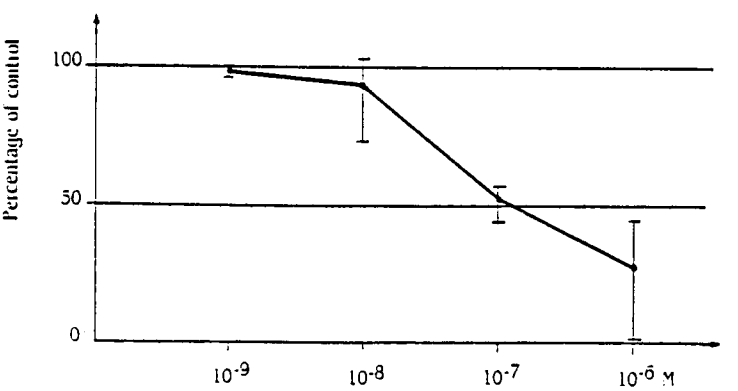

Figure 5. Influence of conjugated T101 ricin A chain of CFU-GEMM. The immunotoxin was incubated with CEM cells and 10^{-2} M NH_4Cl for 24 h at 37°C.

safe approach to the in vitro treatment of bone marrow before ABMT for patients with T-cell leukemias or lymphomas. Six patients have already been treated with T101 IT in a phase I pilot study and showed a normal hematological recovery (NC Gorin et al., unpublished data).

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Four Rat Cytotoxic Monoclonal Antibodies for the In Vitro Treatment of Bone Marrow Autografts in Non-T Non-B Acute Lymphoblastic Leukemias

A. M. LeBacq-Verheyden, Y. Humblet, A. Neirynck, A. Ravoet, and M. Symann

INTRODUCTION

Prolonged disease-free survival may be achieved in second remissions of acute lymphoblastic leukemia (ALL) by following high-dose cyclophosphamide therapy and total body irradiation with allogeneic bone marrow transplantation (1,2). In the absence of appropriate donors, autologous bone marrow transplants can be used, especially if the marrow has been cleared in vitro of detectable and undetectable leukemic cells. This purging has been attempted with drugs (3), with monoclonal antibodies and complement (4), and with monoclonal antibodies coupled to the A chain of ricin (5). Cocktails of monoclonal antibodies that bind to distinct membrane molecules can have a higher lytic efficiency than antibodies aimed at a single surface structure (6,7). The aim of this paper is to describe a cytotoxic cocktail of four rat monoclonal antibodies. AL2 and AL3 (8) define the common acute lymphoblastic leukemia antigen (CALLA) gp100; AL6 (description submitted for publication), like BA2 (9), binds to p24, while AL1a (description submitted for publication) labels a so far unreported heavy polymeric protein (p120, 220, 280) that is similar but not identical to the molecule (p30[?]) defined by BA1 (10).

MATERIALS AND METHODS

BA1 and BA2 were purchased from Hybritech. Rat monoclonal antibodies were prepared, characterized, and purified as described earlier (8). For cytotoxicity, rat antibodies and control IgG2b, IgG2c and IgM were extensively dialyzed against phosphate-buffered saline, diluted to 0.4 mg/ml in human antibody serum, and stored at -80°C.

The diagnosis of non-T non-B acute lymphoblastic malignancy rested on classical bone marrow morphology, as well as upon complementary immunological analyses (8). All samples with at least 85% leukemic blasts were cryopreserved in liquid nitrogen. After thawing, cell viability and blast counts were above 85%. The Nalm-6 leukemic cell line was cultured as has been described (8).

Indirect immunofluorescence with rat and mouse monoclonal antibodies was done as previously described (8). Tests were repeated several times, and the percentages of labeled cells were averaged.

Microcytotoxicity was performed as previously described (11). In brief, 2 x 10^3 cells were incubated for 30 min at 20°C in 2 µl of minimum essential medium (MEM) containing 20 mM HEPES (pH 7.2), 10% human antibody serum, and various amounts of purified rat monoclonal antibodies or immunoglobulins. After 1 h incubation at 20°C with 4 µl of locally prepared rabbit complement (11), dead cells were revealed by eosin uptake.

Granulocyte-macrophage colony-forming units (CFU-GM) and erythroid burst-forming units (BFU-E) were determined as previously described (12, 13). Culture conditions for mixed hematopoietic colonies (CFU-GEMM) were as described by Fauser et al (14).

RESULTS AND DISCUSSION

Results of immunofluorescence assays are summarized in Table 1. On average, 77-86% of cells were labeled in positive samples by each antibody; 92-96% of samples reacted with BA1/AL1a and BA2/AL6; 76% of samples

Table 1. Binding of monoclonal antibodies to 25 non-T non-B acute lymphoblastic leukemias

Diagnosis[1]	Number positive[2] / Number tested with				
	BA1	ALla	BA2	AL6	AL2, AL3
Null ALL	5/6	5/6	6/6	6/6	0/6
Common ALL	16/16	16/16	14/16	14/16	16/16
Lymphoid AUL	2/2	1/2	2/2	1/1	2/2
CML Lymphoid blast crisis	1/1	1/1	1/1	1/1	1/1
Total (% positive samples)	24/25 (96%)	23/25 (92%)	23/25 (92%)	22/24 (92%)	19/25 (76%)
Mean ± SEM % of fluorescent cells in positive samples	83.1 ± 15.1	77.3 ± 23.6	80.6 ± 13.7	77.1 ± 16.1	86.0 ± 11.8

(1) ALL: Acute lymphoblastic leukemia; AUL: Acute undifferentiated leukemia; CML: chronic myeloid leukemia
(2) Positive samples contain at least 20% of fluorescent cells

Table 2. Complement-Mediated Cytotoxicity on Hematopoietic Colony-Forming Cells

Pre-treatment	CFU-GEMM (number)	BFU-E (%)*	CFU-GM (%)*
Culture medium	5	100(1)	100 (2)
C'(3)	4	96.6	82.6
C' + AL1	4	74.0	101.5
C' + AL2 + AL3	3	69.4	83.8
C' + AL6	2	98.4	97.3
C' + AL1a + AL2 + AL3 + AL6	5	87.3	94.5
C' + HL1(4)	0	0.6	0

Note: *CFU-GEMM, human pluripotent stem cell; BFU-E, erythroid burst-forming unit; CFU-GM, granulocyte-macrophage colony-forming unit.*

**Percentage recovery.*

(1) *141.8 ± 9.7/1.5 x 10^5 cells (mean ± SEM).*

(2) *117.4 ± 10.8/2 x 10^5 cells (mean ± SEM).*

(3) *C', rabbit complement.*

(4) *HL1, anti-HLA class I rat monoclonal antibody.*

were labeled by AL2 and AL3, the two anti-CALLA reagents; and only one null ALL cell sample exclusively bound BA2/AL6. Cocktails of 2, 3, or 4 rat monoclonal antibodies might be prepared in accordance with the leukemic phenotype.

Purified antibodies were titrated in the presence of complement on Nalm-6 cells, which express considerable amounts of all three antigens. Control titration curves performed in the presence of complement with purified IgG2b, IgG2c, and IgM yielded less than 5% background killing. By contrast, 100% of cells were killed with minimal doses of 0.02 µg/ml AL3 (an IgM), 0.2 µg/ml AL6 (an IgG2a or IgG2b), and 2 µg/ml AL2 (an IgG2b). As much as 200 µg/ml AL1a (an IgG2c) were required to achieve a 90% lysis, indicating that AL1a was intrinsically less cytotoxic than the three others. When the cocktail of four monoclonal antibodies was titrated in the presence of complement, 0.02 µg/ml of total antibody (i.e., 0.005 µg/ml of each individual antibody) did as well as 0.02 µg/ml of the best antibody. Synergy between two or more antibodies could not be demonstrated when one antibody sufficed to kill the entire leukemic population. However, in limiting conditions, for example when using human rather than rabbit complement, a synergy between two or more antibodies could be observed. Clearly, the gain of cytotoxicity expected from combinations of antibodies needs to be critically evaluated on a series of leukemic samples.

The results of complement-mediated cytotoxicity on hematopoietic progenitor cells are shown in Table 2. Cultures preincubated with medium yielded the 100% recovery values. No significant reduction was observed after incubation with complement alone or with complement and AL1a, AL2, AL3, and AL6; however, incubation with HL1 and complement

inhibited colony formation, thus demonstrating that progenitor cells expressing human leukocyte antigen class I antigens had been efficiently killed. The data show that the cocktail of AL1a, AL2, AL3, and AL6 does not prevent the in vitro development of CFU-GEMM, BFU-E, and CFU-GM. A full hematopoietic recovery was already observed in three of four patients who had received a bone marrow transplant purged with AL2 plus AL3 and complement (15); further eligible patients will be transplanted with autografts treated in vitro with AL1a, AL2, AL3, AL6, and nontoxic rabbit complement.

ACKNOWLEDGMENTS

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Bone Marrow Transplantation after In Vitro Purging with T101 Ricin A-Chain Immunotoxin: A Phase I Clinical Study

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INTRODUCTION

T101 immunotoxin (CLIN-MIDY/SANOFI) is a hybrid molecule coupling the A chain of ricin to the T101 monoclonal antibody (T101 IT). T101 reacts with the T65 antigen present on mature T cells and most malignant T cells (1). Specific fixation of T101 IT allows the incorporation into the target cells of ricin A chain, which inhibits protein synthesis on ribosomes and induces arrest of cell division and cell death (2).

T101 is not expressed on hematopoietic precursors (3); therefore, T101 IT can be used in bone marrow transplantation (BMT) for purging to remove malignant T cells before autologous bone marrow transplantation (ABMT) or normal T cells before allogeneic BMT.

METHODS AND PATIENTS

T101 IT Bone Marrow Purging

Optimal conditions for clinical use of T101 IT have been previously defined (4; P Casellas et al., unpublished results; see the chapter "Optimal Elimination of Leukemia T Cells from Human Bone Marrow with T101 Ricin A-Chain Immunotoxin"). In brief, unprocessed marrow cells were adjusted, if necessary, to a final concentration of 1.2×10^7 cells/ml. T101 IT was added to the marrow cells at a final concentration of 10^{-8} M of A chain in the presence of NH_4Cl (10^{-2} M) for 18-24 h at 37°C, with constant gentle shaking.

For allogeneic transplantation, treated bone marrow cells were directly transfused, without washing, after incubation. For ABMT, treated bone marrow was processed on a Hemonetics V50 before being frozen in dimethyl sulfoxide and plasma. Later, bone marrow was rapidly thawed in a water bath and immediately transfused to the patient.

Treated and untreated marrow cells were separated by centrifugation and buffy-coat cells were subsequently removed. Cells at 2×10^5 were plated in triplicate in soft agar with an optimal dose of human placenta-conditioned medium. Colonies with more than 50 cells were scored after 14 days' incubation in a fully humidified atmosphere of air and 4-5% CO_2.

Indirect immunofluorescence assay was performed with a panel of anti-T monoclonal antibodies and FITC-conjugated goat anti-mouse antibody.

Conditioning Regimens and Supportive Care

All patients (Table 1) received fractionated total body irradiation (TBI) of 2.2 Gy/day for 5 days with lung shielding after a lung dose of 8 Gy. TBI was done with a linear accelerator (Saturne) at a low-dose rate (< 5 cGY/min).

Patients 2 and 3 received 120 mg/kg cyclosphosphamide (Cy) with the TBI. Patient 1 received, in association with Cy-TBI, VP-16-213 (150 mg/m²) and cytarabine (500 mg/m²) per day in continuous infusion for 5 days. Patient 4 received 140 mg/m² melphalan in association with TBI. Recipients of allogeneic BMT (human leukocyte antigen fully-matched siblings) had, in addition to T-cell depletion, conventional prophylaxis of graft-versus-host disease (GVHD) with methotrexate (patient 3) or cyclosporin A (patient 4).

Table 1. Patient Data

Pt n°	Age	Sex	Diagnosis	Status Preparative at BMT regimen	BMT	NC/kg x 10^8	Days to recovery of Gr >500	Plts >50000	OUTCOME
1	14	M	T-ALL	2nd CR VP16 AraC Cy TBI	Auto	1.57	24	39	Alive d90+
2	42	M	T-ALL	1st CR Cy TBI	Auto	1.35	12	27	Alive d45+
3	22	M	H D Stage IV BM	P.D. Cy TBI	Allo	2.14	43	90	Deadsepsis d 90 GVH III
4	6	M	ALL	5th CR HDM-TBI	Allo	1.5	18	25	Alive d65+ GVH 0

Note: *M, male; BM, bone marrow; PD, progressive disease; CR, complete remission; HDM, high-dose melphalan; NC, nucleated cells; Gr, granulocytes; Plts, platelets; d, day; HD, Hodgkin's disease.*

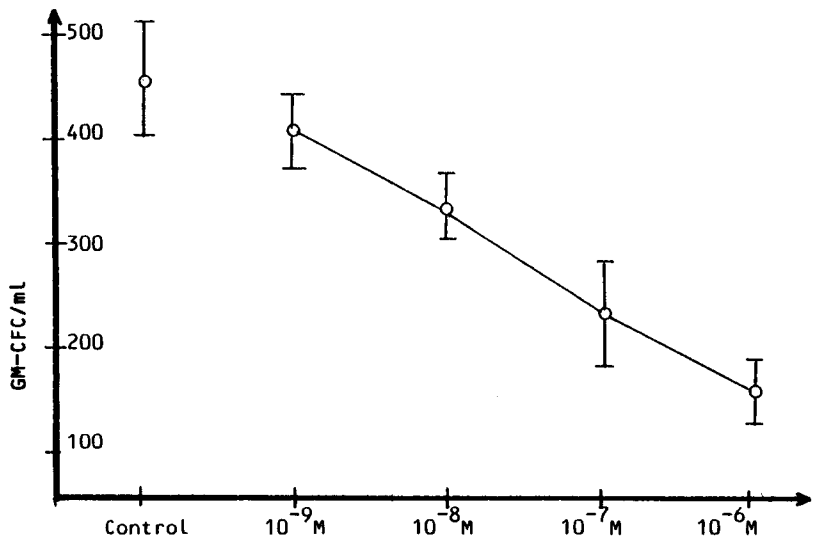

Figure 1. Effects of 24-h incubation of normal marrow cells with different concentrations of immunotoxin in the presence of NH_4Cl (10^{-2} M).

Table 2. Results of Ex Vivo Treatment of Patients' Bone Marrow

	COLLECTED			STORED			TRANSFUSED		
Pt n°	Nucl. cells x 10^9	GM-CFC x 10^4	T65 positive cells	Nucl. cells x 10^9	GM-CFC x 10^4	T65 positive cells	Nucl. cells x 10^9	GM-CFC x 10^4	T65 positive cells
1	12.9	19.3	6%	5.7	17	<1%	4	10.8	–
2	21.8	32.7	37%	10.5	10.5	<1%	9.6	5.7	–
3	20	90	–	–	–	–	12.6	38	–
4	5	NE	46%	–	–	–	3	NE	< 1%

Three of four patients were treated in laminar air-flow rooms after gut decontamination. One patient (patient 4) underwent transplantation in a conventional hospital room.

Two patients (patients 1 and 2) had high-risk T-cell acute lymphocytic leukemia (T-ALL) with hyperleukocytosis and tumor masses at diagnosis. Durations of the last complete remissions (CRs) before ABMT were 7 and 11 months, respectively.

The two recipients of allogeneic BMT had poor prognostic factors (refractory Hodgkin's disease and ALL in fifth CR).

RESULTS

Results are shown in Fig 1. In the presence of NH_4Cl (10^{-2} M), after 24-h incubation at 37°C, no major toxicity was observed up to a concentration of 10^{-8} M of A chain. At this concentration, granulocyte-macrophage colony-forming unit (CFU-GM) recovery was slightly decreased (73% of controls). Furthermore, nonspecific T101 IT toxicity appears to be linked to A-chain toxicity. These findings confirm others (4).

CFU-GM assays carried out on the whole bone marrow showed acceptable recovery (Table 2). Immunofluorescence studies demonstrated that less than 1% of T65-positive cells were transplanted to the patients.

Three of the patients were febrile during marrow transfusion (patients 1, 2, and 4). No other side effect was observed. All the patients engrafted had full reticulocyte, granulocyte, and lymphocyte recoveries. Patient 3, despite megakaryocyte engraftment, had prolonged thrombocytopenia (see Table 1).

DISCUSSION

These results confirm that T101 IT can be used to purge bone marrow without major damage to hematopoietic progenitors (NC Gorin, L Douay, JP Laporte et al., unpublished observations). Clinical feasibility of the method is demonstrated by the hematological recovery of the patients. However, additional prospective studies are needed to assess reproducibility and efficacy of T101 IT bone marrow purging.

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The Effect of Cryopreservation and of Purging with Asta Z 7557 on Pluripotent Stem Cells of the Mouse and Human Granulocyte-Macrophage Colony-Forming Units

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INTRODUCTION

The successful outcome of autologous bone marrow transplantation (ABMT) depends on both the in vivo eradication of the tumor prior to transplantation and the elimination of minimal residual clonogenic leukemic cells that contaminate the remission marrow (1). Sharkis et al (2) have reported an in vitro method in a rat model for purging bone marrow of leukemia cells without impairing the bone marrow's hematopoietic repopulating potential by means of a congener of cyclophosphamide, 4-hydroperoxycyclophosphamide (4-HC). Clinical studies with human ABMT and data from animal models (2) show that marrow cell suspensions treated in vitro with 4-HC retain their ability to restore hematopoiesis after total body irradiation despite the absence of committed progenitor cells, which are granulocyte-macrophage colony-forming units (CFU-GM) (3) and erythroid burst-forming units (BFU-E) (4) in the suspensions.

Recent reports have shown that in vitro chemical manipulations of marrow cell suspensions with 4-HC or Asta Z 7557 decrease the human multipotential hematopoietic stem cell (CFU-GEMM) content as well as the CFU-GM and BFU-E content (4). These recent data suggest that the CFU-GEMM is unlikely to be the cell capable of repopulating the hematopoietic system in ABMT when the marrow cells have been purged chemically with 4-HC.

The sensitivity of the pluripotent stem cell, or spleen colony-forming units (CFU-S), to the cyclophosphamide derivatives can be tested in mice by the spleen colony assay (5). Recently Magli et al (6) reported the interesting observation that in this widely used assay, colonies of days 7 and 8 were mainly erythroid and lacked primitive precursors, whereas 11- and 12-day-old colonies had several multilineage cells and contained primitive multipotential precursors. Accordingly, we have studied the effect on day 8 and day 12 CFU-S of a newly derived cyclophosphamide analogue named Asta Z 7557. The results indicate that in the murine model Asta Z 7557 has a selective toxic effect on committed progenitor cells, whereas it spares the pluripotent stem cells.

MATERIALS AND METHODS

NMRI female mice, 10-14 weeks old, were used throughout the study. Asta Z 7557 was kindly supplied by Asta-Werke AG, Degussa Pharma Gruppe (Bielefeld, West Germany).

The bone marrow suspension was incubated for 30 min at 37°C in a water bath with graded doses (12-100 μg/ml) of Asta Z 7557. Control suspensions were incubated in the same way without adding the drug. The cells were then washed three times with TC-199 medium. Part of the suspension was injected intravenously into lethally irradiated recipients to assay the surviving CFU-S, and part was plated in agar for the CFU-GM assay. Spleen colonies were evaluated on day 8, as described by Till and McCulloch (5), and pluripotent stem cells were evaluated on day 12, as described by Magli et al (6). Statistical analyses were performed using Student's t-test.

After incubation with Asta Z 7557, as described above, the cell population was mixed at +10°C with an equal volume of the cryopreservation medium containing 20% dimethyl sulfoxide, 40% serum antibody, and 40% Hanks' balanced salt solution. Two milliliters of the final cell suspension were transferred to polypropylene screw-cap sterile vials. The vials

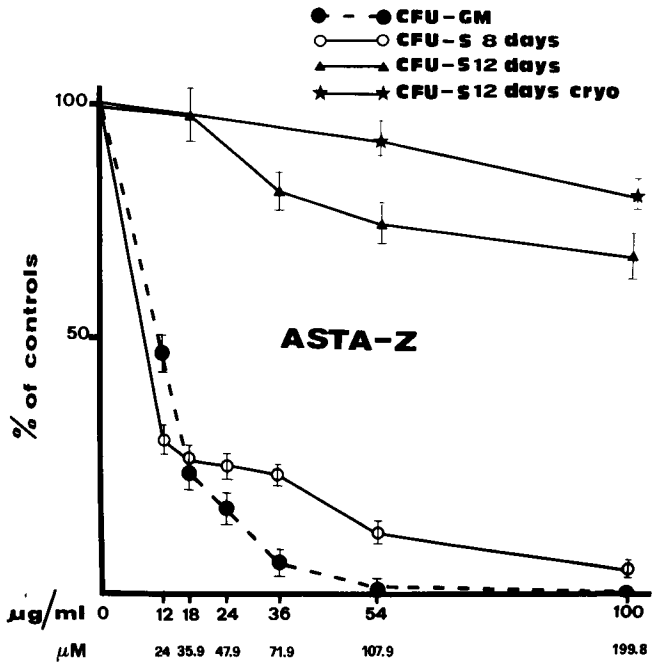

Figure 1. Effect of cryopreservation and increasing doses of Asta Z 7557 on survival of granulocyte-macrophage colony-forming units (CFU-GM) and early and late spleen colony-forming units (CFU-S). Values are the means (+ SE) from three to six experiments. CFU-S and CFU-GM are derived from spleen colony assays in 22-31 surviving recipient mice. Results are expressed as percentages of values from control suspensions.

were then transferred to a Nicool biologic electronic freezing unit set at a programmed freezing rate. Our standard freezing schedule was as follows: starting temperature 10°C; liquid cooling rate 1°C/min, start of phase change -4°C, 5°C/min; third-phase freezing rate 7°C/min, end cycle -140°C. At the end of the freezing cycle, the vials were rapidly placed into a liquid nitrogen container at -196°C. Vials removed from the liquid nitrogen were rapidly thawed in a 37°C water bath and tested for nucleated cell numbers after the supernatant was washed.

RESULTS AND DISCUSSION

Results are shown in Figs 1 and 2. The survival of CFU-S after marrow purging is of great importance to the transplant procedure. However, in humans it is only possible to quantify the CFU-GM, BFU-E, and CFU-GEMM cells. The present results indicate that the murine model may be useful in evaluating the response of CFU-GM, BFU-E, and CFU-S to cytotoxic drugs in vitro. A modest dose of Asta Z 7557 (12 µg/ml) caused more than 50% inhibition of CFU-GM. With an increase to 100 µg/ml, a dose used in clinical practice, no colony formation was observed, and similar results were noted in the day 8 CFU-S assay.

In contrast, the day 12 CFU-S were relatively resistant to Asta Z 7557 treatment at the highest dose tested (100 µg/ml); 60.7% of the cells were detected by the 12-day colony assay.

Recent studies have shown that marrow cell suspensions devoid of up to 85% of CFU-GM and approximately 99% of BFU-E and CFU-GEMM cells retain their marrow repopulating potential (3). These data suggest that the CFU-GM, BFU-E, and CFU-GEMM cells are not responsible for in vivo marrow reconstitution after myeloablative treatment (4). The marrow regeneration probably results from a more primitive stem cell that cannot be measured

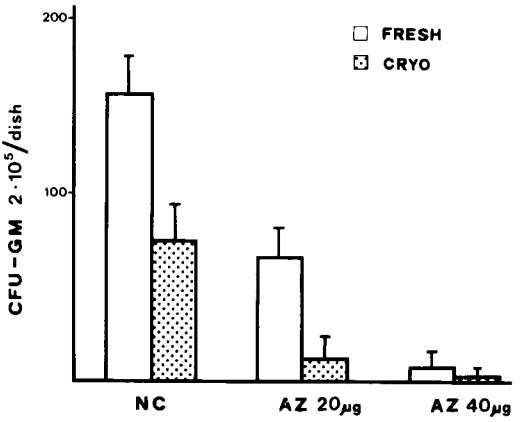

Figure 2. Effect of cryopreservation and Asta Z 7557 (AZ) on survival of human granulocyte-macrophage colony-forming units (CFU-GM). Values are the means (± SE) from 10 experiments. Results are expressed as colonies per plate. NC, no chemotherapy.

by present techniques. Day 12 CFU-S were slightly affected by the freezing-thawing procedures, whereas this procedure can account for an additional 60% decrease of the CFU-GM cells.

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Colloidal Immunomagnetic Fluids for Cell Separation

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C. L. Reading, and K. A. Dicke

INTRODUCTION

The concept of a magnetic fluid has intrigued scientists since the 18th century, but attempts to make one by heating various magnetic materials above their melting points have been unsuccessful because the Curie temperature of all known magnetic materials is below that of the melting point. Work begun in the 1960's (1) showed that magnetic particles made small enough would remain in colloidal suspension. Early magnetic fluids were composed of magnetite particles suspended in various surfactants, usually organic solvents. Such colloidal magnetic fluids exhibit a number of interesting properties, in particular, superparamagnetism, which is the rapid response and return to a nonmagnetic state after the external stimulus is withdrawn, a property that has been exploited in a number of industrial applications (2).

From a biological standpoint, the most interesting property of colloidal particles (magnetic or nonmagnetic) is their ability to bind surfactant molecules to their surface, virtually irreversibly. This binding has been exploited with colloidal gold or silver, both of which have been used as particulate markers for electron microscopy since the 1960's (3). The binding is due to the ability of biologic macromolecules (such as polysaccharides and proteins) to alter their conformation and hence distribute their electrical charge heterogeneously, so that they bind strongly to the surface of the colloidal particles. The binding of the surfactant (in this case, protein) to the surface of the particle is essentially electrostatic and depends on the forces at the surfaces involved (both the surface tension and the zeta potential at the interface). The interactive forces involved between particles in colloidal suspension can be defined by the DLVO theory (4).

To prevent dissociation effectively, we have exploited this surfactant ability by using human albumin adsorbed around colloidal cobalt/magnetite particles, which is then crosslinked with glutaraldehyde to form a cage, as it were, of albumin around each particle. Immunoglobulins (monoclonal or polyclonal) are then covalently bound to the albumin with p-benzoquinone (5), thus creating the final immunomagnetic fluid. Figure 1 shows the relationships of the magnetic colloid particle, the carrier protein (human albumin), and the antibody.

Immunogold has been shown to be a highly specific immunologic marker for microscopy (6) and electron microscopy, so that even antigen-binding sites can be quantitated (7). One of the inherent advantages in a colloidal system is the high surface area-to-volume ratio of the particles, which allows a potentially high concentration of antibody to be presented to the reactive cells. The development of methods to maximize the magnetic gradients without having to resort to extremely high magnetic fields allows efficient separation of colloid-coated cells. Using the principle of high gradient magnetic separation, even deoxygenated RBCs have been retained in a magnetic separation (8).

MATERIALS AND METHODS

Preparation of the Immunomagnetic Colloid

The detailed preparation of the immunomagnetic colloid is the subject of another report (Poynton and Reading, manuscript in preparation). In brief, magnetite particles are formed from ferrous and ferric chlorides in alkaline solution (9) and dispersed to form a colloidal suspension in tetramethylammonium hydroxide. This is added to a human albumin solution

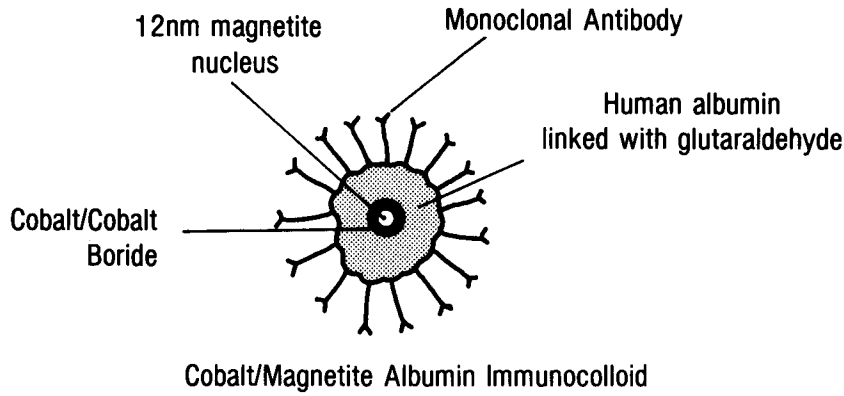

Figure 1. Diagrammatic representation of an individual colloidal cobalt/magnetite particle (core diameter, approximately 40 nm) surrounded by cross-linked albumin and antibody.

and a cobalt chloride, which forms a shell around the magnetite when it is reduced with sodium borohydride. The albumin is crosslinked around the particles with glutaraldehyde, and antibody is bound to the albumin with *p*-benzoquinone.

Magnetic Separation of Cells

An equal volume of immunomagnetic colloid (sterile-filtered through a 0.22-μm filter) is added to the cells in phosphate-buffered saline (PBS) pH 7.4 (PBS 7.4), so that the final cell concentration is between 1 and 2 x 10^8/ml. After a 30-min incubation at 4°C with rocking, the cells are washed and resuspended in PBS 7.4 and passed through the magnet at a rate of 2 ml/min. The magnetic system is shown in Fig 2. The first stage of the system (produced by Crucible Magnetics, Elizabethtown, KY) consists of 11 alternating pole face cobalt/samarium magnets interrupted by steel pole pieces with concave edges. The second stage uses fine paramagnetic wire in the tube in which magnetism is induced by an externally applied cobalt/samarium magnet.

Detection of Cells

Experiments were done in which rhesus-positive RBCs were first incubated with an IgG human anti-D, washed, and then incubated with either a goat anti-human magnetic colloid or a goat anti-mouse magnetic colloid as a control. Emerging cells were counted in a hemocytometer or on a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

Other experiments were done in which equal numbers of RBCs and K562 cells were mixed and added to a total concentration of 10^8/ml. The K562 cells were then removed magnetically after incubation with a CF-1 antibody magnetic colloid. The CF-1 monoclonal antibody (supplied by Centocor, Inc., Malvern, PA) reacted with approximately 100% of the K562 cells. Emerging cells were detected by light microscopy. After counting, the RBCs were lysed, leaving the K562 cells to be counted alone after further concentration by centrifugation.

RESULTS

We have attempted to characterize each stage of the immunocolloid formation. Although the particles of the cobalt magnetite immunocolloid

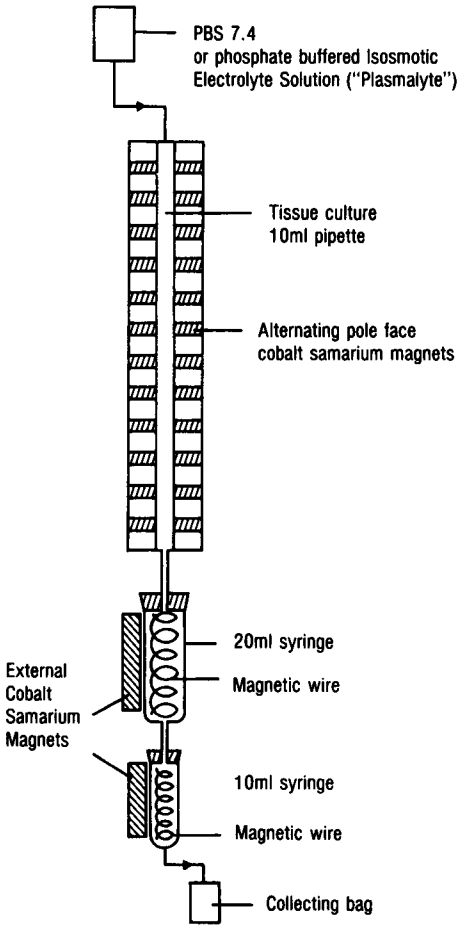

Figure 2. Flow system used for high gradient magnetic separation of immunomagnetic colloid-coated cells. All magnets are cobalt/samarium. The first stage removes the bulk of the positive cells, while the second and third stages act as fine magnetic filters to remove any remaining positive cells.

are irregularly shaped, they are approximately spherical. Their sizes range from approximately 20-60 nm. Emission spectroscopy analysis shows a ratio of Fe:B:Co:Cr of 32.4:1.3:1:0.48 by weight. In molar terms, the amount of boron present is too high to be accounted for entirely by cobalt boride, and other borides such as iron boride are, therefore, likely to be present.

The binding of ^{125}I-labeled antibody (IgG fraction goat anti-mouse immunoglobulins) to the albumin around the magnetic particles was also studied. Over a wide range of immunoglobulin concentrations greater than 50% of the benzoquinone-activated antibody was bound to the colloid until it reached greater than 1 mg/ml. The concentration of the albumin on the colloid was presumably limiting. Dissociation of antibody from the surface of the particles was also analyzed. The conjugate, which was stable over a 3-month period, was kept sterile at 4°C, without the colloid settling out or the antibody dissociating. The results were comparable to those with colloidal gold, in which approximately 90% of the antibody remained bound after storage for extended periods of time. Using these immunocolloids and the high gradient magnetic separation depicted in Fig 2 and discussed below, we achieved as much as a 4-log reduction in antibody-positive cells. (See Table 1 for some of the data.) However, these cell separations are somewhat artificial in that they do not reflect the cell

Table 1. In Vitro Cell Separation Data

Cells and Immunomagnetic Colloid	Cells Passing Through Magnet	% Cell Removal or Recovery
A. 1.78×10^{10} Rhesus-positive RBCs coated with human anti-B:		
Goat anti-human colloid	$4.4 \pm 0.34 \times 10^{5}$/ml	99.99753 ± 0.00019
Goat anti-mouse colloid (control)	$1.19 \pm 0.48 \times 10^{10}$/ml	67 ± 27
B. 5×10^{9} K562 + 5×10^{9} RBC: CF-1 monoclonal antibody colloid		
K562:	$2.68 \pm 2.58 \times 10^{7}$	99.465 ± 0.515
RBC:	$3.2 \pm 0.85 \times 10^{9}$	64 ± 17

Mean of 10 separation experiments in each case. See text details.

Table 2. Initial Clinical Separations

	Patient 1	Patient 2	Patient 3
J5 Positive Cells			
Before - Fraction 1[a]	4	8	12.2
After	4	1	1.6
Before - Fraction 2	4	8	12.2
After	0	1	1.8
Before - Fraction 3	4		
After	0		
GM-CFC			
Before	59.5	15.5	20
After - (i)	103.5	13	148
After - (ii)	77.5	78.5	
BFU-E			
Before	17.5	3	0
After - (i)	31.6	1	8.5
After - (ii)	30	3.5	
Days to			
1,000/μl granulocytes	22	40	28
100,000/μl platelets	23	35	30

[a] Bone marrow samples separated into 2 or 3 portions prior to separation.

situation in a clinical bone marrow buffy-coat sample in which the total concentration, including RBCs, may be more than 10^{12}/l.

Data from our pilot clinical study, in which we assessed any toxicity of the procedure, are shown in Table 2. This purging of bone marrow technique (i.e., using J5 antibody and a goat anti-mouse colloid) was used in three patients with acute lymphoblastic leukemia in second and subsequent remissions (10). Detection of residual leukemic cells was by

immunofluorescence. The patients then received high-dose cyclophosphamide/1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU)/VP-16-213, followed by autologous bone marrow transplantation. Three patients reengrafted promptly with no evidence of toxicity from this procedure.

DISCUSSION

The magnetic force (Fm) on an individual colloid particle is small and for a spherical particle (D) with magnetization, I_s in a field gradient dH is given by $Fm = I_s \times dH\ \pi D^3/6$.

Thus, cells rendered magnetic by colloid on their surface will experience a magnetic force in the z direction when placed in a magnetic field B_o, with field gradient dB_o/dz, according to the following equation:

$$Fm = (\chi\ V/\mu_o)B_o(dB_o/dz)$$

where χ is the magnetic susceptibility of the cell; V its volume; and μ_o, the permeability of free space.

It is clear that the magnetic gradient (dB_o/dz) is an important factor in imparting a velocity to the magnetic cells. We attempted to design a permanent magnet that incorporated some of these features (Fig 2). The magnetic pole pieces focus the magnetic gradient into the tube lying in the inferior groove. Gradients are also induced along the fine paramagnetic wire in the second stage.

Biological applications of magnetic fluids are in their infancy because biological compatibility and lack of toxicity are only now being fully established. The development of nontoxic immunomagnetic fluids may, therefore, be useful as cell surface markers and for cell separation in clinical bone marrow transplantation (11). Further improvements to the stability of our magnetic fluids will need to be made before full-scale clinical studies can be done with reproducible results.

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Purging of Bone Marrow with Immunomagnetic Beads: Studies with Neuroblastoma as a Model System

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INTRODUCTION

Selective purging of cell populations from marrow may be necessary to remove alloreactive cells (for allogeneic transplantation) or malignant cells (for autologous transplantation). A number of materials and methods for selective removal of cells from marrow have been reported, including monoclonal antibodies and complement (1,2), lectin agglutination (3), immunotoxins (4), cytotoxic drugs (5,6), and physical separation by equilibrium centrifugation (7,8). Recently, a novel approach to physical separation has been reported in which monoclonal antibodies are used to attach magnetic microspheres, or magnetic colloid, to the target cells, which are then removed by magnets (9,10).

We have established a model system to study methods for purging bone marrow. Neuroblastoma offers several unique properties that make it ideal for study as a model system. Especially attractive are the intracellular and extracellular antigens of neuroblastoma, which are not found in normal marrow, permitting sensitive detection of residual cells after purging (11). Furthermore, the improvement in survival seen in neuroblastoma patients treated with intensive therapy and marrow transplant should promote an increase in the use of this type of therapy for patients with widespread disease (12-14). Use of autologous marrow in treating such patients will require purging of tumor cells from the marrow (15,16).

The model system we have devised involves seeding a known number of premarked neuroblastoma cells into normal bone marrow (16). Premarking the cells with a supravital DNA stain allows sensitive detection of as few as one viable tumor cell per million marrow cells (11). For detection of cells in actual clinical marrow samples, a sensitive immunoperoxidase assay is employed that can detect as few as 1 neuroblastoma cell per 100,000 marrow cells (17).

We have used both detection methods to study the purging of neuroblastoma cells from bone marrow with immunomagnetic beads and monoclonal antibodies. The sensitive quantitation of cell removal that is possible using this model system has allowed us to determine the effect of small changes in the procedure on the efficiency of immunomagnetic purging. We review here the effects of various parameters on immunomagnetic purging, including the quantity of antibody bound to the target cells, the number of immunomagnetic beads per tumor cell used, and the use of one or two cycles of treatment.

MATERIALS AND METHODS

Human neuroblastoma cell lines used in this study were established in the laboratories of the authors (5,18) or were obtained from the originators of the cell line (19). Cell lines were maintained in RPMI 1640 supplemented with 15% fetal calf serum. Monoclonal antibodies 390 (20) and HSAN 1.2 (21) were purified from tissue culture supernatant or mouse ascites using staphylococcal protein A affinity chromatography. Antibodies 459 (22) and RB 21-7 were purified by precipitation with ammonium sulphate and then size filtration through Sephacryl S-300. Antibody preparations were assessed for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Purified antibodies BA-1 and BA-2 (23,24) were provided by Hybritech, Inc., and purified Leu-7 (HNK-1) (25) was purchased from Becton-Dickinson.

These monoclonal antibodies have been characterized as follows: 390, antihuman Thy-1 (20); 459, antihuman fetal brain (22); HSAN 1.2, antihuman neuroblastoma (21); RB 21-7, antirat fetal brain; BA-1 and BA-2, anti-B cell lineage (23,24), which also bind to neuroblastoma (26,27); Leu-7, antinatural killer antibody (25), which also binds to myelin-associated glycoprotein (28) and to neuroblastoma (26). Analysis of the binding of these antibodies to neuroblastoma cells has been presented elsewhere (16,26). Antibodies 390, 459, HSAN 1.2, and RB 21-7 do not bind to normal human bone marrow (16,20-22). Antibodies BA-1 and BA-2 bind to cells in normal marrow but are known from laboratory and clinical data not to bind to hematopoietic stem cells (2,23). Antibodies in these experiments, whether used as single antibodies or in a cocktail, were used at saturating concentration. Determination of antibody binding and titration of the monoclonal antibodies were primarily by flow cytometry, as described elsewhere (16,26).

Polystyrene magnetic beads (prepared from styrene-divinyl benzene polymer, 3 μm in diameter, magnetite content corresponding to 27.4% by weight of iron) were prepared as previously described (29,30). Affinity purified goat antimouse immunoglobulin (GAM) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a concentration of 200 μm/mg of beads, was incubated with the beads (1 mg/ml) in 0.3 M phosphate buffer (pH 7.4) on a rotating wheel at 22°C for 18 h. Unbound GAM was removed by washing (15,16).

Normal human bone marrow was obtained from vertebral bodies and cryopreserved (31), thawed, and the viable mononuclear cells separated by density centrifugation with Ficoll-Hypaque (32). Neuroblastoma cells, which had been marked with the viable DNA stain Hoechst 33342 (H342), were then seeded into the marrow (11,33). Neuroblastoma cells seeded into marrow included single cells and small-to-moderate-sized clumps, thus accurately simulating metastatic neuroblastoma in the marrow. The premarking of the tumor cells with H342 allowed accurate quantitation of the number of tumor cells in the marrow, before and after immunodepletion. The fluorescence of the H342-labeled cells is sufficiently bright to allow detection of one marked tumor cell per million marrow cells. Counterstaining the tumor-marrow mixture with the vital dye trypan blue limits the detection of tumor cells to only viable cells, because trypan blue quenches the nuclear H342 fluorescence (11). Quantitation of tumor cells in marrow from patients was done by immunoperoxidase staining using rabbit antiserum to neuron-specific enolase and monoclonal antibodies to cell surface antigens (17). This method accurately detects 1 tumor cell per 100,000 marrow cells.

For purging experiments, 50 or 100 million bone marrow cells were seeded with 10 to 20% H342 stained neuroblastoma cells. The cell mixture was incubated in monoclonal antibodies for 1 h at 4°C, washed twice in RPMI 1640 and 10% fetal calf serum, and mixed with GAM-coated beads (200/tumor cell) for 1 h at 4°C on a rotating wheel. The sample was then diluted threefold and passed over 15 x 48-mm samarium cobalt magnets (Edmunds Scientific, Barrington, NJ) to retain free beads and bead-coated cells. Quantitation of myeloid stem cells (CFC-GM) was done after 10 days' growth in agar with leukocyte-conditioned medium (34).

RESULTS

The immunomagnetic purging procedure is outlined in Fig 1. The system uses an indirect attachment of beads to the cells. Target cells are first coated with antibodies that do not bind to hematopoietic stem cells. After washing to remove excess antibody, magnetic beads that have GAM attached are incubated with the marrow. The beads attach to the antibody-coated target cells, allowing selective removal of the cells with high energy magnets.

Depletion of neuroblastoma cells from marrow was found to depend on the number of beads per target cell used and the time the beads were incubated with the cells. The effect of bead number and time of incubation on depletion of the neuroblastoma cells is shown in Fig 2. With a constant incubation time of 60 min, increasing the number of beads per target cell

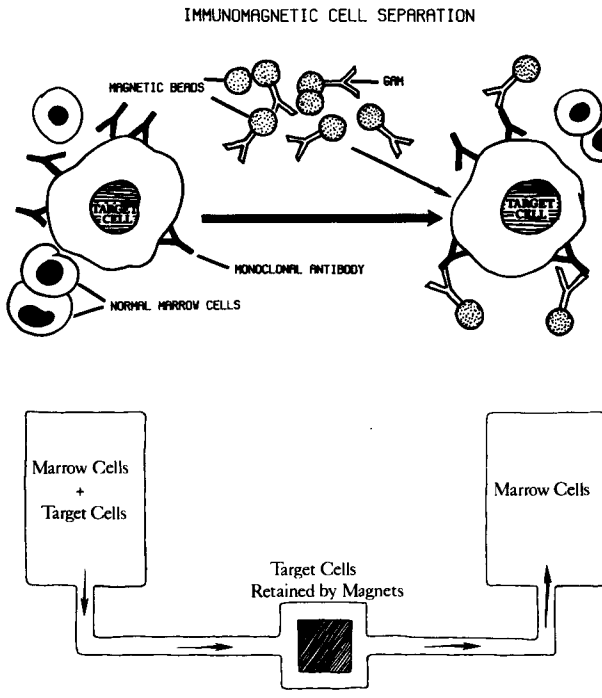

Figure 1. Immunomagnetic purging procedure. Top, *The principles of the separation procedure here have been depicted. Monoclonal antibodies conjugated with magnetic microspheres react with tumor cells present in the bone marrow. These cells, reacting with the monoclonal antibodies, are paramagnetic, so they will be trapped when exposed to a magnetic field.* Bottom, *The clinical separation procedure based on the principles outlined above has been depicted here. Bone marrow cells, potentially contaminated with tumor cells, are incubated with monoclonal antibodies conjugated with microspheres, and thereafter the cells are exposed to a magnetic field. Cells reacting with the monoclonal antibodies will be trapped in the magnetic field, but the antibody nonreactive cells will pass through.*

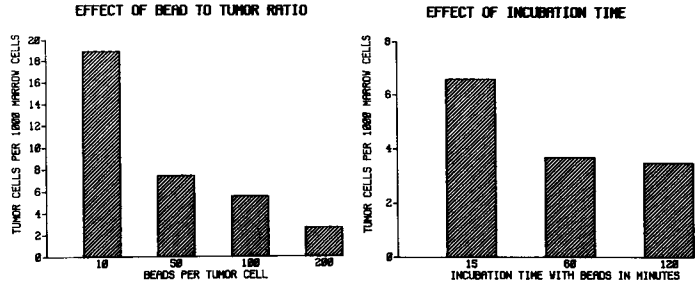

Figure 2. Effect of varying the ratio of beads to tumor cells (time fixed at 60 min) or varying incubation time of marrow with beads (200 beads per tumor cell) on depletion of CHP-100 neuroblastoma cells from marrow.

seeded into the marrow increased the efficacy of depletion. Using 200 beads per target cell, increasing the time of incubation with the beads from 15 min to 1 h also resulted in more effective purging, although no significant improvement was noted beyond 1 h.

Tumor heterogeneity may cause variable expression of cell surface antigens. For this reason it is of theoretical benefit to use several

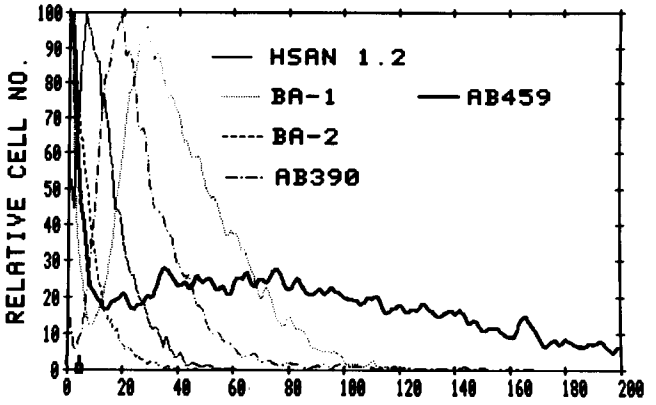

Figure 3. Binding profiles for the individual monoclonal antibodies HSAN 1.2, BA-1, BA-2, antibody 390, and antibody 459 on the SMS-KCNR neuroblastoma cell line. Negative controls for this experiment showed less than 2% of cells beyond channel 5. Staining with both monoclonal antibodies and subsequent staining with FITC-sheep antimouse immunoglobulin was under saturating conditions as previously described (8,32).

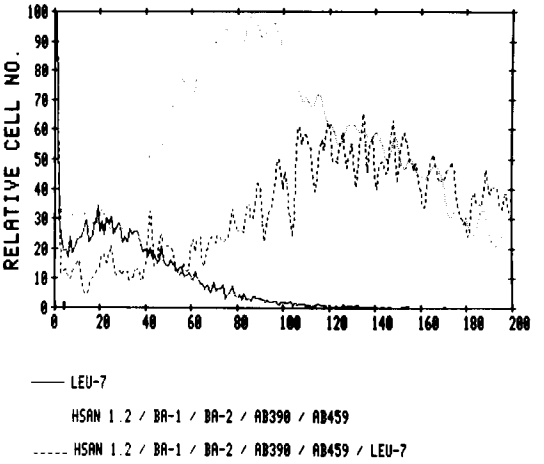

Figure 4. Binding profiles for Leu-7 alone; the cocktail of HSAN 1.2, BA-1, BA-2, antibody 390, and antibody 459; and the same cocktail with the addition of Leu-7 on the SMS-KCNR neuroblastoma cell line. Experimental details as described for Fig 3.

monoclonal antibodies to increase the efficacy of depletion. We found that the most accurate means of quantitating the amount of mouse antibody bound to the tumor cell surfaces was by use of flow cytometry. Figure 3 compares antibody-binding histograms for individual antibodies binding to the SMS-KCNR neuroblastoma cell line. The binding profiles shown in Fig 3 are typical of all human neuroblastoma lines examined to date, with antibody 459 consistently showing the brightest staining. The additive effect of a "cocktail" of multiple antibodies is shown in Fig 4. An increase in mean fluorescence per cell, as well as a decreased number of cells with weak fluorescence, can be seen in the cells stained with cocktails of antibodies. An increase in the mean fluorescence results from addition of Leu-7 to the cocktail of five antibodies (HSAN 1.2, BA-1, BA-2, 390, 459) as shown in Fig 4.

To determine if an increase in the amount of antibody on the cell surface results in a more efficient purging with immunomagnetic beads, we

Table 1.

EFFECT OF ADDING LEU-7 TO THE ANTIBODY COCKTAIL

Pre-Depletion % Tumor	Post-Depletion Tumor Cells/10^6	Log Reduction of Tumor
- Leu-7 14.42+/-1.8	163.6 +/-6.6	2.94
+ Leu-7 26.2 +/-5.0	6.6 +/-2.6	4.6

Depletion of neuroblastoma cell line SMS-KCNR with two cycles of monoclonal antibodies and beads. Antibody cocktail used was 459, 390, HSAN 1.2, BA-1, BA-2.

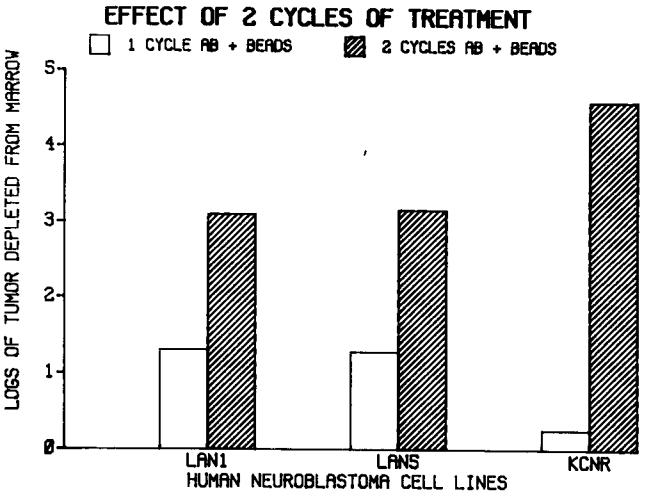

Figure 5. Effect of treating neuroblastoma-contaminated marrow with one or two cycles of monoclonal antibodies and immunomagnetic beads. Monoclonal antibody cocktail of HSAN 1.2, BA-1, BA-2, antibody 390, antibody 459, and Leu-7 was used for each cycle. Immunomagnetic beads at 200 beads per target cell were also used for each cycle. Neuroblastoma cells stained with H342 were seeded into marrow. The initial number and the number of cells remaining after each cycle of depletion were quantitated as previously described (23). Values represent the log of the number of neuroblastoma cells per million marrow cells depleted for each of three cell lines (LA-N-1, LA-N-5, and SMS-KCNR) seeded into normal marrow.

compared the depletion of SMS-KCNR cells from bone marrow using a cocktail of antibodies (HSAN 1.2, BA-1, BA-2, 390, 459) with or without Leu-7. The cocktail with Leu-7 resulted in a greater reduction of tumor cells in the marrow than did the cocktail without Leu-7 (Table 1).

We compared a single treatment with the antibody cocktail and immunomagnetic beads to two sequential treatments. As shown in Fig 5, an additional 1 to 2 logs of tumor was removed with the second treatment. A second treatment with both monoclonal antibodies and beads was required to achieve this effect. Treatment with monoclonal antibodies once, even if the antibody concentration was doubled, and then treatment with immunomagnetic beads twice was not as effective as two treatments with both antibodies and beads.

Some of the antibodies used in the study bind to cells found in normal marrow (BA-1, BA-2, Leu-7). Thus, it was not surprising that normal marrow cells were also depleted in the process of purging tumor from the marrow. To improve the yield of normal marrow cells, a new cocktail was studied in which Leu-7 was replaced with RB 21-7, an antibody that binds to neuroblastoma but not to marrow cells. Furthermore, because of the weak and very heterogeneous binding of BA-2 to neuroblastoma, it was not used in the new cocktail. This new cocktail purged marrow as effectively as the former cocktail (average removal of tumor was 3 logs), yet it resulted in higher recoveries of marrow cells. A representative experiment using the new cocktail is shown in Fig 6. Note that 3 logs of tumor are removed from the marrow, with recovery of nearly one-half of the total starting marrow mononuclear cells and excellent recovery of CFC-GM. The cocktail was also tested with similar results using tumor-contaminated marrow aspirated from a neuroblastoma patient: although the marrow initially contained 15% tumor cells, after purging no neuroblastoma cells were detectable by immunoperoxidase staining for neuron-specific enolase and cell surface monoclonal antibodies (sensitivity 1/100,000). The recovery of total cells in the marrow was 58%, and there was no reduction in the number of CFC-GM.

DISCUSSION

These experiments demonstrate that 3-4 logs of tumor cells can be consistently removed from marrow with monoclonal antibodies and immunomagnetic beads. To achieve this level of removal, one must carefully optimize the purging procedure. This requires a sensitive and rapid assay to detect tumor cells in the marrow. By premarking neuroblastoma cells with a supravital DNA stain, detection down to 1 tumor cell per million marrow cells is possible (11). Seeding normal marrow with such premarked tumor cells provides an excellent model for studying marrow purging procedures.

We have defined several parameters that can affect the success of purging using the immunomagnetic technique. The number of immunomagnetic beads per tumor cell is important as is the time of incubation with the beads. We found 200 beads per target cell and 1 h incubation with the beads to be optimal, under the conditions described here. It is possible that with different target cells or different types of immunomagnetic particles that the parameters for optimal purging will change.

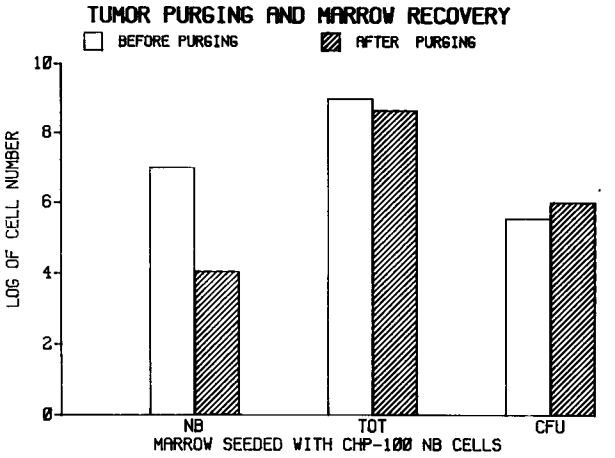

Figure 6. Representative experiment showing effect of purging marrow with antibody cocktail of HSAN 1.2, antibody 390, antibody 459, BA-1, and RB 21-7. Marrow was seeded with H342-stained CHP-100 neuroblastoma cells, and the number of neuroblastoma cells (NB), the total number of mononuclear cells (TOT), and the total CFC-GM (CFU) were quantitated after two cycles of purging.

An important factor governing the efficacy of purging is the amount of antibody on the surface of the target cell. Careful titration of the monoclonal antibodies on viable cells using flow cytometry allows determining optimal concentration of antibody for use in purging (16). Furthermore, use of a cocktail of antibodies has an additive effect (results in more immunoglobulin per cell) and helps overcome tumor heterogeneity of cell surface antigens (15,16). The amount of antibody on the target cells (as determined by flow cytometry) correlated with the ability to deplete the target cells. We demonstrated that addition of a single antibody to the cocktail can increase the amount of antibody bound to the target cells (increased mean fluorescence by cytometry), and also increases the efficacy of purging.

A critical observation provided by use of this model system is the increased removal of tumor seen using two cycles of treatment rather than one. A comparison by number of cycles has not been studied in a previously reported immunomagnetic purging system for neuroblastoma (10). The increased sensitivity of detecting residual tumor cells provided by the model system is no doubt important to our ability to measure the effect of a second cycle of antibody-bead treatment. A significant increase in the number of tumor cells removed in the second cycle is seen consistently, with several different neuroblastoma cell lines, and many of the cell lines used are low-passage lines isolated from bone marrow (5,18). Also, the cells are seeded into marrow in a fashion that closely resembles the clumps and single cells found in the marrow of neuroblastoma patients. Thus, the model system closely resembles the actual clinical situation, suggesting that the second cycle of treatment will contribute significantly to effective purging of clinical marrow samples.

The proportion of tumor cells that is clonogenic in humans is unknown and may vary for different tumors as it does for cell lines in vitro (18). Ideally, the number of viable tumor cells remaining in marrow to be reinfused into the patient should be decreased to the lowest possible level. Marrow with 1% tumor cells harvested for autologous transplant from a 20-kg patient would still contain 2000 to 20,000 tumor cells after 3-4 logs of depletion (15,16). Because the proportion of the cells that can reestablish tumors is unknown, it is critical to remove as many as possible. This is especially true in tumors such as neuroblastoma, which have a tendency to relapse years after cessation of therapy, suggesting that a small residual of viable tumor can reestablish the disease (35).

Use of additional anti-cell surface monoclonal antibodies may provide more effective purging, but other methods may also be required. Our preliminary experiments suggest that physical separation by filtering out tumor cell clumps, treatment with 6-hydroxydopamine (5), or treatment of the marrow after immunomagnetic purging with monoclonal antibodies and complement, can each add an additional log of depletion. The nonimmunological methods should be effective against cells that are not removed by antibodies because of low levels of cell surface antigens. Monoclonal antibodies and immunomagnetic beads could be combined with other tumor cell removal methods for a potential 5-6-log depletion of tumor cells. Effective purging of 5-6 logs of tumor should make it unlikely that tumor cells in the marrow will reestablish disease, even when using autologous marrow harvested from patients with overt tumor in the marrow (15,16).

By use of a well-defined model system, we have demonstrated that this method can reproducibly remove 3-4 logs of tumor cells from bone marrow with good recovery of total marrow cells. The marrow recovered is highly viable as assessed by dye exclusion and by growth of CFC-GM. Although the latter data do not prove viability of pluripotent hematopoietic stem cells, they do suggest that marrow treated in such a manner retains cells necessary for hematological and immunological reconstitution. Our model system should be useful for the study of marrow purging for other types of malignancies and of T-cell depletion prior to allogeneic transplantation.

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Immunotoxins Containing Pokeweed Antiviral Protein: New Strategies for More Effective Autologous Marrow Transplantation in Acute Lymphoblastic Leukemia

F. M. Uckun, S. Ramakrishnan, and L. L. Houston

INTRODUCTION

Bone marrow transplantation is a highly promising approach to the treatment of childhood and adult acute lymphoblastic leukemia (ALL) (1). Allogeneic bone marrow transplantation is limited, however, to the 30-40% of patients who have a human leukocyte antigen-mixed leukocyte culture (HLA-MLC) compatible sibling donor. Furthermore, the transplant-related complications of immunosuppression, graft-versus-host disease (GVHD), and pneumonitis contribute to significant morbidity and mortality in patients receiving allogeneic transplantation. Autologous remission bone marrow represents an alternative source of pluripotent marrow progenitors (2). Autologous bone marrow transplantation (ABMT) avoids GVHD and is not limited by donor availability. A high leukemic relapse rate is the major obstacle to the success of autologous transplants for ALL. Better therapeutic efficacy of ABMT for ALL most likely requires highly effective marrow purging methods to eliminate occult leukemic cells from autologous stem cell grafts ex vivo, as well as novel approaches to improve or intensify pretransplant conditioning regimens to eliminate minimal residual disease in vivo.

One method of purging autologous stem cell grafts involves the ex vivo treatment of marrow with synthetic derivatives of cyclophosphamide, such as 4-hydroperoxycyclophosphamide (4-HC) (3) or mafosfamid. In a recent study we tested the ability of mafosfamid to eliminate lymphoblastic leukemia cells from autologous bone marrow grafts following a 30-min preincubation. Treatment with 50-100 μg mafosfamid/ml eliminated more than 99.99% of contaminating clonogenic tumor cells from a 200-fold excess of normal marrow (4). However, mafosfamid appears to be mostly cytotoxic to rapidly cycling tumor cells and target leukemia cells may escape treatment because of slower proliferation rates, differences in cellular drug metabolism, or both. In this case, combining another purging agent that acts at another stage in the cellular machinery with mafosfamid may improve the ex vivo efficacy. One alternative approach to eliminating malignant cells from human bone marrow is the application of immunotoxins, conjugates of monoclonal antibodies (MAbs) with toxins, such as ricin (5); a portion of the toxin, such as ricin A chain (6); or a hemitoxin, such as pokeweed antiviral protein (PAP) (7,8). The use of PAP immunotoxins is attractive because PAP lacks a binding moiety and PAP immunotoxins are bound to the target cells solely by the specific antibody portion of the conjugate. Furthermore, there are three immunologically distinct PAP species (9) that are equally effective. Although PAP immunotoxins appear not to be as potent as intact ricin conjugates in vitro, they are highly selective marrow purging agents, and they are also potential candidates for eliminating minimal residual leukemia in vivo (10). In the study reported here we used two immunotoxins containing PAP directed against T-ALL and B-ALL cells. We compared their ex vivo efficiency alone with their efficiency in combination with mafosfamid in eliminating lymphoblastic leukemia cells from human marrow.

MATERIALS AND METHODS

The lymphoblastoid leukemia cell lines B-ALL (11) and HSB-2 (7) were used in clonogenic assay. B43 MAb reacts strongly with 99% of B-ALL cells, and the p41 antigen detected by T3-3A1 is present on HSB-2 cells in very high amounts.

B43 MAb (IgG1, K) detects a B-cell specific antigen, which is expressed throughout B-cell differentiation (12). B43 was linked to PAP by a disulfide using SPDP (8).

T3-3A1 MAb (IgG1, K) reacts with a p41 antigen present on 99.6% of HSB-2 cells. T3-3A1 reacts with T-ALL cells (>70%), chronic T-lymphocytic leukemia cells (40-70%), cutaneous T-cell lymphoma cells (<35%), and normal peripheral blood T cells (66%). T3-3A1 was coupled with PAP by a disulfide bond (7).

After treatment, target cell suspensions were prepared in serial dilutions and 100 µl of each dilution were plated in several wells of flat-bottomed tissue culture plates on a monolayer of irradiated bone marrow cells. After 14 days (37°C, 5% CO_2) the most probable number of remaining clonogenic units that survived treatment was estimated by counting the number of wells showing clonogenic growth (13). Under standard conditions 8-log elimination can be measured by this serial dilution assay (14).

Stem cell toxicity of the ex vivo purging procedure was evaluated by modifying the in vitro assay described by Fauser and Messner (15). Bone marrow mononuclear cells (1.5 x 10^5) were suspended in Iscove's modified Dulbecco's medium (IMDM) containing 0.9% methylcellulose, 30% fresh plasma, 5% phytohemagglutinin leukocyte-conditioned medium, and 65 µM 2-mercaptoethanol. Triplicate 1-ml samples were cultured in 35-mm petri dishes for 14 days (37°C, 5% CO_2). One unit of erythropoietin was added per milliliter at the start of the culture.

A total of 100 x 10^6 target B-ALL or HSB-2 cells were treated for 4 h at 37°C with B43-PAP and T3-3A1-PAP, respectively, or three and one-half hours with the relevant immunotoxin and 30 min with mafosfamid (combination treatment). After treatment, cells were washed twice and assayed for clonogenic growth.

RESULTS

Treatment with up to 1000 ng B43-PAP/ml did not induce any significant inhibition of clonogenic growth of B-ALL cells (Fig 1). A dose-dependent elimination of B-ALL cells was observed in the concentration range of 1000-10,000 ng/ml. Treatment with 10,000 ng B43-PAP/ml (4 h, 37°C) eliminated 99.9% of target B-ALL cells.

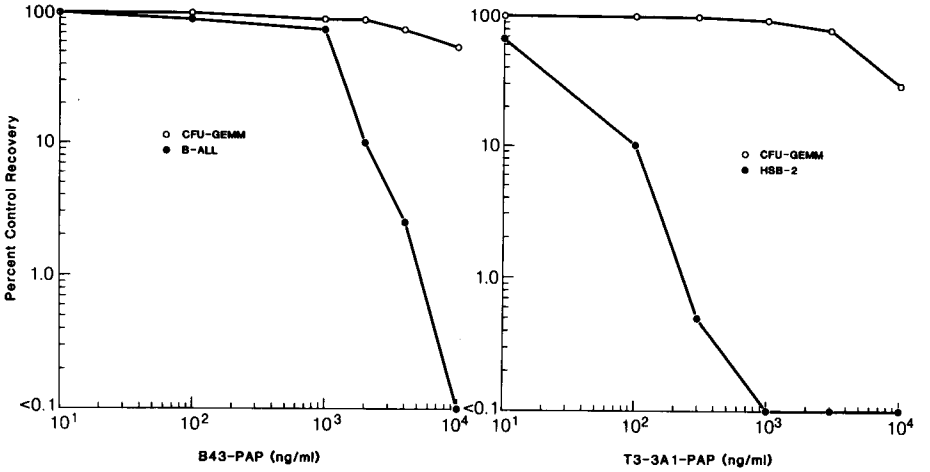

Figure 1. Effects of PAP immunotoxins on target leukemic cells and pluripotent marrow progenitors. A total of 1 x 10^7 leukemic cells (HSB-2 and B-ALL) or 1 x 10^7 Ficoll-Hypaque-isolated bone marrow mononuclear cells were treated with immunotoxin (4 h, 37°C), washed twice, and assayed for colony formation in vitro as described. Data are shown as the percentage of control recovery in which the recovery of treated cells is compared with untreated controls.

TABLE 1

ELIMINATION OF CLONOGENIC B-ALL CELLS BY B43-PAP-IMMUNOTOXIN IN COMBINATION WITH MAFOSFAMID

Mafosfamid (μg/ml)	Without Immunotoxin		With 10μg B43-PAP/ml		With 10μg T3-3A1-PAP/ml	
	CU/ml	LOG Kill	CU/ml	LOG Kill	CU/ml	LOG Kill
0	9.6×10^7	0	8.8×10^4	3.0	9.3×10^7	0
50	4.8×10^3	4.3	1.1×10^1	6.9	4.6×10^3	4.3
100	1.2×10^3	4.9	0	≥7.9	9.2×10^2	5.0

Note: 100×10^6 B-ALL cells were treated for 3½ h at 37°C with immunotoxin, and then with mafosfamid for 30 min at 37° C. The cells were washed twice and assayed for clonogenic growth. Results are the means of two replicate experiments.

TABLE 2

ELIMINATION OF CLONOGENIC HSB-2 CELLS BY T3-3A1-PAP-IMMUNOTOXIN IN COMBINATION WITH MAFOSFAMID

Mafosfamid (μg/ml)	Without Immunotoxin		With 1μg T3-3A1-PAP/ml		With 1μg B43-PAP/ml	
	CU/ml	LOG Kill	CU/ml	LOG Kill	CU/ml	LOG Kill
0	2.5×10^7	0	6.0×10^4	2.6	2.2×10^7	0.1
50	2.4×10^3	4.0	9.6×10^0	6.4	1.8×10^3	4.1
100	8.2×10^2	4.5	0	≥7.4	1.1×10^3	4.4

Note: 100×10^6 HSB-2 cells were treated for 3½ h at 37°C with immunotoxin and then with mafosfamid for 30 min at 37°C. The cells were washed twice and assayed for clonogenic growth. Results are the means of two replicate experiments.

Figure 1 shows that B43-PAP was highly selective in its activity against B-ALL cells and was not significantly toxic to pluripotent stem cells (CFU-GEMM). Less than 50% of pluripotent stem cells were lost under conditions in which 3 logs of target leukemic cells were eliminated.

Significant inhibition of HSB-2 cells was observed at very low concentrations of T3-3A1-PAP (Fig 1). A dose-dependent elimination of target HSB-2 cells was achieved with a maximum kill of 4.8 logs at 10,000 ng immunotoxin/ml. Treatment with 1000 ng T3-3A1-PAP/ml eliminated 99.9% of target tumor cells. If the concentrations of immunotoxins that eliminate 3 logs of target leukemic cells are compared, it is evident that T3-3A1-PAP immunotoxin is ten-fold more potent than B43-PAP immunotoxin against its target cells. The extent of elimination of target leukemic cells was 2 logs better with T3-3A1-PAP immunotoxin than with B43-PAP immunotoxin.

T3-3A1-PAP was not only very potent in killing target leukemic cells, but it also appeared to be a very selective immunotoxin. Only 5% of pluripotent stem cells were lost under conditions in which more than 99.9% of HSB-2 cells were eliminated. Pluripotent stem cells were inhibited by 70% at 10,000 ng T3-3A1-PAP/ml compared with almost 99.999% inhibition of HSB-2 cells.

Target leukemic cells were treated with mafosfamid alone or with mafosfamid in combination with relevant PAP immunotoxins. Table 1 shows that combined treatment with mafosfamid and B43-PAP immunotoxin was superior to mafosfamid alone or B43-PAP alone in eliminating leukemic B-ALL cells. Treatment with 100 μg mafosfamid/ml in combination with 10 μg B43-PAP/ml eliminated almost 8 logs of B-ALL cells. This additive effect appears to be specific since treatment with the irrelevant immunotoxin T3-3A1-PAP in combination with mafosfamid was not more toxic to B-ALL cells than mafosfamid alone. Table 2 shows that the amplification of immunotoxin cytotoxicity by mafosfamid was not restricted to B43-PAP. We used 1000 ng T3-3A1-PAP/ml, which was similar to 10,000 ng B43-PAP/ml in its effectiveness against target leukemic cells. Similar results were obtained with the mafosfamid combination protocol, and treatment with 1000 ng T3-3A1-PAP/ml in combination with 100 μg mafosfamid/ml eliminated more than 7 logs of target leukemic T cells. The concentrations of mafosfamid used in these experiments (50-100 μg/ml) are safe because all patients receiving autologous marrow grafts purged with 100 μg 4-HC/ml showed full hematologic recovery after transplantation (George Santos, personal communication).

CONCLUSIONS AND DISCUSSION

In this study we used two different selective immunotoxins containing pokeweed antiviral protein and found that their efficiency can be enhanced by mafosfamid. The maximum level of kill, which was 7-8 logs with the combination protocol, indicates that this novel approach has clinical potential for ABMT in treating ALL. Nevertheless, the biologic relevance of such marrow purging remains to be evaluated in clinical trials. In view of the high relapse rate observed among ALL patients after ABMT, we believe that improvement is needed both in marrow purging and in cytoreductive preparative regimens.

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Use of a Cocktail of Monoclonal Antibody-Intact Ricin Conjugates to Purge Human Bone Marrow of Leukemic Cells

R. C. Stong, R. J. Youle, J. H. Kersey, E. D. Zanjani, and D. A. Vallera

INTRODUCTION

A variety of strategies to effect the ex vivo purging of leukemic cells from human bone marrow autografts currently is being evaluated. The objective of all strategies is to remove leukemic cells while leaving unharmed the pluripotent hematopoietic cells crucial for engraftment. Our laboratory has demonstrated that immunotoxins (ITs), monoclonal antibodies conjugated to intact ricin, provide the high degree of selective potency required for this (1-3). Our experience using ricin ITs for prophylaxis against graft-versus-host disease in allogeneic bone marrow transplantation has demonstrated that intact ricin ITs are both safe and effective in their activity against target cells (2,4,5). Our approach has proved simple, rapid, and highly reproducible.

In this brief report, we describe our findings from experiments using a highly sensitive colony assay to evaluate the specific cytotoxic effects of two anti-T-cell ITs on leukemic T-cell lines. We selected ITs synthesized with T101 and TA-1 monoclonal antibodies because studies at our institution indicate that these antibodies bind to leukemic T cells in greater than 90% of cases. We also present results of preliminary experiments designed to test the feasibility of using the ITs together in a cocktail for the ex vivo elimination of leukemic cells from autologous bone marrow.

MATERIALS AND METHODS

T101 is an IgG2a monoclonal antibody that recognizes a 65- to 67-kilodalton glycoprotein (6). TA-1, also an IgG2a antibody, recognizes a glycoprotein complex of 170 and 95 kilodaltons (7). The procedure to covalently link intact ricin to monoclonal antibody employing the cross-linking agent M-malimidobenzoyl-N-hydroxysuccinamide ester (MBS) has been described in detail (8).

The leukemic T-cell lines MOLT4 and CEM were obtained from Dr. Jun Minowada, Hines, IL. Binding of unconjugated monoclonal antibody to the cell lines was determined by an indirect immunofluorescence assay using a fluorescence-activated cell sorter (FACS) IV. Normal bone marrow was obtained with informed consent from volunteers, and mononuclear cells were isolated using Ficoll-Hypaque density gradients.

Leukemic cells (10^7/ml) were treated with ITs for 2 h at 37°C, 5% CO_2, and 95% air in RPMI 1640 containing 2% human serum albumin and 100 mM lactose. Lactose was included to block the native galactosyl binding site of ricin B chain and render ITs specific for antigen. Cells were washed twice in lactose medium, counted, and resuspended for clonogenic assay. In experiments of leukemic cell and bone marrow mixtures, the combined cell concentration for IT treatment was 10^7/ml.

The methylcellulose colony assay for human leukemic cell lines has been previously described in detail (1). Results are expressed as a percentage of the control response of lactose-treated cells. In vitro assays to detect human pluripotent stem cells (CFU-GEMM) and erythroid burst-forming units (BFU-E) were conducted as previously described (1,2).

RESULTS AND DISCUSSION

In previous studies, we have shown that anti-T-cell ITs display marked potency and selectivity against T lymphocytes in functional assays

(2,5). In this report we evaluate two anti-T-cell ITs with potential for use in human autologous bone marrow transplantation. The potency and selectivity of one of these, T101 ricin, is shown in Fig 1. The T leukemic cell lines CEM and MOLT4 were selected because immunofluorescence typing indicated that T101 bound to nearly 99% of cells in each case. Treatment with T101 ricin in the presence of lactose inhibited the clonogenic growth of these cell lines in a dose-dependent manner. At 300 ng/ml, greater than 99.9% (3 logs) inhibition was observed. At 1000 ng/ml, 99.9992% (5.1 logs) of CEM and greater than 99.9993% (5.2+ logs) of MOLT4 cells were inhibited. In order to simulate the clinical situation, leukemic cells were then mixed with 20-fold excess bone marrow prior to treatment with the T101 ricin. What is important is that we found that the presence of bone marrow cells did not reduce the specific toxicity of ITs for the leukemic targets.

In parallel experiments we treated normal bone marrow with increasing concentrations of T101 ricin. Treatment with 300 ng/ml, a dose that inhibited 99.9% of leukemic cells, induced no inhibition of stem cell activity as detected in CFU-GEMM and BFU-E in vitro assays. Treatment with 100 ng/ml, which effected a 5-log inhibition of leukemic cells, inhibited stem cell activity only 20-49%. These results show that T101 ricin in the presence of lactose is a highly effective reagent that, when used at select concentrations, inhibits the growth of leukemic cells but is not toxic to stem cells.

In some experiments, rare CEM cells escaped treatment with the highest doses of T101 ricin. The resulting colonies were individually recovered from the methylcellulose, and two were successfully expanded in liquid culture. Immunofluorescence testing indicated that these clones (CEM-6 and CEM-11) were 99% positive with T101. However, the relative antigen density determined by FACS analysis was less for the surviving clones (mean fluorescent channel, 133.7 for CEM-6 and 143.1 for CEM-11). The results of side-by-side testing of the clones and the original population with T101 ricin are shown in Fig 2. Both clones were found to be significantly more resistant to killing by T101 ricin. Their sensitivity to ricin was similar to that of the original population (data not shown). Thus, it is likely that the increased resistance of the clones can be attributed to selection of cells expressing lower densities of p67 antigen and therefore binding less T101 ricin to their surface.

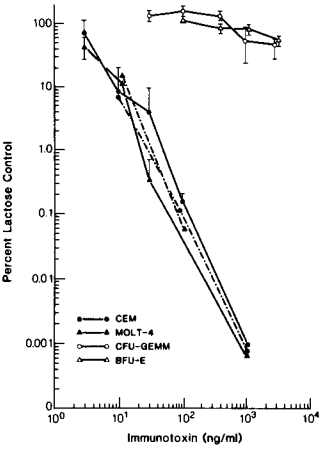

*Figure 1. Effect of T101 ricin/lactose on colony formation by MOLT4 and CEM cell lines and normal hematopoietic stem cells. Cell lines were treated with IT in the presence (*dotted lines*) or absence (*solid lines*) of 20-fold excess bone marrow. Stem cells were measured in parallel experiments. Data are expressed as the percentage of control lactose response and represent the mean plus or minus the standard deviations of at least three independent experiments.*

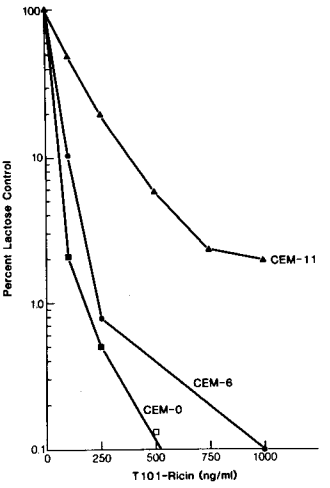

Figure 2. Results of a representative experiment comparing effects of T101 ricin/lactose on colony formation of uncloned CEM-0 population and two clones (CEM-6 and CEM-11), which had previously survived treatment with 100 ng/ml T101 ricin. A minimum estimate of inhibition (open square) *is indicated, the level at which no colony growth was observed under plating conditions adequate to detect the indicated level of killing.*

These results raise the concern that T101 ricin, despite high potency, may not be an adequate treatment for autologous marrow purging. It is well known that leukemic cells freshly isolated from patients are markedly more heterogeneous in antigen expression than are cultured leukemic cell lines. Within the fresh population we might expect the presence of cells with differing antigen densities and the existence of leukemia cell subpopulations that completely lack the targeting determinant. In such cases, use of mixtures of conjugates would offer a greater advantage.

We selected TA-1 for conjugation and further study since this antibody binds to more than 90% of T-cell leukemias. Also, TA-1 ricin exhibits selective and potent toxicity to normal T cells at doses that are not toxic to stem cells (2). The TA-1 ricin conjugate inhibited 35% of clonogenic MOLT4 cells at a concentration of 1000 ng/ml. Thus, it proved to be much less potent than T101 ricin against MOLT4 (Fig 3), most likely because the antibody binds to less than 50% of MOLT4 cells by FACS analysis. Since this pattern of antibody reactivity is one that we might expect to encounter clinically, we decided to test TA-1 ricin and T101 ricin together on MOLT4 cells.

MOLT4 cells were mixed with 20-fold excess bone marrow and treated with an equimolar mixture of T101 ricin and TA-1 ricin. As shown in Fig 3, the cocktail was highly effective in inhibiting clonogenic cell populations. Greater than 4.4 logs of inhibition were observed at a dose of 600 ng/ml. Aliquots of the cocktail-treated marrow were frozen to determine the effect of freezing and thawing on IT potency and selectivity. No decrease in IT potency was observed.

Comparison of the dose-response curves in Fig 3 suggests that the T101 conjugate within the cocktail mediated the majority of killing. The reduced level of killing with the cocktail compared with that of the T101 ricin alone does not, however, preclude use of an IT cocktail. In rodents, elimination of 3 logs of leukemic cells from the marrow grafts prevented leukemia in the transplant recipients (10,11). Using the IT cocktail at a dose of 600 ng/ml, we eliminated greater than 4.4 logs of *partially* reactive leukemic cells. Moreover, as our results with the resistant CEM clones emphasize, the heterogeneity of a patient's leukemic cell population may require more than one IT to effectively eliminate

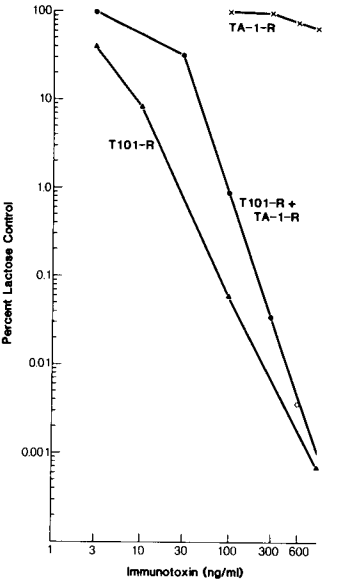

Figure 3. Comparison of the effect of T101 ricin, TA-1 ricin, and an equimolar mixture of these ITs on colony growth of MOLT4 leukemic cells. Cells were treated in the presence of lactose. An open circle represents the minimum estimate of inhibition.

contaminating leukemic cells from the marrow autograft. We are currently evaluating several T-reactive ITs that may prove beneficial when used in a cocktail to purge autologous marrow from T-leukemic patients. Clearly, reproducible techniques to clone fresh leukemic cells are needed to determine which surface determinants are expressed on the clonogenic cell in T-cell leukemias and to select ITs that will eliminate this critical population. Since we do not currently know the phenotype of the clonogenic cells, we believe that treatment with several ITs directed to different determinants increases the likelihood of eliminating this critical population.

After elimination of leukemic cells, the best results that can be expected for autologous marrow transplantation will be the 50% relapse rate observed for syngeneic transplantation (12). Clearly the efficacy of autologous marrow purging will be difficult to evaluate. Evaluation will be improved as more efficient conditioning regimens are developed to guarantee the elimination of all residual leukemic cells in the recipient. Thus, research in autologous marrow transplantation should focus on in vivo treatment of the patient as well as on ex vivo treatment of the bone marrow.

ACKNOWLEDGMENTS

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In Vitro Cytolytic Effects of Asta Z 7557 on Clonogenic Burkitt's Cells: Potential Value for a Bone Marrow Purging Procedure

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INTRODUCTION

In vitro pharmacological treatment of bone marrow before autologous bone marrow transplantation (ABMT) has been proved to be feasible in animal models since 1979 (1). In human leukemia models, results obtained either with 4-hydroperoxycyclophosphamide (4-HC), an active derivative of cyclophosphamide, or stabilized derivative Asta Z 7557 are still controversial. It is not known if the in vitro sensitivity of human leukemic clonogenic cells and normal hematopoietic progenitors is different (2-4). We demonstrated that the purging procedure was necessary in ABMT in Burkitt's lymphoma (BL) (5) and that we can expect a 2- to 3-log BL cell elimination with a purging procedure using three monoclonal antibodies (MAbs) and two sets of complement (see the chapter "Monoclonal Antibodies and Complement as Purging Procedure in Burkitt's Lymphoma"). Hence, we wanted to define the ability of Asta Z to destroy BL cells at doses that do not eliminate the CFC-GM (granulocyte-macrophage colony-forming cell) population completely (3). This drug could then be applied alone or, more probably, in association with an immunological procedure (6). In our experiments, we used three BL cell lines as in vitro models to test the activity of Asta Z. Since this drug is an alkylating agent that causes cell lysis by blocking DNA (deoxyribonucleic acid) synthesis, we used a tritiated thymidine incorporation technique to quantify the efficiency of the procedure (7). Also, the effect on the CFC-GM population was studied.

MATERIALS AND METHODS

BL cell lines--Daudi, Raji, and IARC BL_{63}--were kindly given by G. Lenoir (International Agency for Cancer Research, Lyon, France). Their characteristics, as well as their reactivity with various B-cell MAbs, were previously described (8). Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (Gibco-Europe) at 37°C in a 5% CO_2 atmosphere in air. Cells were harvested for experiments 24 h after medium change. Cell viability was checked with trypan blue exclusion, and the assay was started when viability was more than 80%.

All manipulations were performed in sterile conditions. Clonogenic BL cells were resuspended in Hanks' balanced salt solution (BSS) (pH 7.4) with 10 mM HEPES and 10% heat-inactivated fetal bovine serum. Asta Z 7557 (Asta-Werke, Bielefeld, West Germany) was diluted in Hanks' BSS. A total of 3×10^6 cells/ml were mixed with a 2-ml Asta Z solution at various dilutions, the final quantity of Asta Z varying 0-120 µg/ml for 3×10^7 cells/ml. Samples were incubated for 30 min at 37°C, washed twice in Hanks' BSS, and finally resuspended in culture medium (as described above). Cells were then distributed in a 96-well flat-bottomed cluster plate, a 250-µl cell suspension (8×10^4 cells) being placed in each well without a feeder layer. Cultures were then incubated at 37°C in 5% CO_2 humidified atmosphere in air. Two control samples were done as follows: first, one sample treated without Asta Z was distributed in wells at the same concentration and volume; second, 8×10^2 untreated cells were distributed in each well in 250 µl of medium in order to check that a very low number of cells to be expected after Asta Z exposure will still be able to grow and to incorporate tritiated thymidine. The assay lasted from 18 h to 72 h. The DNA synthesis was determined by tritiated thymidine incorporation at 18 h, 24 h, 48 h, and 72 h.

We used 0.5 µCi of tritiated thymidine (25 Ci/mmol) (Amersham, UK) at a 1:20 dilution (10 µl in each well). The optimum duration of incubation

with tritiated thymidine to label nontreated BL cells was previously determined as 18 h. Therefore, tritiated thymidine was added 18 h before analyzing the results. The tritiated thymidine incorporated in cell DNA was collected automatically from each well on glass fiber strips using individual filters (MASH II, O.S.I., France) and then placed in individual polyethylene vials (Packard Instruments) with 4-ml scintillation fluid (Ready Solv NA, Beckman, France) and counted in a Kontron MR300 light scintillation counter.

The tritiated thymidine incorporation was measured as disintegration per minute (dpm) in the two untreated samples and in the treated samples. The total number of dpm was strictly correlated to the total number of untreated plated cells either at 8×10^4 or 8×10^2 cells per well. The efficiency of the DNA synthesis blocking or the killing was determined as the ratio between the dpm of treated and untreated samples (8×10^4 cells/well) and expressed as a percentage, the dpm of the untreated samples being considered as 100%.

Normal bone marrow mononuclear cells separated on Ficoll gradients and resuspended in RPMI 1640 (1×10^7/ml) were incubated with Asta Z solubilized in RPMI at appropriate concentrations, the total dose of Asta Z varying 0-50 µg/ml for 30 min at 37°C, and washed twice in RPMI 1640. The toxicity on hematopoietic stem cells was evaluated by the CFC-GM recovery from 2×10^5 mononuclear cells (9).

RESULTS

As shown in Fig 1, curve 1, we obtained on BL_{63} cells a maximum killing of 2 logs with an Asta Z dose of 60 µg/ml. With an increasing dose (0-60 µg/ml), the efficiency of the killing was dose dependent, but from 30 µg/ml to 120 µg/ml, the maximum dose studied, we obtained a plateau. This curve was not modified when the observation was continued from 18 h to 72 h. The data presented in Fig 1 are a compilation of the results of 10 experiments. The standard deviations are not shown in the curve but have been calculated to be no more than 0.01. The efficiency of the killing in the case of the Raji cell line (curve 2) was very similar to that obtained for the BL_{63} cell line: a maximum of a 2.5-log killing was reached for 90 µg/ml Asta Z, reaching a plateau for higher doses and without any modification when the assay was continued after 18 h. For the Daudi cell line (curve 3), the plateau was higher, with a maximum killing of 1.5 log with 90 µg/ml Asta Z. This curve was still obtained at 18 h and not modified later.

As shown in Fig 2, the toxicity of the drug was dose dependent, with no CFC-GM recovery from a dose of 50 µg/ml. The break in the curve was obtained at 30 µg/ml Asta Z, with a rapid decrease of CFC-GM from 12% to 0% with a dose increasing to 50 µg/ml. For a concentration of 30 µg/ml Asta Z, the efficiency of killing of BL cells was 1 log and did not increase by more than 0.5 log at a dose of 50 µg/ml.

DISCUSSION

Asta Z treatment of clonogenic BL cells allowed a maximum killing of 1.5-2.5 logs at doses 60-90 µg/ml (3×10^7 cells/ml). The maximum killing and the dose varied from one line to another, but they were very similar for the same line in successive assays. The sensitivity to Asta Z differs for each line (especially for the Daudi and Raji lines); however, for doses lower than 40 µg/ml, we obtained the same killing efficiency of the three lines (i.e., 1.5 log elimination of the cells). The same plateau phenomenon is obvious in recently published data by DeFabritiis et al (6) obtained by lysing clonogenic BL cells mixed with irradiated bone marrow with 4-HC. In this study the efficiency of the purging was quantitated by a clonogenic assay. The plateau, which was rather low (between 2 and 3 logs), was obtained for 90 µg/ml drug. This was probably due to the various sensitivities of the BL cell lines. However, for 40 µg/ml stabilized 4-HC, the efficiency of the killing was 1.5 log, as in our study. This plateau is rather surprising for an alkylating agent with

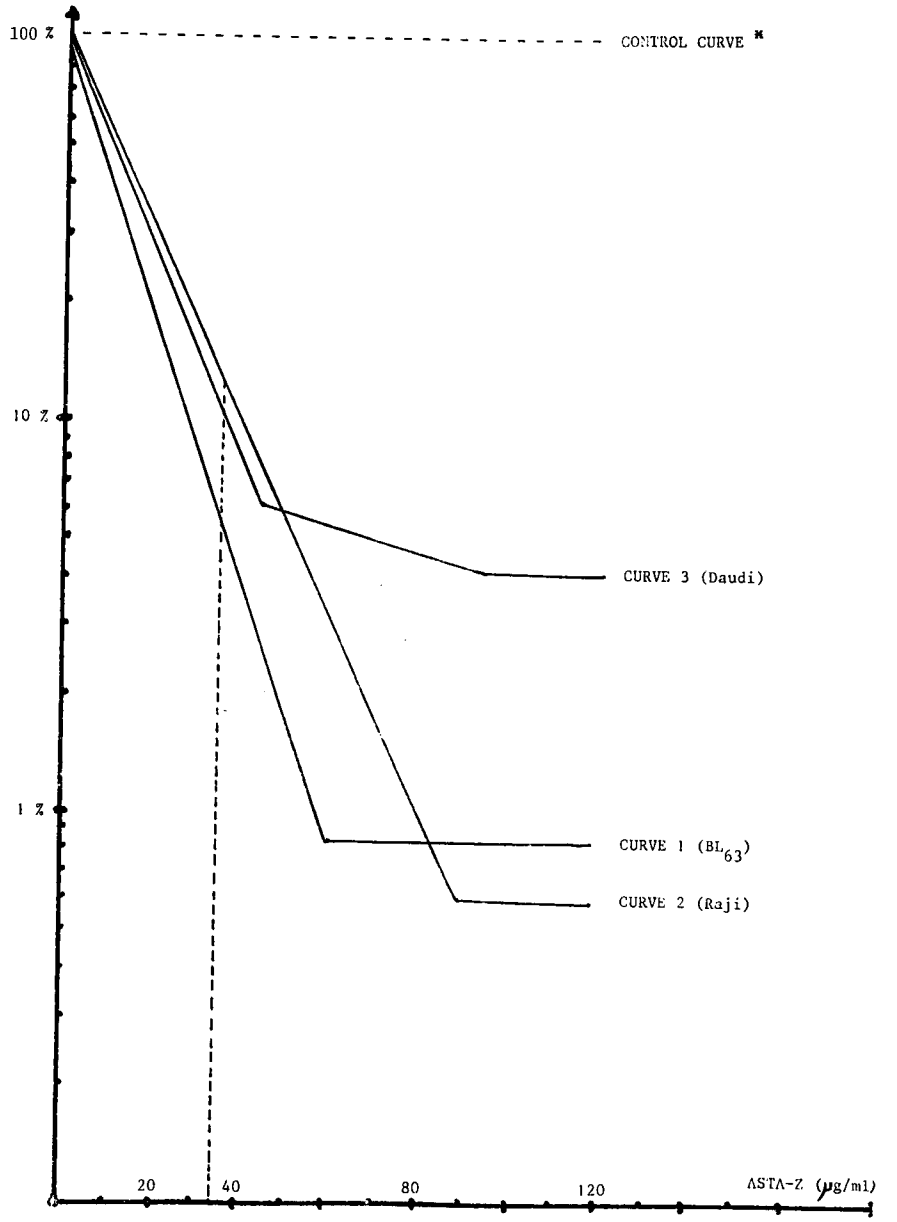

Figure 1. Effect of increasing dose of Asta Z on BL cell lines' lysis. Control curve was identical for untreated sample of 8 x 10^4 cells/ml and samples of 8 x 10^2 cells/ml.

cycle-dependent activity and has not been observed by teams working with myeloid leukemia models. In addition, DeFabritiis (6) showed no increase in cytotoxicity of 4-HC activity by an increased duration of incubation with the drug. This suggests that the mechanism of resistance to cyclophosphamide derivatives is independent of the cell cycle and varies from one BL line to another.

The question then arises whether this sensitivity of BL clonogenic cells to 4-HC can be extrapolated to fresh tumor cells. From clinical experience we know large variations in the therapeutic activity of cyclophosphamide may exist from one BL case to another.

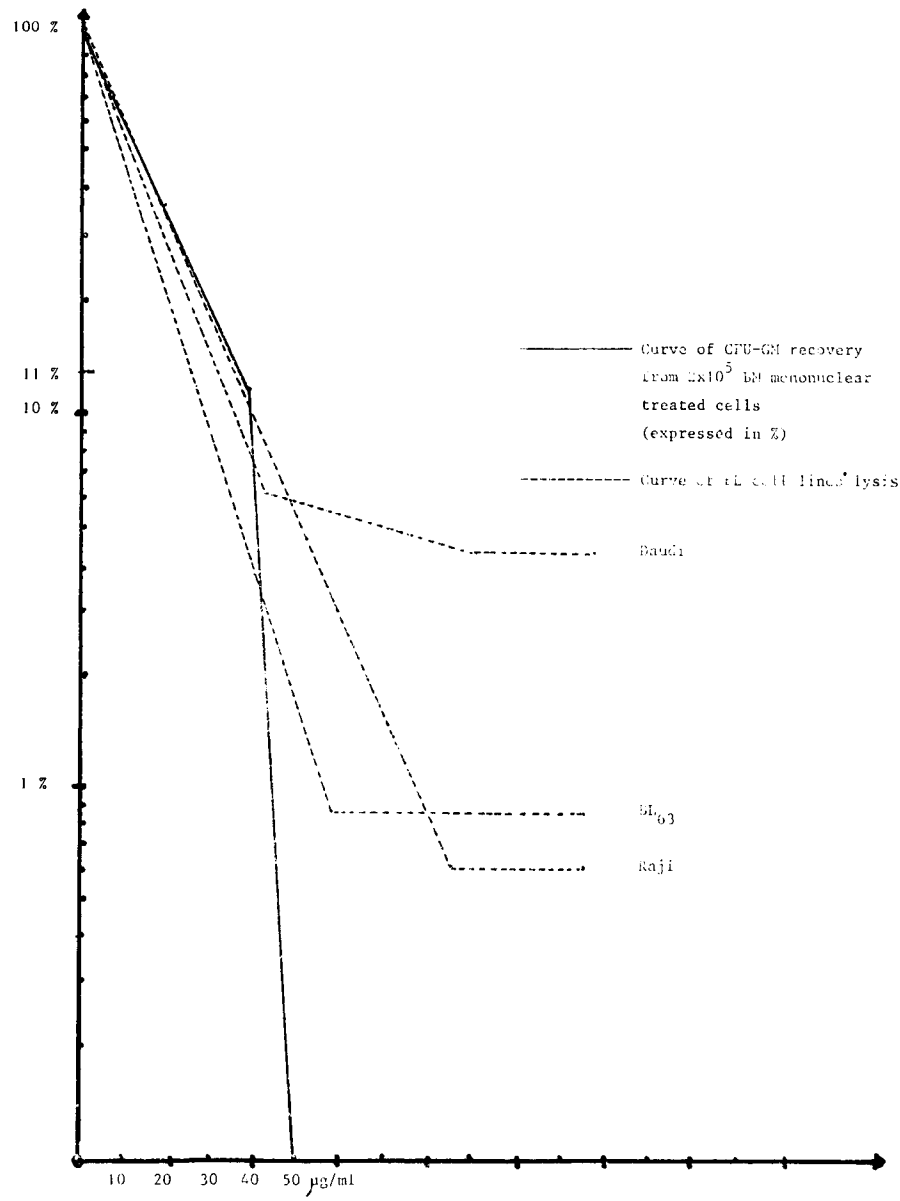

Figure 2. Comparative effect of Asta Z on CFC-GM recovery and on BL cell lines' lysis.

In addition, similar variations in lysis of BL cell lines were observed with a purging procedure using MAbs and complement. The efficiency of this procedure varied between 1 and 4 logs (see the chapter "Monoclonal Antibodies and Complement as Purging Procedure in Burkitt's Lymphoma").

It seems logical to combine chemical and immunological procedures, especially if the subpopulation resistant to one technique is sensitive to the other, as recently has been demonstrated (8). From the point of view of CFC-GM recovery, we will use Asta Z at a 35-µg/ml dose (i.e., 70 µmol/l), and will expect a 1-log more efficient killing with the double procedure than with the immunological procedure alone. This procedure was used in one recent case. Bone marrow was purged with three MAbs and two

complement treatments followed by Asta Z incubation. The total CFC-GM recovery after the procedure was 13%. The patient was grafted recently and at day 15 after transplantation the white blood count was greater than 1000/mm^2. However, a less toxic procedure than Asta Z, either chemical or physical, needs to be developed that could be added to the immunological procedure that would allow at least a 1-log increase in the killing.

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Pharmacological Treatment of Bone Marrow Grafts

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INTRODUCTION

Allogeneic bone marrow transplantation has become the treatment of choice for hematologic malignancies in young patients. This method is limited by the scarcity of identical human leukocyte antigen (HLA) donors; the rate of serious morbidity, which increases as age does; and immunological reactions, such as acute or chronic graft-versus-host disease (GVHD) and graft rejection (1-3). Autologous bone marrow transplantation (ABMT) has been developed as an alternative treatment for patients who do not have a suitable donor or are older.

When the remission duration of autologous and allogeneic bone marrow transplantation was compared in matched patient groups, allogeneic bone marrow transplantation yielded better results (4,5). Efficacy of ABMT for hematologic malignancies may be limited by collected bone marrow's contamination by malignant cells or by damage of lymphohematopoietic stem cells by preceding cytoreductive treatments. Several different approaches have been taken to eliminating malignant cells from marrow grafts: (*a*) immunological separation with monoclonal antibodies having specificity against the malignant cells, (*b*) physical separation utilizing differences in density and size for separation, and (*c*) administration of drugs with different cytotoxicity to malignant and hematopoietic stem cells.

We have utilized methylprednisolone (MP) incubation in an attempt to prevent acute GVHD after allogeneic bone marrow transplantation based on encouraging data in animal models and on the observation that in vitro treatment with high doses of MP eliminated predominantly mature T-lymphocytes and spared hematopoietic stem cells (11,15). The results of this study are relevant to ABMT because high-dose MP could be used for in vitro treatment of patients with lymphoid malignancies.

MATERIALS AND METHODS

Eighteen patients received donor bone marrow pretreated with MP at a dose of 15 mg/ml for 60 min. The patients received allogeneic transplants for malignant lymphoma (one), acute lymphoblastic leukemia (ALL) (six), acute myeloblastic leukemia (AML) (seven), and chronic myeloblastic leukemia (CML) (four). Nine patients received transplants in first to third remission and nine patients at relapse. The conditioning regimen consisted of a combination of cyclophosphamide, 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU), and VP-16-213 (11) in two patients; melphalan and total body irradiation (TBI) in one patient; and piperazinedione and TBI in 15 patients (12,13). No posttransplantation graft-versus-host prophylaxis was administered.

Bone marrow (800-1000 ml) from normal donors was centrifuged at 850 g for 15 min. The bone marrow buffy coats were incubated with 15 mg/ml MP for 60 min at 37°C. The cell suspension contained 50 x 10^6 nucleated cells/ml; 2% autologous serum and 0.025% deoxyribonuclease. After incubation, the bone marrow cells were washed twice with Hanks' balanced salt solution and filtered.

All samples went through the following tests: pre- and posttesting incubation cell count, differential count, granulocyte-macrophage colony-forming cell (CFC-GM), phytohemagglutinin (PHA) response, and lymphocyte surface marker analysis.

The marrow CFC-GM culture was performed as described previously (14). In brief, 1 x 10^5 marrow nucleated cells were cultured in 1 ml of minimum essential medium containing 15% fetal calf serum, 0.3% agar, and 10% human placenta condition media. All samples were plated in triplicate and incubated in 5% CO_2 at 37°C for 12 days.

One milliliter of bone marrow mononuclear cells (1 x 10^6 cells in RPMI 1640 and 10% fetal calf serum) was mixed with 1 ml of 0.5% sheep red blood cells (SRBCs) pretreated with 2-aminoethylisothiuronium bromide and then centrifuged at 60 g for 5 min at room temperature. The pellet was kept overnight at 4°C. The mixture was then gently resuspended by end-over-end rotation. The percentage of cells binding three or more SRBCs were counted in a hemocytometer after staining with toluidine blue dye. Approximately 200 cells were examined for each assay.

The proliferative response of the MP-treated marrow mononucleated cells was monitored using a mitogen assay in the presence of 1% PHA (GIBCO, Grand Island, NY). Mononuclear cells were added to each well of Corning 96-well microtiter plates (Corning, NY) at a cell concentration of 1 x 10^5 per well. Medium (RPMI 1640 with 10% fetal calf serum) with or without PHA was added to the wells to yield a final volume of 0.2 ml and 1% PHA per well. The cells were incubated in 5% CO_2 at 37°C for 72 h prior to the addition of 1 μCi of [^{3}H]-thymidine per well. After an additional 10 h of incubation, cells were harvested onto a filter and counted by the TriCarb 300.

The mean reduction of mononucleated cells after MP incubation was 50% (range, 8-65%). The PHA response decreased from 75 x 10^3 to a baseline level of 0.35 x 10^3. Erythrocyte-rosette formations were reduced from 21 to 0%. CFC-GM per 10^5 cells plated decreased from a median of 33 to 0.

Cell surface marker analysis was attempted with OKT3, OKT4, OKT7, OKT11, Leu-1, Leu-7, Leu-11, and monoclonal antibodies. After MP treatment, nonspecific binding was noted in 20-50% mononuclear cells. The control assay was performed with monoclonal antibody directed against melanoma cells. The monoclonal antibody reacted with less than 1% of normal bone marrow cells and gave a nonspecific binding of 50% in bone marrow cells after MP treatment.

Eighteen patients received transplants of MP-treated bone marrow grafts. Two patients died before engraftment could be documented. Fourteen patients had evidence of engraftment within 3 weeks. Two patients' transplants failed to engraft, and the patients required a second transplantation. Clinically significant GVHD (grades II-IV) occurred in eight patients: grade II in four patients, grade III in two patients, and grade IV in two patients.

The time to hematopoietic recovery of patients receiving bone marrow treated with 15 mg/ml MP is shown in Fig 1. The mean granulocyte count was 500/μl within 3 weeks and 1500/μl within 4 weeks.

DISCUSSION

This pilot study shows that transplantation with MP-treated bone marrow grafts is feasible. Engraftment was obtained with bone marrow treated with high doses of MP in the absence of CFC-GM. This treatment, however, did not lead to a significant reduction in the incidence of acute GVHD. Liquid culture studies of MP-treated bone marrow cells have shown that mature T cells could be regenerated in 8-12 days in the presence of interleukin-2 and PHA (J Yau, personal communication). It is likely that mature T cells are regenerated from pre-T-lymphocytes residing in the bone marrow and surviving the MP treatment. More study is necessary to discover whether mature T cells can be regenerated after treatment with monoclonal antibodies.

The in vitro exposure to 15 mg/ml of MP equals a 3-4 log increase over concentrations achieved with conventional doses of corticosteroids. It is likely that the mechanism of action of high-dose MP is different from that of conventional doses of corticosteroids. Cytotoxicity is quickly observed after in vitro MP treatment. Within the first 15 min cytoplasmic changes are seen with electron microscopy. In vitro incubation with high doses of corticosteroids could be particularly effective in lymphoid malignancies. Preliminary data showed selective killing of CEM cell line cells over bone marrow cells in clonogenic assays and thymidine incorporation tests.

Drugs used for pharmacological in vitro treatment should be active against the malignant cells and be not at all or only moderately toxic to

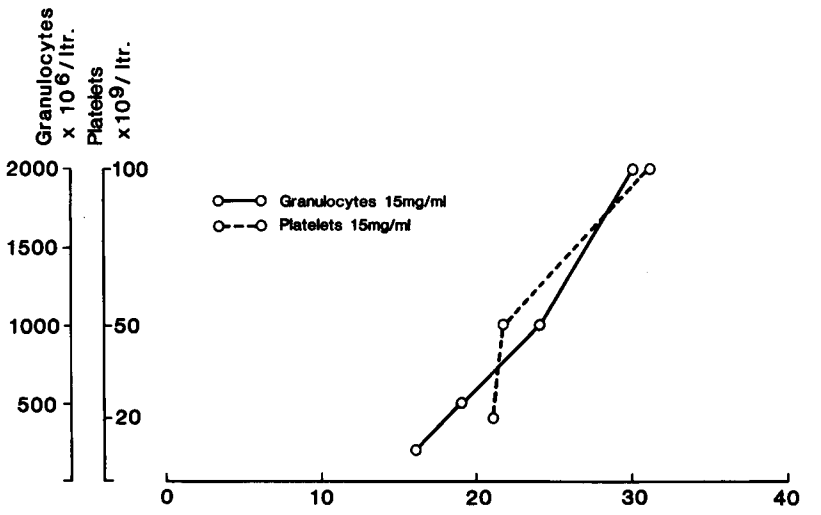

Figure 1. Hematopoietic recovery following transplantation with MP-treated donor marrow. The horizontal axis indicates days posttransplant.

normal hematopoietic stem cells. The short-term in vitro incubation allows the exploration of drug treatments limited by nonmyeloid toxicity in clinical applications. Clinically, nonmyelosuppressive potentially useful drugs are L-asparaginase, bleomycin, cisplatin, corticosteroids, and spirogermanium. Even moderately myelosuppressive drugs could be used such as anthracyclines, epipodophyllotoxins, 4-hydroperoxycyclophosphamide (4-HC) and 5-fluorouracil. Asta Z 7557 and 4-HC, derivatives of cyclophosphamide, have been used for treatment of bone marrow autografts (6-9).

Animal models have been used to evaluate new drugs with this approach (9,10). These models are not helpful for selecting a specific drug in clinical transplantation because of the different sensitivities and growth kinetics of human malignancies. Cytotoxicity studies of human cell lines derived from malignancies are of questionable relevance too, since changes in sensitivity can be acquired by continuous in vitro propagation. Testing drugs against untreated malignant cells using various assays for cytotoxicity might not reflect clonogenic malignant cells. Even if we culture untreated malignant cells in vitro, we face a few problems: 1) the reproducibility of the assay, 2) the difficulty in documenting that the cloned cells are of malignant origin, and 3) the question of whether the clonogenic tumor cells are identical to tumor stem cells. The most important question pertaining to the contaminating malignant cells found in the remission marrow is whether the clonogenic cells have the same sensitivity as cells found on first presentation of the disease or whether they are resistant to the cytoreductive treatment. In case of resistance, drugs other than those proven effective in induction treatment would be needed to eradicate the tumor cells. In this situation, cytotoxicity assays done on pretreatment marrow samples would not predict the optimal choice of drugs for the pharmacological treatment.

It appears feasible to explore the use of MP and other corticosteroids in the in vitro treatment of bone marrow cells from patients with lymphohematopoietic malignancies.

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Panel Discussion: Session IX

A. H. Filipovich and C. L. Reading, Moderators

Dr. Alexandra Filipovich: I would like to raise the issue of to what extent hematologic recovery is a function of the marrow that is infused. Does anyone have any comments on this?

Dr. Gary Spitzer: Dr. Kaizer, since the AML data are so interesting, could you tell us what concentration most of those patients were treated with?

Dr. Herbert Kaizer: Yes, there were 12 acute myelogenous leukemia (AML) patients. Four of those patients were treated with concentrations below 100 μg/ml; eight of them were treated at 100 μg/ml.

Dr. Spitzer: How many are still disease free? What were they treated with?

Dr. Kaizer: Six remain disease free. One was treated at 60 μg/ml, one was treated at 80 μg/ml, and the rest were treated at 100 μg/ml.

Dr. Spitzer: So your longer survivors must have received your lowest doses of in vitro treatment.

Dr. Kaizer: That's correct.

Dr. Filipovich: Those of us who treat marrow in various ways for allogeneic transplantation have found that this significantly changes the outcome in that we have observed very prompt autologous recovery in some patients who received so-called superlethal conditioning. It would seem that manipulating autologous marrow might also change the component contribution of true autologous regeneration as well as engraftment from the autologous transplant. Can we really look at hematologic recovery as the in vivo model for stem cells? Would anyone else like to comment on that at this point? Are there questions about other separation techniques?

Dr. Karel Dicke: I want to ask Dr. Laurent how stable and how pure the A-chain of his ricin preparation is.

Dr. Guy Laurent: B-chain contamination in the preparation leads to the reconstitution of ricin. In order to assess the presence of B-chain, the most sensitive approach is to compare the toxicity of whole ricin and the toxicity of A-chain using protein synthesis inhibition assay: respectively 2×10^{-12} and 2×10^{-7} M produce 50% inhibition on CEM cells. Therefore it can be postulated that the contamination of B-chain is less than 10^{-6} M.

Dr. Daniel Vallera: We have A-chain conjugates sent to us from Franz Jansen in Montpellier. They have remained stable over the course of a year at 4°C. I think that both intact ricin immunotoxins and A-chain ricin immunotoxins have been kept for a year without any problem. Relative to purity, it is much more difficult to obtain pure preparations of A-chain immunotoxins. This is a matter of the differences in chemical procedures and column purification.

Dr. John Kemshead: Could you tell me about the rate of endocytosis of T101 whole ricin and A-chain?

Dr. Vallera: The rates of endocytosis have not been tested. However, we have looked at kinetics using protein synthesis inhibition assays with cell lines. We have found that T101 intact ricin conjugates in the presence of lactose have much faster kinetics than A-chain immunotoxins.

Dr. Kemshead: So you actually are not certain that when you reinfuse marrow after 2 h you may not be reinfusing cells coated with Ig that you may then opsonize into the liver. Do you have an opsinizing effect with the conjugates, actually adding to the killing effect on the tumor cells?

Dr. Filipovich: Well, I think the answer is that we don't know if there is an effect. You are quite right in that not all of the antibody or immunotoxin has been internalized at the time of the infusion because we can detect it if we come in with a second fluorescent antibody; we can see that there are T101-positive cells among the marrow that we infused. The subclass of T101 is IgG-2A.

Dr. Christopher Reading: Is it the 2A or the 2B subclass that is so effective with mononuclear cells in vivo?

Dr. Filipovich: Just to clarify that, despite the several washing procedures and the time that elapses from the incubation until the infusion, there are detectable antibody moieties on the surface of the cells that are infused. So we cannot exclude the possibility of some other effect or mechanism contributing, hopefully to the beneficial effect.

Dr. C. Patrick Reynolds: If I could just answer that question with some data from Paul Martin at Seattle. In the paper, which he presented at the last UCLA bone marrow meetings and has since been published, he looked at purging marrows of T cells with nine different monoclonal antibodies to T cells in patients with identical haploid chromosome constitutions trying to abrogate graft-versus-host disease (GVHD). The bone marrow cells were treated with the antibodies without complement and then injected into the patients. He saw no effect on the incidence of GVHD. So that would argue against there being any sort of effect of just having Ig on the surface (i.e., opsonization).

Dr. Filipovich: Well, I think there was no significant effect (in GVHD prevention) in terms of that uncontrolled study because the patient outcome was not satisfactory. That isn't to say that any one of those antibodies might not have mediated a little bit of removal of the cells.

Dr. Reynolds: In vivo efficacy may in fact depend on the individual that is receiving the coated cells. These patients have essentially received lethal doses of radiation and chemotherapy for conditioning, and they are suffering from cancer. And you know I am not quite sure whether you can rely on the immune system of the host to actually supply anything that would speed up lysis of the cells.

Dr. Vallera: I wanted to actually get back to a point that Karel Dicke had raised earlier. In regards to the efficacy of "antibody plus complement" versus "immunotoxins," I do think that complement does have one serious drawback. It is the fact that it can be inconsistent in activity. Standardization often takes fastidious screening and problems still occur. Immunotoxins, on the other hand, are very consistent.

Dr. Filipovich: Are there any other questions or comments regarding any subject?

Dr. Robert Bast: In the assay which I described, a 20-fold excess of irradiated bone marrow cells are left in contact with the surviving cells at all dilutions. If you are going to extend the assay by another log or 2, you will have to dilute below the incredibly high cell concentration of the order of 5×10^7/ml. When you are diluting that much you may indeed have real problems with bystander effects. I think you do need to show that the assay is linear if you are going to extend it to those higher levels, even with the limiting dilution technique.

X. Supportive Care

Treatment of Herpes Simplex Virus Infection in Bone Marrow Transplantation Patients

W. H. Burns and R. Saral

Detailed studies of herpes virus infections in autologous bone marrow transplantation patients have not been published. However, we found that reactivated herpes simplex virus (HSV) infections in allogeneic bone marrow transplantation patients (1) and intensively treated patients with acute leukemia (2) are comparable in severity and timing. Infection in both populations occurs in the majority of HSV seropositive patients during the third week after beginning the treatment regimen. In prospective randomized studies with placebo controls, we demonstrated that the prophylactic use of intravenously administered acyclovir (ACV) completely suppresses reactivation of the virus during the time that the drug is given to these patients (1,2).

In our transplantation patients, we routinely start the drug 4 days before transplantation and continue it until the time of discharge. A frequently voiced concern relating to this practice is the development of resistant virus. We addressed this concern recently in the Lancet (3). We found seven cases of resistant virus, and in all cases the patients had established HSV infection before ACV administration. We have never seen resistant virus develop following prophylactic treatment and in fact have never cultured the virus from patients treated prophylactically while they remain on the drug. We believe that prophylactic use of the drug takes advantage of the special relationship of the latent virus with its host. In that situation, the evidence to date suggests very little transcription, translation, or replication of the viral genome. If thymidine kinase should be produced in the latently infected cell the accumulation of the triphosphate of ACV should inhibit virus production at a time of the smallest possible genomic load. If the physician waits for extensive infection before beginning the drug, there exist millions more viral genomes from which resistant virus can be selected. Thymidine kinase-deficient virus is often detectable in clinical isolates from patients never before treated with ACV. We have previously made the analogy to the situation of treatment of tuberculosis with isoniazid. Treatment is effective and resistance is infrequently encountered if the mycobacterial burden is low, but when isoniazid is used to treat cavitary lesions with a high mycobacterial load, the likelihood of encountering resistance is high.

Since we believe strongly that selection for resistant virus is minimized by prophylactic treatment compared to treatment of established HSV infections, we strongly recommend this treatment for patients at high risk in a well-defined time period, such as those being treated for acute leukemia and BMT patients of all types. Two factors arguing against this recommendation deserve merit. One is the cost, which for an average size adult patient runs about $60 per day. One has to balance the cost with the high morbidity and occasional mortality seen with HSV infection in these patients. Herpetic lesions with secondary bacterial and fungal infections during a time of granulocytopenia heal slowly and with difficulty, resulting in discomfort and often malnutrition. We believe the cost of ACV prophylaxis is justified in this situation. The second consideration is the possible decrease in cell-mediated immunity (4) or antibody responses (5) to HSV in ACV-treated patients when they are tested later. Presumably, the decreased presence of HSV antigens in the ACV-treated patient results in a diminished stimulation of the developing immune system after transplantation. The significance of these laboratory findings is not clear, and we have not seen any obvious increased severity in HSV infections in our patients when infection has occurred after cessation of ACV. If this were to be a demonstrable clinical problem, one could consider HSV immunization with viral proteins or killed virus during the posttransplantation period while the patients are on ACV therapy.

In conclusion, we strongly advocate the prophylactic use of ACV for HSV seropositive transplantation patients. We believe prophylaxis is a strategy that maximizes antiviral efficacy and minimizes emergence of resistant virus. This strategy should also be followed for other virus infections in this patient population if effective antiviral agents have been developed for them. In fact, it is especially true for cytomegalovirus (CMV), since CMV interstitial pneumonitis can develop with such rapidity and lead to the death of nearly one third of such patients within 3 days. Prophylactic use of a very highly effective antiviral for CMV may even be required for success. Finally, if delay of viral antigen presentation to the immune system becomes a clinical problem, then immunizations with the appropriate viral antigens should be tried.

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Predictive Value of Progenitor Assays for Time of Hematopoietic Recovery after Autologous Bone Marrow Transplantation

L. Vellekoop, G. Spitzer, S. L. Tucker, S. Tindle, and K. A. Dicke

INTRODUCTION

Autologous bone marrow transplantation after high-dose cytoreductive regimens has been employed in the treatment of leukemia and a variety of solid tumors for the last 25 years (1). Although it is still an investigational therapeutic approach to cancer, there is a need for consensus on quality control issues, especially regarding the repopulation capacity of the frozen marrow. In the past it has been shown that growth of granulocyte-macrophage colony-forming cells (CFC-GM) from bone marrow thawed at the time of transplantation after 7 days in agar with human placenta-conditioned medium (HPCM) correlated with in vivo recovery of granulocytes after piperazinedione and total body irradiation (TBI) but not with in vivo recovery of platelets and lymphocytes (2). In the studies of Abrams et al, CFC-GM growth in agar correlated with both neutrophil and platelet recovery (3). However, in Kaizer's studies of in vitro treatment of bone marrow with 4-hydroperoxycyclophosphamide, supralethal treatment of patients was prevented by bone marrow that did not show in vitro growth of CFC-GM (4). Because the cell that forms a granulocyte-macrophage colony is already committed to myeloid differentiation, CFC-GM growth may not reflect the full repopulation capacity of the marrow to be used. Therefore, we investigated whether in vitro growth in methylcellulose of mixed colonies of granulocyte-macrophage erythroid megakaryocyte colony-forming cells (CFC-GMEM), erythroid burst-forming units (BFU-E), and CFC-GM from nonpurged bone marrow after thawing correlates with time until full hematopoietic recovery.

MATERIALS AND METHODS

Twelve patients undergoing autologous transplantation for acute leukemia or lymphoma were studied. Six patients received 1.020 Gy of fractionated TBI and chemotherapy as part of their cytoreductive regimens, and six received only high-dose combination chemotherapy (Table 1). Autologous bone marrow had been harvested previously and stored in liquid nitrogen. Storage time varied from 1 month to 3 years, with a median storage time of 10 months. After autologous marrow infusion patients had daily peripheral blood counts.

At the time of autologous marrow transplantation, an aliquot of the thawed bone marrow solution was used for in vitro culture of BFU-E, CFC-GM, and CFC-GMEM. After density centrifugation on Ficoll-Hypaque (d = 1.077 g/ml), 1×10^5 of the interface cells were plated in a one-layer system with 0.8% methylcellulose, 1% bovine serum albumin, 20% fetal bovine serum, 10% phytohemagglutinin-leukocyte-conditioned medium (PHA-LCM), 1×10^{-5} M α-thioglycerol, and 1 unit erythropoietin. Colonies were scored on day 14.

Time until recovery of peripheral blood counts (total white blood cell [WBC] count, 1500/mm^3; absolute neutrophil count, 1000/mm^3; lymphocyte count, 500/mm^3; and platelet count, 50,000/mm^3) and number of colonies in vitro were correlated using the Spearman rank test. A regression model was developed to predict hematopoietic recovery time.

RESULTS

Growth of BFU-E and CFC-GM in vitro as well as hematopoietic recovery data are shown in Table 1 and their correlation coefficients in Table 2.

Table 1. Days Posttransplant until Hematological Recovery

Transplant		Time until Recovery (days)				
BFU-E x 10^8 Infused	CFC-GM x 10^5 Infused	WBC 1500/mm^3	Neutrophils 1000/mm^3	Lymphocytes 500/mm^3	Platelets 50,000/mm^3	Treatment[a]
21.8	25	11	15	11	21	CPA + TBI
12.5	92	21	23	17	23	CPA + TBI
12.4	70	39	>39	>39	>39	CPA + TBI
0.2	3.0	50	180	42	>180	MEL + TBI
0	0.4	38	38	24	38	CPA + TBI
0	0.3	>128	>128	>128	>128	CPA + TBI
15.9	54	19	19	18	16	CBV
4.3	6	36	36	28	27	AMSA + BV
2.5	18	22	35	21	26	CBV
0.6	12	35	38	24	35	CBV
0	6	46	51	52	41	CBV
0	0	32	32	40	32	CBV

[a]*CPA, cyclophosphamide; MEL, melphalan; CBV, cyclophosphamide, 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU), and VP-16-213; AMSA + BV, amsacrine (AMSA), BCNU, and VP-16-213.*

CFC-GMEM growth did not correlate with time until hematopoietic recovery, probably because of very low numbers of such colonies in our assay system. Growth of both BFU-E and CFC-GM showed negative correlations with time until recovery, BFU-E growth correlating most strongly with all hematopoietic recovery parameters. A simple model was fitted to these data, using BFU-E growth as the predictor of hematopoietic recovery. For WBCs, the equation is *ln (time)* = 3.668 - .046 (BFU-E); for neutrophils, *ln (time)* = 3.938 - .055 (BFU-E); for lymphocytes, *ln (time)* = 3.545 - .043 (BFU-E); and for platelets, *ln (time)* = 3.798 - .043 (BFU-E). Adding CFC-GM results to the equations as a second variable did not improve the accuracy of prediction significantly. The fit of the model to neutrophil recovery and platelet count based on BFU-E growth alone is shown in Table 3. Patients who died before their blood counts recovered to the levels mentioned are not included in the table.

DISCUSSION

These preliminary data show that current BFU-E growth in vitro predicts best for both neutrophil and platelet recovery, although CFC-GM growth is also correlated with time until recovery. However, according to our results, growth of CFC-GMEM from thawed bone marrow and recovery times do not correlate. The spread of the data suggests that other factors besides BFU-E and CFC-GM growth also affect the individual responses. More patients need to be studied to determine the usefulness of this model and

Table 2. Correlation Coefficients between Hematopoietic Recovery Time and CFC-GM and BFU-E Infused

Colony	WBC 1500/mm³	Neutrophils 1000/mm³	Lymphocytes 500/mm³	Platelets 50,000/mm³
CFC-GM	-.646 (.025)	-.530 (.067)	-.637 (.027)	-.471 (.103)
BFU-E	-.708 (.019)	-.617 (.041)	-.719 (.017)	-.580 (.055)

Note: P *values in parentheses.*

Table 3. Neutrophil and Platelet Count Recovery: Fit of the Model

Neutrophils (1000/mm³)		Platelets (50,000/mm³)	
Predicted Time (days)	Actual Time (days)	Predicted Time (days)	Actual Time (days)
15.5	15	22.5	16
21.4	19	17.5	21
25.8	23	26.1	23
51.3	32	40.1	26
44.7	35	37.1	27
40.8	36	44.6	32
49.7	38	43.5	35
51.3	38	44.6	38
51.3	51	44.6	41
50.8	>180		

Note: *Recovery values are indicated parenthetically.*

if there are also other variables, such as T-cell colony formation and the liquid culture system, that may have an impact on this model.

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Interstitial Pneumonia after Bone Marrow Transplantation

D. J. Winston, W. G. Ho,
R. P. Gale, and R. E. Champlin

INTRODUCTION

Interstitial pneumonia is a common and often fatal complication of bone marrow transplantation (1-5). The epidemiology, treatment, and prevention of interstitial pneumonia have been extensively studied in patients receiving either allogeneic or syngeneic marrow grafts from appropriately matched donors. Nevertheless, much of the information from these studies is also applicable to patients undergoing autologous bone marrow transplantation (ABMT).

Interstitial pneumonia occurred in approximately 30-40% of all patients who underwent allogeneic marrow transplants and in approximately 15% of the patients who had syngeneic or twin transplants (1-5). The overall mortality rate of the interstitial pneumonia was 70% in allogeneic recipients and 40% in syngeneic recipients. Most cases occurred within the initial 120 days after transplantation. Older patients (i.e., patients conditioned with total body irradiation (TBI) before transplantation), and patients with severe graft-versus-host disease (GVHD) were especially at risk for interstitial pneumonia. Cytomegalovirus (CMV) was the organism most frequently associated with interstitial pneumonia after allogeneic bone marrow transplantation and was identified in 50-70% of the cases (1-5). Most of the other pneumonias were idiopathic. In contrast, CMV was an uncommon cause of interstitial pneumonia after syngeneic bone marrow transplantation in which idiopathic interstitial pneumonitis predominated (1). These latter relationships suggested that the idiopathic pneumonias were related to pulmonary toxicity from radiation and chemotherapy and had little to do with GVHD or its treatment. *Pneumocystis carinii*, adenovirus, herpes simplex virus (HSV), and varicella-zoster virus were unusual pathogens in patients with interstitial pneumonia, especially when prophylactic trimethoprim-sulfamethoxazole was administered for the prevention of pneumocystic infections (1-5).

In ABMT treatment, the risk factors for interstitial pneumonia are somewhat analogous to those factors associated with syngeneic bone marrow transplantation (i.e., in the absence of GVHD, most interstitial pneumonias after ABMT are idiopathic and are presumably due to the pulmonary toxicity of the radiation and chemotherapy used to condition the patient for transplantation [6-11]). Indeed, two distinct clinical presentations of interstitial pneumonia occur in ABMT recipients. Within the first 3 weeks after transplantation and before engraftment occurs, patients may develop an acute interstitial pneumonitis, characterized by the sudden onset of dyspnea, tachycardia, hypoxemia, and diffused bilateral pulmonary interstitial infiltrates. These symptoms are often accompanied by some evidence of cardiac failure (7). The incidence of this acute cardiopulmonary failure depends upon the type and intensity of the radiation and chemotherapy used to prepare the patient for transplantation and varies between 0 and 50% (6-8,10). Open lung biopsy or autopsy usually fails to establish an infectious etiology, and both the pulmonary and cardiac failures are likely representations of toxic reactions to drugs and radiation. On the other hand, ABMT recipients, who experience successful marrow engraftment and survive more than 3 weeks after the transplant, may develop a late-onset interstitial pneumonia similar to allogeneic transplant recipients. This late-onset interstitial pneumonia occurs infrequently (<15% of all patients), is not associated with cardiac failure, and is idiopathic or rarely due to CMV (6-8,10). Acute cardiopulmonary toxicity following ABMT is usually fatal. Patients who develop the late-onset interstitial pneumonia may have a better survival rate.

Since CMV has been the most common pathogen isolated from the lungs and other sites of patients with interstitial pneumonia, several studies

have focused on the epidemiology of CMV infection after marrow transplantation. Both primary and reactivated CMV infections are likely to occur (2,3,5,12). Bone marrow transplant recipients usually receive an enormous number of blood transfusions that can transmit latent CMV and be a source of primary infection (13,14). Patients who have been given leukocyte transfusions are especially at increased risk for CMV infection (15,16). On the other hand, some patients have a tendency to harbor their own latent CMV, which can be reactivated by the immunosuppressive effects of the bone marrow transplant procedure. The finding that bone marrow transplant recipients can develop posttransplant CMV pneumonia and other infections caused by CMV strains that were previously present in pretransplant urine cultures is consistent with the concept that some CMV infections after marrow transplantation are caused by preexisting latent or persistent viral strains (17).

METHODS

The treatment of interstitial pneumonia after bone marrow transplantation has been, for the most part, very unrewarding (5). This negative experience may partly reflect the irreparable damage to the lungs caused by radiation, drugs, or the large quantities of pathogenic organisms present in established cases of pneumonia. For this reason, the effectiveness of any therapy may be severely limited, and efforts at prevention are likely to be more successful. Similarly, therapy should not be compromised by delays in making a specific etiologic diagnosis. Open lung biopsy is generally preferred over bronchoscopy and other diagnostic procedures that are generally not sensitive or reliable (18). Serologic tests are also unreliable for diagnosis (3,4). Recently, the applications of indirect immunofluorescence with CMV monoclonal antibodies and DNA hybridization with radiolabeled CMV DNA probes have improved the sensitivity of the diagnosis of CMV infection from pulmonary specimens (19).

Most attempts to treat interstitial pneumonia after marrow transplantation have focused on CMV infection. Unfortunately, treatment with antiviral drugs that are presently available has been ineffective. Use of adenine arabinoside, interferon, acyclovir (ACV), and combinations of these agents have all been unsuccessful in the treatment of CMV pneumonia, which continues to have a mortality rate of 80-90% (5,20-26).

A new guanosine analog, 9-(1,3-dihydroxy-2-propoxymethyl) guanine or DHPG, has recently been synthesized and has much greater potency against CMV and other herpes viruses than that of ACV (27). DHPG inhibits HSV type 1, HSV type 2, CMV, and Epstein-Barr virus replication by more than 50% at concentrations that do not inhibit cell growth in culture (27). Thus, it is anticipated that DHPG may be a more effective agent for the treatment and possible prevention of CMV pneumonia. Preliminary clinical testing of DHPG in selected high-risk patients with CMV infections is now under way. The mechanism of action of DHPG has not been totally elucidated, but it appears that the drug is phosphorylated by CMV-induced DNA polymerase (28,39).

For those few cases of interstitial pneumonia caused by *P. carinii*, trimethoprim-sulfamethoxazole is the drug of choice (30). Similarly, intravenous ACV is effective treatment for pneumonia and other infections caused by HSV or varicella-zoster virus (31,32). On the other hand, there is no therapy of proven value for patients with idiopathic interstitial pneumonia caused by the toxicity of radiation or chemotherapy. Both controlled and uncontrolled observations suggest that corticosteroids and other anti-inflammatory agents have no benefit (4,5).

RESULTS

Antiviral drug therapy, for the prevention of CMV infection and interstitial pneumonia after bone marrow transplantation, has been as unsuccessful as the treatment. In controlled clinical trials, neither

Table 1. Prophylactic Immune Plasma or Globulin
for Prevention of Cytomegalovirus Infection and Pneumonia after Bone Marrow Transplantation

Reference	Therapy	CMV Infection[a]		CMV Pneumonia[b]	
		Controls	Treated	Controls	Treated
UCLA (Winston [37])	CMV immune plasma	15/24 (63%)	12/24 (50%)	8/24 (33%)	3/24 (13%)
			(Patients not given WBC transfusions)		
		9/18 (50%)	7/17 (41%)	6/18 (33%)	0/17
Seattle (Meyers [38])	CMV immune globulin	14/32 (44%)	10/30 (33%)	3/32 (9%)	2/33 (7%)
			(Patients not given WBC transfusions)		
		8/19 (42%)	2/17 (12%)	1/19 (5%)	0/17
Memorial Sloan-Kettering (O'Reilly [39])	CMV immune globulin	10/25 (45%)	0/14	6/22 (27%)	0/14
UCLA (Winston [36])	i.v. immune globulin (Gamimune, Cutter Biologicals)	10/18 (55%)	8/18 (44%)	8/18 (44%)	3/18 (17%)

Note: *CMV, cytomegalovirus; UCLA, University of California at Los Angeles; WBC, white blood cell.*

[a]*Number of patients with CMV infection/total number of patients (%).*

[b]*Number of patients with CMV pneumonia/total number of patients (%).*

adenine arabinoside nor interferon was able to prevent CMV infection or pneumonia effectively. ACV has not been formally evaluated as a prophylactic agent for CMV infection, but clinical studies using intravenous ACV for the prevention or treatment of herpes simplex and varicella-zoster viral infections have found no ACV effect on either the incident or the severity of CMV infection (31,32). Indeed, two patients developed fatal CMV interstitial pneumonia while receiving intravenous ACV for a previous varicella-zoster infection (34). If preliminary clinical testing establishes the safety of DHPG, the prophylactic administration of this new antiviral compound may be useful.

The limitations of antiviral drug therapy in bone marrow transplant recipients have led to attempts to prevent or modify CMV infection by passive immunization with immune plasma or globulin. These attempts were also prompted by the observations that marrow recipients with either a very high pretransplant CMV complement-fixation antibody titer or an ability to respond to CMV infection with antibody synthesis have a better prognosis than patients with a low or absent pretransplant CMV complement-fixation antibody titer or those who fail to manifest an antibody response (3,4). In addition, animal studies suggest that passive immunization modifies CMV infection (35). The results from four separate controlled trials of CMV immune plasma or globulin are summarized in Table 1. In the two trials at the University of California at Los Angeles, CMV-immune plasma or intravenous immune globulin did not prevent CMV infection but modified the severity of infection and prevented interstitial pneumonia (36,37). In the Seattle and Memorial Sloan-Kettering trials, both CMV infections and interstitial pneumonia were prevented by CMV immune globulin (38,39). The combined results of these passive immunization trials suggest that CMV pneumonia can be prevented by the prophylactic administration of CMV immune plasma or globulin.

Other possible approaches for preventing CMV infection after bone marrow transplantation are the exclusive use of blood donors who are seronegative for CMV antibody and active immunization with a CMV vaccine (40,41). A substantial reduction of CMV infection occurs in CMV-seronegative neonates when only blood products from CMV-seronegative blood donors are administered (41), and a similar approach in CMV-seronegative marrow transplant recipients is recommended. However, active immunization in marrow recipients with a CMV vaccine is considered unwise because of the potential risk for reactivation of a vaccine virus.

Analyses from several bone marrow transplant centers suggested a relationship between the incident of idiopathic interstitial pneumonia caused by radiation and drug toxicity and the method of delivery of a pretransplant radiation therapy (42-44). Lowering the absorbed radiation dose to the lungs by shielding the lungs, fractionating the TBI, and decreasing the dose-rate of radiation therapy may prevent some cases of interstitial pneumonia. Improvement in the results of ABMT will also likely occur by developing chemotherapeutic and radiation regimens that have minimal toxicity to the lungs and other nonmarrow sites.

Trimethoprim-sulfamethoxazole is extremely effective for the prevention of *P. carinii* pneumonia after bone marrow transplantation (5). Intravenous or oral ACV can prevent HSV infections; however, a significant number of patients experience HSV infections after the drug has been discontinued (45,46). There is also concern that the use of prophylactic ACV may contribute to the development of ACV-resistant strains of HSV (47). Thus, ACV should not be used for routine prophylaxis, but rather should be reserved for the treatment of well-documented and clinically significant HSV infections.

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Clinical Relevance of Serial Bacteriologic Monitoring for Prediction and Diagnosis of Infection in Managing Granulocytopenia in Patients in Strict Gnotobiotic Isolation

U. Jehn and G. Ruckdeschel

INTRODUCTION

Advances in chemotherapy of malignant diseases, particularly bone marrow transplantation (BMT), have resulted in an increased number of patients whose defenses against infection are severely impaired. Infection is the major cause of morbidity and mortality in patients with granulocytopenia. Programs that might prevent infection depend on the knowledge of the type and site of infections and whether these infections arise from the endogenous flora of the patient. Colonization with certain bacterial and fungal pathogens has been linked to a high risk of subsequent infection (1-3) and thus, surveillance cultures would be clinically useful if detection of colonization reliably predicted infection. However, opinions of the predictive relevance of such cultures remain controversial (4-6).

The purpose of this study is to present our experience with microbiologic surveillance cultures from patients treated for acute leukemia using both conventional chemotherapy and BMT in a protective environment of two laminar air-flow rooms (LAFRs).

MATERIAL AND METHODS

Study Population

Twenty-three consecutive patients were admitted to the hospital for a total of 25 treatment episodes in the protective environment or LAFRs. Conventional chemotherapy was given 16 times and nine patients received allogeneic BMT. The patients' characteristics are summarized in Table 1. Most patients placed in the unit for chemotherapy were at high risk (acute nonlymphocytic leukemia [ANLL] relapse, three patients; acute lymphoblastic leukemia [ALL] relapse, two patients; T-cell ALL with large mediastinal mass, three patients; and severe infections, three patients). The average number of days per treatment episode was 61. Patients undergoing BMT were in the protective environment longer than those receiving conventional treatment. Accordingly, the number of days with granulocytopenia and the percentage of days with fever were less in the latter group.

Protective Environment

Patients were kept in single rooms for 3 days before entering the LAFRs. During that time, selective gut decontamination with nonabsorbable antibiotics was started and daily povidone-iodine baths were given and continued during treatment episodes in the LAFR. Patients received sterile foods and liquids in the LAFRs. Once in the LAFR, patients never left for special examinations. Only patients with BMT could leave since they had to be brought to the total body irradiation (TBI) facilities. TBI was performed on 1 day under aseptic conditions. Patients requiring a respirator because of respiratory failure were transferred to the intensive care unit and excluded from the study. All patients included in this series were cared for by the same medical and nursing teams.

Oral Antibiotic Regimen

The decontaminating protocol used consisted of 2×10^6 U of colistin sulfate/day plus 2.6×10^6 U of neomycin sulfate/day divided by 4 doses/

Table 1. Patient Characteristics

Characteristics	Chemotherapy	BMT	Both
Treatment episodes	16	9	25
Diagnosis			
ANLL	5	4	9
ALL	9	3	12
AA	-	2	2
Age (median)	16-55 (29)	19-35 (29)	16-55 (29)
Males/females	9:5	5:4	14:9
Days on study (mean per treatment episode)	790 (49)	744 (83)	1534 (61)
Number of granulocytopenic episodes ($<1 \times 10^9/l$)	17	12	29
Days on study with $<1 \times 10^9/l$ granulocytes	46	62	108
Percentage of days on study with $<0.5 \times 10^9/l$ granulocytes	16	24	20
Percentage of days with fever $>38.5^{\circ}C$	16	36	26
Percentage of patients with fever admitted to protected environment (LAFR)	3	2	5

Note: *BMT, bone marrow transplantation; ANLL, acute nonlymphocytic leukemia; ALL, acute lymphoblastic leukemia; AA, aplastic anemia; LAFR, laminar air-flow room.*

24 h; in addition, dosages for fungal infection included 3×10^6 U of nystatin (tablets) three times daily plus 2.4 g of amphotericin B suspension (liquid). Furthermore, mouthwashes with pyocyanine or chlorhexidine gluconate solutions were given several times a day. Only patients with BMT received 320 mg trimethoprim + 1.600 mg of sulfamethoxazole daily, in addition to the prophylaxis of infection with *Pneumocystis carinii.*

Microbiologic Surveillance

Quantitative aerobic, anaerobic, and fungal stool and urine cultures were done. Semiquantitative cultures on body orifices (nose, ear, throat, vagina, rectum) and skin (axillae, groin, perianal region, umbilicus) were done before the patient entered the LAFR and at least once a week during the course of treatment. Other bacteriologic studies, particularly blood cultures, were done if they were clinically indicated. Routine bacteriologic surveillance (twice weekly) also included several sites of the room such as the sink, floor, wall, and toilet.

Patient Evaluation

Fever was defined as a rectal temperature of $38.5^{\circ}C$ on two occasions in 1 day or lasting longer than 24 h and not being related to blood products or pyogenic drug administration. Clinical infection was defined as definite signs and symptoms of infection, whether they were microbiologi-

cally confirmed or not. Bacteriologic documentation of infection was defined as definite signs and symptoms of infection with microbiologic documentation. Bacteremia was defined as the finding of one or more blood cultures that were positive for an organism considered to represent a pathogen. Urinary tract infection was defined as urinary frequency, urgency, dysuria, and a positive urine culture (10^5 organisms/ml). Death resulting from infection was assumed when an infection was clinically or bacteriologically documented and was present prior to death with no evidence of other immediate cause of death, such as hemorrhage or organ failure.

For bacteriologic evaluation, positive cultures were considered only once for each patient and organ involved and were independent of the number of cultures investigated and the nature of the organism identified. Cultures that were microbiologically suspicious were excluded from analysis. Gram-negative rods and *Candida* species were counted in the same way. However, because of their higher infectious potential, they were evaluated without the above-mentioned qualitative criteria.

Sensitivity, specificity, predictive values, and prevalence of disease were calculated as described elsewhere (7).

RESULTS

The median age of patients in both treatment groups was 29 years (range, 16-55 years). Fourteen of these patients were men. A total of 1534 days of study was evaluated for microbiologic surveillance (average of 61 days per treatment episode) (Table 1). The total number of cultures investigated was 434, or 17 per episode. Since BMT patients stayed in the protective environment almost twice as long as those treated with conventional chemotherapy (BMT 83 days vs. chemotherapy 49 days), the total number of cultures and the number per treatment episode were accordingly higher (Table 2). Bacteremias, including those caused by gram-negative organisms, were equal in both groups. *Staphylococcus aureus* and *Pseudomonas aeruginosa* caused no serious infection. Oropharyngitis and infections of the respiratory tract were more frequent in the BMT group, probably because of the preceding TBI in which no lung shielding was employed. Four patients died of opportunistic infections after BMT (three died of *Pneumocystis carinii* pneumonia, one died of cerebral toxoplasmosis). One patient in the other treatment category died of septicemia with *Candida* despite proper antifungal prophylaxis. Airborne infections with *Aspergillus*, more effectively prevented by laminar air flow, were observed in none of the cases.

Rates of colonization and subsequent bacteremia are summarized in Table 3. The organisms responsible include enterococci, *Salmonella typhimurium*, and yeasts. Three patients developed bacteremia caused by coagulase-negative staphylococci, one by *Streptococcus pyogenes* and one by *Corynebacteria* without being colonized before bacteremia occurred. *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, although frequently found in the surveillance cultures (72%, 24%, and 24%, respectively), did not cause bacteremia. In one patient (*Salmonella typhimurium*), the first positive surveillance culture was the one immediately preceding bacteremia. The relative risk of developing bacteremia among colonized patients ranged from 0.3 to 12-fold greater than the risk for noncolonized patients.

When cultures taken from any site either before entry or during treatment in the LAFRs were considered, all organisms causing bacteremia were identified by surveillance cultures--and the sensitivity of these was high. In contrast, the specificity ranged from 13 to 21% only. The positive predictive values for enterococci and yeasts were low (5%) because few of the high percentage of colonized patients developed bacteremia. Since the only patient colonized with *Salmonella typhimurium* subsequently developed a bacteremia, the positive prediction was 100%. The negative predictive values remained nearly 100% throughout. By analyzing the duration of colonization, the rate of colonization among patients who subsequently developed bacteremia was not higher with increased time in the protective environment.

Table 2. Frequency and Sites of Documented Infections

Infections	Chemotherapy	BMT	Both
No. of cultures investigated (per treatment episode)	187 (12)	247 (30)	434 (17)
Type of infection with all organisms (number of infections per 100 days)			
Bacteremia	2 (0.3)	3 (0.4)	5 (0.3)
Respiratory tract	1 (0.1)	4 (0.5)	5 (0.3)
Oropharyngitis	2 (0.3)	4 (0.5)	6 (0.4)
Urinary tract	2 (0.3)	3 (0.4)	5 (0.3)
Gram-negative and *Candida* species (number of infections per 100 days)			
Bacteremia	1 (0.1)	--	1 (0.07)
Respiratory tract	1 (0.1)	2 (0.3)	3 (0.2)
Oropharyngitis	--	2 (0.3)	2 (0.1)
Urinary tract	1 (0.1)	3 (0.4)	4 (0.3)
Other	1 (0.1)	2 (0.3)	3 (0.2)
Number of deaths caused by infection			
Pneumocystis	0	3	3
Toxoplasmosis	0	1	1
Candida species	1	0	1

Note: *BMT, bone marrow transplantation.*

DISCUSSION

Only 37% of the patients who developed bacteremia were colonized with the infecting organism before, usually in the gastrointestinal tract, whereas in 63% of the patients with bacteremia, organisms were identified differently from those in the colonized patients. Since the incident of bacteremia was low among the high percentage of patients colonized with enterococci and yeast, specificity and the positive predictive value of a positive surveillance culture were low. In these instances, cultures failed to identify the organisms in patients who were at risk of infection. The high percentage of colonization with enterococci was not surprising since selective gut decontamination, using a combination of neomycin and colistin, preserved anaerobic rods and enterococci.

Because the pathogens that are predominantly nosocomial, i.e., *Staphylococcus aureus* and *Pseudomonas aeruginosa*, have been reported (1) to cause up to 40% of serious infections in patients with granulocytopenia, particular emphasis was placed on detecting early colonization by these bacteria. In our study, colonization occurred in 24% of the cases, which did not present any problems for serious infections. In contrast, coagulase-negative staphylococci, which are a major component of the normal skin and mucosal flora, were not found as colonizing bacteria by our survey after the decontamination procedure. Instead they caused 3/8 (38%) of the bacteremias to be observed. This is in good agreement with a recent observation (8) that coagulase-negative staphylococci are an increasing cause of infection in patients with granulocytopenia, probably because of the central venous catheters (9).

The value of fungal surveillance cultures as predictors of systemic fungal infections has been stressed (10). In our experience, however, positive surveillance data correlated poorly with disease, and the positive prediction was only 5%. Also surprising was the high rate of colonization with *Candida* species despite an antifungal prophylaxis consisting of nystatin and amphotericin B.

Table 3. Treatment Episodes (N = 25)

Organism	No. Colonized (%)	No. with Bacteremia Colonized (%)	No. with Bacteremia Not Colonized (%)	Relative Risk	Sensitivity	Specificity	Predictive Value		Prevalence of Disease
							Positive	Negative	
Staphylococcus aureus	6 (24)	-	-	-	-	.76	-	1.00	-
Staphylococci coagulase-negative[a]	-	-	3 (12)	-	-	-	-	0.89	1.00
Streptococcus pyogenes	1 (4)	-	1 (4)	-	-	.96	-	0.96	0.50
Streptococcus agalactiae	2 (8)	-	-	-	-	.92	-	1.00	-
Streptococci α-hemolytic	1 (4)	-	-	-	-	.96	-	1.00	-
Enterococci	22 (88)	1 (4)	-	0.3	1.00	.13	0.05	1.00	0.33
Corynebacteria[a]	-	-	1 (4)	-	-	-	-	0.96	0.04
Escherichia coli	18 (72)	-	-	-	-	.28	-	1.00	-
Klebsiella pneumoniae	2 (8)	-	-	-	-	.92	-	1.00	-
Proteus mirabilis	1 (4)	-	-	-	-	.96	-	1.00	-
Salmonella typhimurium	1 (4)	1 (100)	-	12.0	1.00	-	1.00	-	1.00
Pseudomonas aeruginosa	6 (24)	-	-	-	-	.76	-	1.00	-
Acinetobacter calcoaceticus	1 (4)	-	-	-	-	.96	-	1.00	-
Yeasts	20 (80)	1 (5)	-	0.5	1.00	.21	0.05	1.00	0.04

[a] *Both groups could not be detected after swabbing in direct and enrichment cultures.*

Serial bacteriologic examinations of patients with persistent fever were helpful in defining the type of infectious etiology in 50% of the cases. Although routine body surveillance cultures were rather insensitive and thus unlikely to influence antimicrobial therapy, they may be justified for patients receiving maximum supportive care. However, such cultures are costly and their value remains doubtful. Based on our experience, it seems to be more important to intensify the diagnostic survey while infections are clinically suspected rather than perform routine surveillance cultures from many different sites.

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The Use of Central Venous Catheters in Patients Undergoing Intensive Therapy for Malignancy

H. M. Lazarus and R. H. Herzig

INTRODUCTION

Right atrial Silastic catheters are commonly used in patients undergoing intensive chemotherapy and radiotherapy. The use of these catheters, however, may contribute to the infectious and hemorrhagic complications in patients with pancytopenia. Our experience has been with silicone rubber atrial (Broviac) catheters (1). These catheters could be inserted by a percutaneous puncture technique with few problems, even in pancytopenic patients. We noted, however, a change in the spectrum of infection with a preponderance of gram-positive bacteremias and fungemias (2,3). Since many neutropenic patients continue to remain febrile despite the use of broad-spectrum antibacterial agents, we undertook a randomized prospective study to determine the appropriate time for catheter removal and the usefulness of empiric administration of amphotericin B (2).

METHODS

From January 1, 1979 to June 30, 1982, 136 central venous Broviac catheters were inserted into 122 cancer patients undergoing bone marrow transplantation or intensive antitumor therapy at University Hospitals of Cleveland, Ohio. Informed written consent was obtained from all patients, and the studies were approved by the Institutional Review Board for Human Investigation.

Before beginning intensive chemotherapy and radiotherapy treatment, the central venous catheter was inserted into the patient during surgery by one of two senior surgeons. The majority of the catheters were inserted by dissection in the deltopectoral groove with direct exposure, venotomy, and cannulation of the cephalic vein. However, 46 were inserted by direct subclavian puncture through a large-bore split needle. In 33 of these patients (37 catheter insertions), the platelet count was less than or equal to 96,000/μl. Patients with platelet counts less than 50,000/μl received 6-8 units of platelets intraoperatively, just before the procedure was performed. During hospitalization, the catheter exit site was cleaned with povidone-iodine and hydrogen peroxide. Povidone-iodine ointment was then applied to the catheter site and covered with sterile gauze. Patients received divided dosages of oral nystatin suspensions and trimethoprim-sulfamethoxazole daily. The catheters were used for infusion of chemotherapeutic agents, parenteral alimentation solutions, bone marrow, other blood products, and antibiotics. Routine blood drawing was usually accomplished by peripheral venipuncture. In neutropenic patients who developed an oral temperature of >38°C, appropriate cultures were obtained and a search for the fever source was done. Then, ticarcillin disodium, tobramycin sulfate, and nafcillin sodium were intravenously and empirically infused and continued until the peripheral blood neutrophil count exceeded 500/μl. Forty-two patients entered a randomized study for an evaluation of the role of the catheter in persistent, unexplained fever. Those patients, in whom fever never occurred or in whom the source of fever was identified, were not eligible for randomization of catheter removal. Patients who had neutropenia and an unexplained fever for 5 days despite broad-spectrum antibiotics or those who became afebrile initially after empiric antibiotic therapy but later developed an unexplained fever for 5 days comprised this study group. They were randomized into two groups, one in which the catheter was left in place and the patients were treated with amphotericin B, and the other in which the catheter was removed. Both groups continued to receive broad-spectrum antibacterial agents. Patients in whom the catheter was removed were reevaluated 3 days

later; those who had become afebrile continued to receive antibiotics until the granulocyte count exceeded 500/μl but those with persistent fever received amphotericin B intravenously, in addition to antibacterial drugs. A 10-mg test dose of amphotericin B was administered, followed by 25 mg on the same or next day, then doses of 50 mg/day to a maximum of 60 mg/day at a dose schedule of 0.7-1.0 mg/kg/day. Amphotericin B was generally discontinued when the peripheral blood neutrophil count exceeded 500/μl for 2 consecutive days. All catheter tips were cultured and histologically examined upon removal at randomization, completion of therapy, or death.

RESULTS

The study population included 122 patients (67 men and 55 women) in whom 136 catheters were placed. The ages ranged from 15 years to 69 years (median, 47 years). Patients were to receive intensive chemotherapy and radiotherapy (with or without bone marrow transplantation) for malignancy.

Percutaneous Catheter Placement

Twenty-three of the 37 catheter insertions were successfully placed with the first puncture attempt. Platelet count in the 33 patients with thrombocytopenia ranged from 2000/μl to 96,000/μl, with a median of 34,000/μl. Fifty-nine percent of the procedures (22/37) took place when the platelet count was less than or equal to 30,000/μl. Of these 33 patients, 20 had a peripheral blood neutrophil count less than 500/μl.

There was no mortality related to catheter placement. Complications included three arterial punctures without sequelae and one large transient cervical hematoma. Three patients experienced minor postoperative bleeding, which was easily controlled with the transfusion of platelets.

Occlusion and Infection in Catheters

Catheter occlusion, which occurred in four patients, initially manifested as difficulty in infusing intravenous fluids. Of the four patients, two were receiving intravenous phenytoin via the catheter at the time the occlusion was noted. Erythema, drainage, and tenderness over the catheter insertion site or along the chest wall developed in 15 patients.

Ten gram-negative bacteremias were observed in the study population. Six episodes were due to *Escherichia coli* and two bacteremias with *Klebsiella pneumoniae* were observed. Bloodstream infections with gram-positive organisms occurred in 12 patients: *Staphylococcus epidermidis* in five, *S. aureus* in three, *Streptococcus viridans* in four, Corynebacteria species in two, and miliary tuberculosis in one. Fungemias occurred in eight patients with various *Candida* species.

Randomized Catheter Study

Of 42 patients (46 catheters), seven patients never became febrile, even though 6/7 had neutrophil counts less than 1000/μl. In seven other patients, the source of the fever was identified within the 5-day observation period and it included bacteremia (*S. aureus*, *Strep. viridans*, and *Corynebacterium sp.*), *Candida* fungemia (two patients), and biopsy-proven, culture-negative interstitial pneumonitis (two patients). Thirty-two patients with neutropenia and unexplained fever received empiric antibiotic therapy and were observed. Twenty-two patients became afebrile after 5 days, 12 within 24 h. Four of these 22 patients had a recrudescence of unexplained fever that lasted for 5 days, and 10 of the original 32 patients had persistent fever that also lasted for 5 days; thus, 14 patients were eligible for randomization. Six of the 14 patients had the catheter removed and eight received amphotericin B (in addition to ticarcillin, tobramycin, and nafcillin) through the indwelling central venous catheter.

Table 1. Results of Catheter Removal versus Amphotericin B Therapy and Dose of Antifungal Therapy and Fever Outcome

Group	N	Response: No. of Patients Defervesced	Response: No. of Patients Remaining Febrile	Amphotericin B Dose[a]: Until Afebrile	Amphotericin B Dose[a]: Total
Catheter removal	6	0	6[b]	35 mg, 135 mg	285 mg (135-1960 mg)
Amphotericin B (iv)	8	6	1[c]	85 mg (35-95 mg)	560 mg (285-1085 mg)

[a] *Median; range in parentheses.*

[b] *Two patients subsequently became afebrile when amphotericin B was administered intravenously.*

[c] *Persistent fever in one patient; one patient became afebrile after 2 days of amphotericin B therapy and the PMN count >500/μl.*

The results of the randomization are shown in Table 1. None of the six patients who underwent catheter removal became afebrile. In contrast, the fever resolved in six of eight patients who received amphotericin B empiric therapy with the catheter left in place (0/6 vs. 6/8, $P < .01$, Fischer's exact test). In the amphotericin B group, one patient had a persistent fever, presumably caused by leukemia; another patient became afebrile after 2 days of antifungal therapy, which was coincidental with a return of a granulocyte count greater than 500/μl.

Amphotericin B was administered until patients became afebrile. None of six patients became afebrile within 3 days after the catheter was removed--these patients received amphotericin B; two patients became afebrile after receiving 35 mg and 135 mg of amphotericin B. In the four patients who had persistent fever despite both catheter removal, and later, amphotericin B therapy, one patient's fever was apparently due to miliary tuberculosis and two patients' fevers were secondary to malignancy (malignant melanoma and chronic myelogenous leukemia, blast crises). The remaining patient became afebrile after all drugs were discontinued, which suggested that drug fever was the cause. The median total dose of amphotericin B administered to this group of patients was 285 mg, and ranged from 135 to 1960 mg.

Of the group of patients receiving antifungal treatment with the catheter in place, the median dose necessary to achieve resolution of fever was 85 mg and ranged from 35 to 95 mg. The median total dose of amphotericin B for this group was 560 mg, and ranged from 285 to 1085 mg.

Histologic evaluation of sectioned catheter tips showed fibrin deposition and stranding to varying degrees on the luminal surface of most of the catheters. Microorganisms could not be identified on luminal surfaces or within fibrin deposits.

DISCUSSION

Our experience with 136 Broviac catheters in cancer patients receiving intensive therapy was reviewed in relation to problems of catheter placement, occlusion and infection, and indications for removal. Although catheters could be inserted by direct exposure into the cephalic vein, percutaneous placement minimized the wound dissection in patients with

pancytopenia and was less time-consuming than other approaches. With appropriate precautions, including intraoperative platelet transfusions, percutaneous placement of Silastic catheters can be performed safely, even in patients with severe thrombocytopenia and neutropenia (TA Stellato, MWL Gauderer, HM Lazarus et al., unpublished observations).

Occlusion of catheters is likely to occur if a drug (e.g., phenytoin) precipitates in solution and is infused through the catheter. Catheter obstruction in other situations can be resolved with the infusion of heparinized saline solution under high pressure or the use of streptokinase through the catheter.

Local infections caused by the catheter may be manifest by erythema, drainage, or tenderness over the insertion site or chest wall. The catheter may need to be removed under these circumstances. Bacteremias and fungemias may occur from the use of catheters. In our series, 10 of 36 (27%) bacteremias or fungemias were caused by gram-negative organisms, while 17 of 36 (45%) were caused by gram-positive bacteria and 8 of 36 (24%) were caused by fungi. Because of the increase in gram-positive infections, which were apparently caused by the catheter, specific anti-staphylococcal agents should be added to the initial antibiotic regimen.

It is difficult to distinguish primary catheter infections from secondary ones. The former, however, are significantly more common and bloodstream infections can be cleared with the catheter in place with antibiotics alone. In patients with catheter occlusion, or both local and disseminated infection, the catheter should be removed and reinserted at a later time.

We undertook our randomized study to more clearly delineate the role of central venous catheters with respect to fever and infections in patients with extended immunocompromised periods. Persistent, unexplained fever in patients with neutropenia in this study was not due to the presence of the catheter, since none of the patients became afebrile after its removal, and histologic examination of the catheter did not support a causal role. The present study suggests that the catheter should not be removed; it should instead be left in place for administration of empiric amphotericin B therapy. This drug, which was very sclerosing to peripheral veins, was extremely effective therapy in our study population.

This study refutes the notion that a foreign body in a persistently febrile neutropenic host must be removed, unless local difficulties or bacteremia with skin-commensal organisms occur. The indwelling central venous catheter can be left in place for the administration of blood products, antibacterial drugs, and amphotericin B.

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Newer Antibacterial Regimens for Infections in Patients with Neutropenia

K. V. I. Rolston

INTRODUCTION

Major advances have been made in recent years in the treatment of patients with malignant diseases. These advances, including intensive chemotherapy, improved surgical and radiotherapeutic techniques, immunotherapy, and bone marrow transplantation, have produced a population of patients in whom infections are the leading cause of death. Most bacterial infections occur during periods of neutropenia and are caused by enteric gram-negative bacilli (Enterobacteriaceae, *Pseudomonas aeruginosa*). Gram-positive organisms have become prominent in recent years, the most common being coagulase-positive and -negative staphylococci, enterococci, and JK diphtheroids.

Patients with neutropenia are often unable to mount an adequate inflammatory response, and since infection can disseminate rapidly in such patients, empiric antimicrobial therapy has become standard practice for the initial management of the febrile patient with neutropenia. Empiric regimens have traditionally employed an aminoglycoside (gentamicin, amikacin) in combination with a β-lactam agent (usually the antipseudomonal penicillin, ticarcillin) to provide broad initial antibacterial coverage, but with a few notable gaps (JK diphtheroids, methicillin-resistant staphylococci). The availability of several newer classes of such antibiotics as third-generation cephalosporins, the ureidopenicillins, the β-lactamase inhibitors, and the monobactams have enabled us to devise newer regimens that are potentially more effective and less toxic than the "standard" regimens. This chapter will deal primarily with factors that influence the selection of antibacterial regimens and with experience gained at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston using several of the newer antibiotics.

FACTORS INFLUENCING ANTIBIOTIC SELECTION

Several factors influence the selection of antibiotics used in empiric regimens in patients with neutropenia. These factors include the following: antibacterial spectrum, bactericidal activity, potential for synergism, favorable pharmacokinetic properties, efficacy in patients with neutropenia, wide therapeutic index (margin between serum concentration and inhibitory concentration), toxicity and safety factors, local resistance patterns, local endemicity of particular pathogens, and cost. The regimen should provide a broad spectrum of activity against a wide variety of gram-positive (*Staphylococcus aureus*, coagulase-negative staphylococci, enterococci, corynebacteria-CDC-JK, *Bacillus* spp.) and gram-negative organisms (*Escherichia coli*; *Pseudomonas aeruginosa*; *Klebsiella*, *Enterobacter*, *Serratia* spp.; *Proteus* spp., *Acinetobacter calcoaceticus*, *Aeromonas*). A single antibiotic is not likely to provide such broad-spectrum coverage. Although, as indicated before, two drug regimens leave some gaps in coverage, three-drug regimens do not seem to increase efficacy in prospective randomized trials (1).

Bactericidal antibiotics seem to be superior to bacteriostatic agents in patients with neutropenia. This is due to the fact that neutropenic patients must rely entirely upon antibiotic therapy to eradicate the infection in the absence of an adequate inflammatory response. There is also some evidence that, in this situation, antibiotic combinations that interact synergistically are more effective clinically than combinations that are not synergistic. This was demonstrated by Klastersky et al in a retrospective study: 80% of infections were cured by synergistic combinations, whereas only 49% were cured if the combinations did not exhibit synergy (2).

Certain pharmacokinetic properties might influence the selection of antibiotics. These are protein binding, diffusion into target tissues, serum half-life, and major excretory pathway. Extremely high serum protein binding leaves less "free drug" to exert its activity. Diffusion into target tissues is particularly important in infections such as meningitis, in which the antibiotic must traverse the blood-brain barrier, and pneumonia, in which poor diffusion into the lung and bronchial secretions may produce poorer results. A long serum half-life results in therapeutic serum levels of the drug for sustained periods of time, and the major excretory pathway becomes important in renal or hepatic failure.

Certain antibiotics (β-lactams) are more effective in patients with neutropenia than others (aminoglycosides). This has led to the development of combinations of two β-lactam antibiotics instead of the traditional aminoglycoside-β-lactam duo. Aminoglycosides should certainly not be used as the only agents in the event of neutropenia.

Local factors also influence the selection of antibiotics. Certain pathogens may be endemic in a particular hospital and must be kept in mind when empiric regimens are designed. Local susceptibility-resistance patterns must also be considered carefully. Amikacin should be the aminoglycoside of choice in hospitals that have a greater frequency of gentamicin- or tobramycin-resistant isolates. The routine use of amikacin does not increase the frequency of resistance to it. Conversely, the routine use of amikacin reduces the incidence of resistance to other aminoglycosides (3). It is therefore not necessary to "save" amikacin only for resistant isolates.

A wide therapeutic margin is preferable to a narrow one. Serum levels of the new β-lactam agents are often several hundredfold higher than the inhibitory concentration. This is frequently not so with aminoglycoside therapy, in which far lower serum levels are obtained. This fact might also account for the poorer efficacy of the aminoglycoside.

Finally, toxicity and relative cost of the various antibiotics influence their selection. Cephalothin appears to potentiate the nephrotoxicity of aminoglycosides, whereas other cephalosporins apparently do not have this effect (4). All other factors being equal, the least expensive antibiotic should be employed.

SELECTED ANTIBIOTIC REGIMENS USED IN TREATMENT OF PATIENTS WITH NEUTROPENIA

Many investigators recommend the routine use of aminoglycosides in antibiotic regimens designed for patients with neutropenia (Table 1). There are, however, several problems with aminoglycoside administration. These agents have a potential for oto- and nephrotoxicity that may be increased by the administration of other therapeutic agents such as cephalosporins and cisplatin. The relative lack of efficacy in patients with neutropenia and the need for frequent monitoring of serum levels and renal function parameters also render aminoglycosides less than ideal. Prospective randomized trials comparing double β-lactam combinations with standard β-lactam-aminoglycoside combinations have shown no difference in therapeutic efficacy. A recent study at our institution compared ticarcillin plus moxalactam to tobramycin plus moxalactam. Overall cure rates of 81% and 74%, respectively, were achieved in bacteriologically documented infections, demonstrating the efficacy of the double β-lactam regimen to be equivalent, if not superior to, the β-lactam-aminoglycoside regimen (5). In general, infections with gram-negative organisms responded better than those with gram-positive organisms. The use of moxalactam was associated with an increased incidence of enterococcal superinfection and coagulation abnormalities. Other cephalosporins such as cefotaxime may not be associated with these side effects.

In another study at this institution, ceftazidime alone was as effective as ceftazidime plus tobramycin (72% each) in all documented infections. This clearly demonstrated the fact that the addition of tobramycin was not associated with an improved outcome (6). This study also suggested that monotherapy (i.e., the use of only one antibiotic) might be just as effective as combination therapy. However, both regimens

Table 1. Selected Antibiotic Combinations Used to Treat Infections in Patients with Neutropenia

Regimen (Reference)	Infections			
	Gram-Negative		Gram-Positive	
	N	% Response	*N*	% Response
Mox-Ticar	25	80	26	73
vs.				
Mox-Tobra (5)	39	82	19	53
Ceftazidime	23	92	12	41
vs.				
Ceftazidime + Tobra (6)	35	86	14	57
Vancomycin + Piperacillin	44	73	14	86
Vancomycin + Timentin	9	78	6	100
SMX-TMP + Ticarcillin (7)	23	87	-	-

Note: *Mox, moxalactam; Ticar, ticarcillin; Tobra, tobramycin; SMX-TMP, sulfamethoxazole-trimethoprim. Data on vancomycin combined with piperacillin or timentin are from unpublished reports.*

had much better response rates for gram-negative infections (92% and 86%) than for gram-positive infections (41% and 57%). Since about 20-30% of infections at M. D. Anderson Hospital are caused by gram-positive organisms, we include vancomycin in our empiric regimens.

Vancomycin in combination with piperacillin produced a cure rate of 86% in gram-positive and 73% in gram-negative infections, once again supporting the concept of monotherapy for gram-negative bacillary infections (L Jadeja, R Bolivar, W Grose, unpublished data).

One of the major mechanisms of resistance is the production of β-lactamases by gram-negative bacilli. This led to the development of timentin (ticarcillin plus clavulanic acid--a β-lactamase inhibitor) in an attempt to broaden the spectrum of ticarcillin against β-lactamase-producing isolates. An overall response rate of 85% was achieved when timentin and vancomycin were used in conjunction. Significantly, 100% of gram-positive infections were cured.

Finally, sulfamethoxazole-trimethoprim, an entirely different class of antibiotic, has been used along with ticarcillin at our institution. The results of this regimen were very encouraging, with an 88% cure rate in infections whose causative organism was identified (7).

SUMMARY

It is evident from the multiplicity of regimens discussed above that no single regimen is appropriate under all circumstances. From studies performed at this institution and at others, we can conclude that amino-

glycosides are not essential in the therapy of infections occurring during neutropenia. The question of whether synergistic combinations offer an advantage over nonsynergistic regimens is still open to debate. Studies done at our institution using only one effective agent against gram-negative bacilli and achieving cure rates comparable to regimens with potentially synergistic combinations seem to indicate that synergism may not be a major therapeutic factor. Although monotherapy seems adequate for gram-negative infections, no single agent has a broad enough spectrum to also cover adequately the gram-positive organisms that occur frequently in neutropenia. We have, therefore, incorporated vancomycin into our neutropenic regimens. The spectrum of infections might differ in other hospitals and require different strategies.

The question we must ask ourselves is whether we have achieved the maximum from antibiotic therapy. If indeed we have, then are there other modes of therapy that can help reduce mortality? Strengthening the host-defense mechanisms with the use of J-5 antiserum or hyperimmune plasma for specific pathogens or the use of monoclonal antibodies and granulocyte transfusions may provide the answer.

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Panel Discussion: Session X

W. H. Burns and L. Vellekoop, Moderators

Dr. Winston Ho: Dr. Burns, in your presentation you stated that viremia was not predictive for infections and had no effect on incidence. This is actually not what we found. We found that one could actually predict that 10 days later most of those patients who had viremia would come down with cytomegalovirus (CMV) infection and interstitial pneumonia.

Dr. William Burns: It is almost significant in our hands. John Zayer's data at City of Hope and our data (and I think Seattle's data now) show that it is not at the .05 level. It was close.

Dr. Subhash Gulati: I wanted to ask if you have major trouble getting all your donors typed for CMV. I was wondering how many institutions actually look for CMV antibody in the blood products before they are given?

Dr. Burns: We do at Hopkins. We've screened over 1,000 donors' blood products and set up a bank of people seronegative for CMV, and we give negative blood products to patients who are seronegative with seronegative donors.

Dr. William Peters: That is the tradition at the Farber as well. I would like to ask a question. At ASCO this year, Trudy Elien in one study out of London reported that acyclovir prophylaxis can, in fact, help prevent CMV pneumonia. I wonder if anyone else has been able to confirm that observation? Clearly, that carries a great deal of importance.

Dr. Ho: I would like to reply to that. I think there are two published reports of CMV pneumonias developing in patients who have been treated with acyclovir either for herpes simplex or herpes zoster infections. Therefore, this casts doubt on the potential efficacy of acyclovir in the treatment of CMV infections. One or both reports were in the *New England Journal of Medicine*, one last year and one the year before.

Dr. Burns: They were just cases, but it was only from Elaine Gluckman that I know of a study producing evidence that acyclovir is active against CMV. And actually there is a study going on that we are participating in looking at high-dose acyclovir for prophylaxis against CMV and interstitial pneumonitis.

Dr. Anton Hagenbeek: I would like to address the question of the role of radiation in the induction of interstitial pneumonitis (Fig 1). First of all, there is a significant lack of information on the effect of low-dose rate irradiation on the human lung. This is about all we can find from the literature in terms of the effect of radiation on the lungs, piled up by van Dyk et al at St. Margaret's Hospital, Toronto. It indicates the incidence of interstitial pneumonitis was related to the dose to the lung. But, mind you, these were high dose rates: between 0.50 and 4.0 Gy/min in patients treated for lung metastases or primary bronchogenic carcinoma. The LD50 is on the order of 9.20 Gy, but that is not to be extrapolated to the transplantation situation, where the dose rates employed are much over 0.04-0.10 Gy/min.

Figure 2 shows you how we think we could extrapolate from all the data available now what the role of radiation is in the etiology of idiopathic interstitial pneumonitis. Here we see curves for both rat and mice for the high-dose-rate situation (i.e., between 0.80 and 1.10 Gy/min). The curve shifts to the right if the dose rate is decreased. If you take the LD50's for both the high- and the low-dose-rate curves and simply divide them you come up with a factor of 1.7 chemotherapy to be added to total body irradiation (TBI). Now, if we apply this factor of 1.7 to the

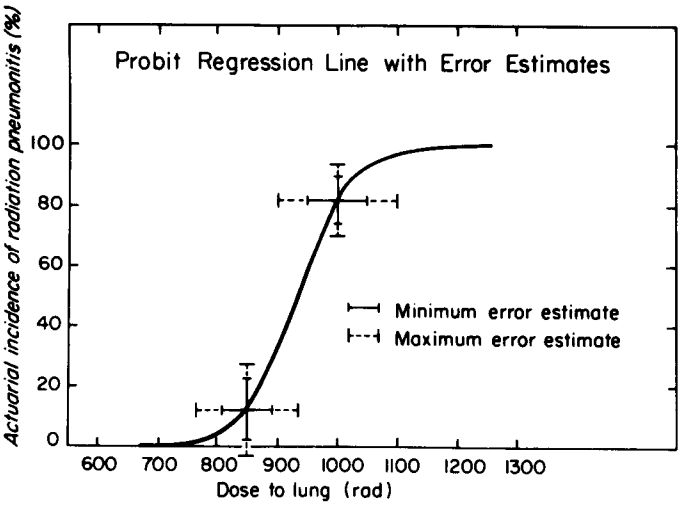
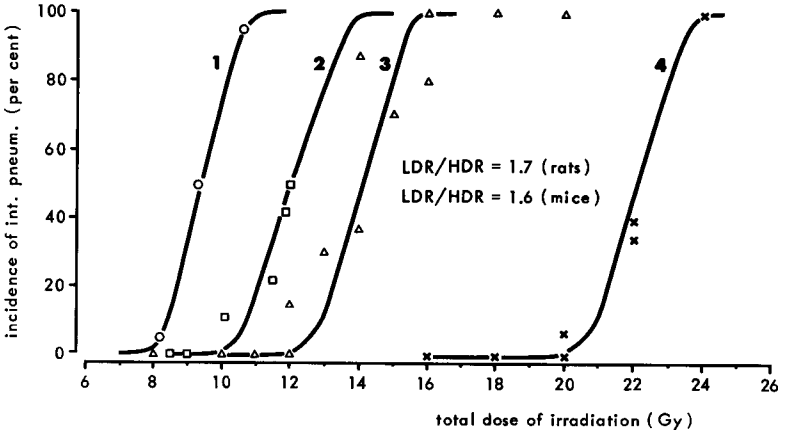


Figure 1. Incidence of radiation-induced pneumonitis. (Data from van Dyk et al, 1981.)



1. human (0.5 - 4.0 Gy/min; van Dyk et al., 1981)
2. human TBI + chemo (0.05 Gy/min; Keane et al., 1981)
3. rats + mice (0.8 - 1.1 Gy/min)
4. rats + mice (0.05 Gy/min)

Figure 2. Incidence of interstitial pneumonitis in man compared with incidence in experimental animal models. LDR, low-dose-rate irradiation; HDR, high-dose-rate irradiation; int. pneum., interstitial pneumonia.

data available for high-dose-rate irradiation, we can come up with a theoretical curve that should describe the influence of low-dose-rate irradiation alone to the incidence of interstitial pneumonitis. You see that the LD50 extrapolated for low-dose-rate irradiation is on the order of 15 or 16 Gy. That means to me that radiation alone is certainly not responsible for the induction of idiopathic interstitial pneumonitis, because the total dose to the lungs that we use is at the most 10 Gy. And that is the point I would like to make. It is also clear from Fig 3 that adding chemotherapy and a bone marrow graft with all its complications (middle curve from Keane et al, Toronto) makes the (hypothetical) low-dose irradiation curve shift to the left.

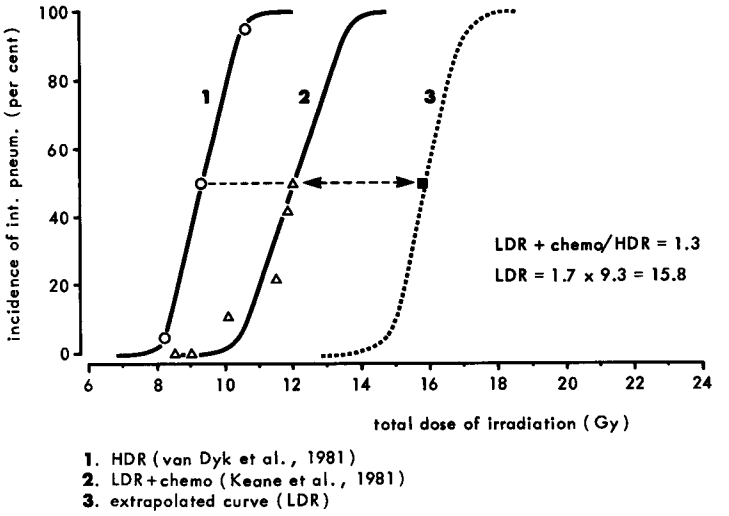


Figure 3. Incidence of interstitial pneumonitis in man after low-dose-rate lung irradiation (extrapolated from animal data). LDR, low-dose-rate irradiation; HDR, high-dose-rate irradiation; int. pneum., interstitial pneumonia.

Dr. Burns: I just want to make one comment about the radiation and CMV interstitial pneumonitis. When we used one-sitting TBI for aplastic anemia, we saw a lot more CMV interstitial pneumonitis and idiopathic interstitial pneumonitis than we see now using fractionated TBI. In fact, when we use the busulfan-cyclosphosphamide regimen instead of the cyclophosphamide-fractionated TBI regimen, the CMV interstitial pneumonitis incidence is the same. So even with a non-TBI regimen, you are still going to see a lot of CMV and interstitial pneumonitis. I just make the point that this is a multifactorial syndrome. You know we are waiting for people to explain to us the allogeneic effect and setting and its very heavy influence in this syndrome.

Dr. Joseph Fay: Since all your patients are treated with prophylactic acyclovir, have you been able to determine whether the incidence of CMV pneumonia was diminished?

Dr. Hagenbeek: The only point I wanted to make is that it seems unlikely that radiation alone is responsible for the occurrence of idiopathic interstitial pneumonitis.

Dr. Robert Gale: Since we are really talking just about radiation being a factor in the idiopathic interstitial pneumonias, which is about 10% of patients, I thought your curve actually looked quite reasonable, that is, a 10% fatality rate from interstitial pneumonia.

Dr. Pierre Biron: You mention the 10% incidence, but there are numerous reports of people indicating a much higher incidence of idiopathic interstitial pneumonitis.

Dr. Gale: No, I think if you take 100 patients who get transplants following radiation, about 10% of them will have interstitial pneumonia of undetermined etiology.

Dr. Herzig: I would like to mention our experience with interstitial pneumonitis in the allografting setting. In our study we compared patients who had 10 Gy as a single fraction and received methotrexate after transplantation with the patients that got fractionated radiation and did not receive methotrexate prophylaxis for graft-versus-host disease (GVHD).

The group of patients that had methotrexate and single-fraction radiation had a higher incidence of interstitial pneumonitis. I can't tell you which one is which, but posttransplantation methotrexate may also add to interstitial pneumonitis.

Dr. Biron: What is your position concerning diagnosis of pneumonitis by bronchial lavage vs. open lung biopsy?

Dr. Ho: I wonder if that question has been prompted by the recent report of the efficacy of lavage that came out in the *New England Journal of Medicine*? We actually have carried out various studies to try and compare the efficacy of diagnostic modalities: bronchoscopy vs. bronchoscopy with lavage vs. bronchoscopy and biopsy. The end point is that the "gold standard" is still an open lung biopsy for definitive diagnostic procedure; all the other procedures are going to give some false-negative results.

Since I have the microphone, can I make a comment? A word of caution about routine screening of your blood donors for CMV: The testing is very simple to do, but since a fair number of your donors are going to turn out to be seropositive, there is an ethical question about what you do with the blood that you have collected from those donors. Are you going to discard it? Or are you going to give it to other patients?

Dr. Burns: I'm not sure on that point.

Dr. Ho: I think, ethically, if you don't know whether the blood is seropositive or seronegative, you can give it to somebody. If you collect a unit of blood and you get your results later on, what will you do with that unit? If you are going to discard it, you are going to discard a lot of blood that you have collected.

Dr. Burns: We don't have the luxury of discarding blood, so we have to give it.

XI. Summary

Combined Intensive Alkylating Agents with Autologous Bone Marrow Transplantation for Metastatic Solid Tumors

E. Frei III

The effectiveness of bone marrow transplantation in the treatment of leukemia and lymphoma has been extensively studied with allogeneic bone marrow transplantation and, more recently, with autologous bone marrow transplantation (ABMT). Dr. William Peters of the Dana-Farber Cancer Institute has presented our initial studies involving ABMT for patients with solid tumors, and I will limit my discussion to that area. The therapeutic treatment of solid tumors in general has lagged very substantially behind those of the hematologic malignancies. In contrast to the hematologic malignancies, such advanced approaches as those involving bone marrow transplantation have been extraordinarily limited in solid tumor therapeutics.

Bone marrow transplantation represents an extraordinary effort for the patient and for medical personnel and material resources. It may be beset with considerable morbidity and some mortality; therefore, a program, for example, in the solid tumor area is not justifiable in my view unless a case can be made that treatment with curative intent is deliverable--not necessarily in the first study, but in projected studies. The synthesis of several recent experimental studies and clinical studies provides a conceptual framework and hypothesis, in my judgment, for curative-intent treatment employing autologous bone marrow rescue in patients with solid tumors. This is as follows.

Perhaps my discussion can best be constructed around an analogy to a successful curative program, that is, the MOPP (mechlorethamine, vincristine, procarbazine, and prednisone) program for Hodgkin's disease. Combination chemotherapy is the name of the game if one is to achieve a major therapeutic effect and indeed a curative effect. All highly effective treatment, in fact, with chemotherapy involves combinations for a variety of reasons, including particularly the increasing and compelling evidence for tumor cell heterogeneity. One axiom of combination chemotherapy is that the agents have qualitatively different mechanisms of action. Perhaps the most important biologic expression of this, in addition to clinical studies, relates to cross-resistance patterns. If one produces resistance to drug A in vivo or in vitro and there is cross-resistance to B, a similar mechanism of action, or at least of resistance, is assumed, and combination therapy is not rational. If there is no cross-resistance, then combination therapy may follow. Inasmuch as the MOPP regimen includes qualitatively different structures and mechanisms and almost certainly mechanisms of resistance, this criterion applies. It is well known that the alkylating agents are heterogeneous, but it has long been assumed, from the time that alkylating agents were initially classed under the rubric of *radiomimetic*, that their mechanisms of action and resistance were common. This dogma was thoroughly undermined by studies by Frank Schabel, who demonstrated that for both L1210 and P388 leukemia in mice, the production of resistance to one alkylating agent was not in the main associated with cross-resistance to the others. Indeed, after multiple studies, he derived the generalization that cross-resistance among alkylating agents was the exception rather than the rule. Most studies of the mechanism of transport, intracellular metabolism, DNA binding, cross-linking, repair processes, and other similar processes of the alkylating agents in recent years support the position that cross-resistance would be the exception. Following Schabel's observations 3 years ago, we initiated studies of human cell lines in vitro and demonstrated also that the production of resistance to one clinically important alkylating agent was in the main not associated with cross-resistance to the others. These studies provide a rationale for the use of alkylating agents in combination, and indeed Dr. Schabel demonstrated that synergism for combinations of alkylating agents commonly occurs.

Let us go back to the MOPP program. A second principle embodied in the MOPP program and of combination chemotherapy generally is that dose, wherever possible, should not be sacrificed. This is possible for programs such as the MOPP program, wherein dose-limiting toxicity is qualitatively different. It does not apply to the alkylating agents employed in combination, except in a setting of bone marrow rescue. This is because dose-limiting toxicity for the alkylating agents is myelosuppression, and anywhere from a three-fold to a 10-fold increase, depending upon the alkylating agent, in a setting of autologous marrow rescue can be accomplished before nonmyelosuppressive toxicity becomes dose-limiting. This is in contrast to many other antitumor agents (for example, methotrexate, 6-mercaptopurine, Adriamycin [doxorubicin]) wherein dose-limiting bowel and other toxicity occurs at about the same level as myelosuppression.

A third principle of combination chemotherapy is that the agents must be individually effective against the disease in question. The individual components of the MOPP program produce partial responses in 50-60% of patients with Hodgkin's disease, responses that are slow in onset and of relatively brief duration. The alkylating agents for solid tumors in conventional doses are far less potent. However, in intensive treatment with a single alkylating agent with autologous bone marrow rescue, substantial effectiveness has been achieved. The most definitive information with respect to case numbers relates to melanoma. Melanoma is marginally sensitive to alkylating agents at standard doses. However, it has been adequately confirmed that metastatic melanoma treated with either melphalan or 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU) at substantially higher doses in the ABMT setting will produce tumor regression in 40-70% of patients. The figures are higher for patients with no former treatment and good performance status, and on the lower side for the remainder. It is not known what cyclophosphamide in transplant doses would accomplish in metastatic melanoma, but let us assume that a substantial (upward of 50%) tumor regression would occur. In short, individual alkylating agents in transplant doses produce response rates in melanoma comparable to those achieved by the MOPP components in Hodgkin's disease. It is highly probable that even higher response rates would be achieved with individual alkylating agents at transplant doses in tumors more sensitive to alkylating agents, tumors such as metastatic breast cancer, metastatic ovarian cancer, and small cell lung cancer.

The next issue returns to dose. Is it possible that the individual alkylating agents at transplant doses could be employed in combination without significant compromise in dose? For other combination regimens in the nontransplant setting, this is possible when dose-limiting toxicities are qualitatively dissimilar. For four prototype alkylating agents, nonmyelosuppressive dose-limiting toxicity occurs primarily in the heart for cyclophosphamide, in the gastrointestinal tract for phenylalanine mustard, the lung for BCNU, and the kidney for cisplatin. To be sure, other toxicities may occur. Nevertheless, the primary and specific hypothesis addressed by Dr. Peters in his combined alkylating agent study was whether or not in this phase I study full doses, or nearly full doses, of three to four of the aforementioned agents in fact could be delivered. While the answer as yet is incompletely known because the study is in progress, it would appear that for at least three of the agents, full or nearly full transplant doses can be achieved.

If the above hypothesis or objective can, in fact, be achieved, it is reasonable, in my judgment to project the potential of combined intensive alkylating agents with autologous bone marrow protecting programs that might be curative for diseases such as metastatic melanoma and perhaps particularly for metastatic breast cancer. Parallel research must include more appropriate alkylating agents (for example, carboplatin in place of cisplatin); the possible introduction of cytotoxic hexatols, such as dibromodulcitol, busulfan, and perhaps other agents (in the phase II studies on patients with no prior treatment and a relatively limited tumor burden), should enhance the effectiveness of the program. Preclinical studies should emphasize the nature of the dose-response curve when carried up to very high doses, models for evaluating interactive toxicology and therapeutic synergism, and finally the potential modulation of alkylating agent effect with agents that influence DNA repair or other

aspects of alkylating agent effect, including polyamine synthesis inhibitors; 3-aminobenzoic acid; nicotinamide analogs; and more direct inhibitors of DNA repair, such as ara-C and methylxanthines.

Summary Keynote Address

E. J Freireich

The proceedings of this symposium have impressed me--in both the extent of accomplishment, as reflected in the data presented, and the broad interests of the medical community in this particular field of clinical research. It seems clear that the field of autologous bone marrow transplantation has enormous potential for extending the therapeutic range of cancer treatments that are now available. As I listened to the impressive data presented during this meeting, I recalled my earliest experiences in clinical cancer research when, in the early 1950's, I was caring for children with acute leukemia at the National Cancer Institute in Bethesda, Maryland. In that era, the chemotherapy that we had to offer such patients produced complete, but temporary, remissions of the disease in 25-35% of the patients treated. The major cause of morbidity and mortality, attendant to the chemotherapy, was clinically visible hemorrhage. One of the earliest clinical investigative efforts that I undertook was an approach to control this dreaded complication. This led to the early work with transfusion of fresh allogeneic platelets from healthy volunteer donors, which after a number of technical breakthroughs, became a standard of treatment for patients with bone marrow failure. Certainly, no one would undertake the treatment of patients with major myelosuppression from whatever cause without the availability of fresh platelets from allogeneic volunteer donors to control the hemorrhagic diathesis associated with thrombocytopenia.

The progress in platelet replacement transfusion soon called attention to the importance of infectious complications. The crucial role of the leukocyte in host defense to infection was evident. The major approach to infection control has been and continues to be the discovery of new potent antimicrobial agents and progress in antimicrobial chemotherapy has been not only rapid, but continuous. Effective infection control is also a major component of the treatment of any patient with myelosuppressive disorders. I mention these experiences only to recall that it was obvious that the control of morbidity with platelet transfusions, with antibiotics, and even with leukocyte transfusions (for their limited effectiveness) improved the therapeutic outcome for those patients undergoing chemotherapy with myelosuppressive dose-limiting toxicity.

The introduction of combination chemotherapy and the discovery of new chemotherapeutic agents have rapidly improved the situation with childhood leukemia--to the point at which approximately 50% of such patients can be cured of that disease. What was an initially palliative "supportive care" soon became an essential integrated part in the effective application of treatment that is curative for leukemia. In the case of adult acute leukemia, dominantly myeloblastic leukemia, treatment was essentially palliative and supportive prior to 1967. Over the last 17 years, we have seen the volution of two major breakthroughs in the treatment of adult leukemia. One breakthrough was the new combination chemotherapy regimens that can result in the cure of between 15 and 20% of all adults, and the second was the important work with allogeneic bone marrow transplantation, which demonstrated that approximately one-half of the patients treated in first remission can be rendered essentially cured of their disease. For both of these treatment modalities, supportive treatment (i.e., in the form of management of the hemorrhagic and infectious complications attendant to myelosuppression) is of fundamental importance to success.

Replacement transfusion with platelets, the use of antibiotics, and even the additive usefulness of leukocyte transfusion are all treatment modalities that were highly effective in the short run, but became progressively less effective for reasons that included isosensitization and resistance of microbial organs to chosen treatments. In general, our supportive treatment modalities are progressively less successful the longer we have to maintain patients without evidence of bone marrow recovery. To me, that is where the autologous bone marrow program becomes

so attractive and important. Autologous transplantation shares the requirement for effective treatment of complications during the 2-4-week period with allogeneic transplantation, while the injected stem cells repopulate the marrow and generate formed elements in the peripheral blood. However, the great power of the autologous transplant is the limited time of myelosuppression.

The methods of support for the management of thrombopenia and leukopenia are in an area of active and important clinical investigation. Regardless of what progress is made in that field, the identification of a technique that allows the regular recovery of marrow function to be based on autologous stem cells is an attractive method for extending the therapeutic range of cancer treatments that are limited by myelosuppression. Thus, autologous marrow transplantation is an extended and complimentary form of supportive therapy for myelosuppressive toxicity. To date, it is not in itself therapeutic for the malignancy, but it does allow the clinical scientist to significantly extend the range of agents that are limited by myelosuppressive toxicity. Since most of the chemotherapeutic agents that have been identified as effective in cancer therapy have steep dose-response curves, the extension of the tolerated dose by any device will almost certainly improve the effectiveness of treatment. As has already been conclusively demonstrated for allogeneic bone marrow transplantation, treatments such as cyclophosphamide and total body irradiation which have not in themselves been demonstrated to be active against adult acute leukemia can, when supported with allogeneic bone marrow transplantation, become curative treatments.

A great virtue of the autologous bone marrow procedure is that it avoids the incompatibility between graft and host and the complications, which are referred to in the transplantation literature as "graft-versus-host disease." In exchange, we have the difficult problem of separating tumor cells that contaminate bone marrow from the blood product used to salvage our patients. But even here--the exciting potential for treating the bone marrow in vitro with doses and schedules of drugs that could not be achieved in the patient offers an exciting vista for improving therapeutic effectiveness of this modality of treatment.

One can almost never talk about the future without alluding to the monoclonal antibodies and hoping that, while they may not detect unique tumor-specific antigens, there will be sufficient heterogeneity between tumor cells and the essential bone marrow stem cells to allow significant gains in therapeutic benefit. Thus, the autologous bone marrow transplantation field provides, in addition to the potential for extending the tolerable dose of agents that would otherwise be less effective or not effective at all, the potential for in vitro therapy in an effort to improve the therapeutic index. As the techniques for collection and preservation improve, it may even be possible to conduct multiple therapy efforts with autologous bone marrow. These efforts would be based on either the collection of adequate numbers of stem cells to permit several courses of different treatments in vitro and in vivo or the potential collection of adequate numbers of stem cells from patients who had a previous autologous marrow transplant that effectively repopulated to the level of which we could collect an adequate transplantation dose for subsequent treatment.

Another important view for this field is the possibility of integrating the autologous bone marrow procedure with a multimodal treatment plan. Several investigations that attempt to use intensive therapy with autologous marrow to accomplish substantial palliation and other already-established modalities of treatment are under way. One such treatment is induction chemotherapy, which is intended to improve the outcome that potentially leads to curative regimens.

As clearly revealed by important summary papers during the symposium, these clinical studies must be conducted against a background with a clear understanding of the natural history of the disease under investigation. In the case of adult acute leukemia, it is now demonstrated that the risk of patients developing recurrent leukemia while they are in complete remission diminishes progressively the longer a patient is in remission after completing his first year of remission. As a result, if we are to study intensification regimens in patients in remission, we must be care-

ful to be aware of and informed about the probability of the patient already being cured, who is otherwise free of disease and at risk of relapse.

A major contribution to this field is represented by the use of models. Particularly useful are logistic regression models, which are based on factors present both at diagnosis and during the disease-free state. The objective and quantitative analysis of these data allows clear identification of the groups at risk. Therefore, it becomes possible to offer a high-risk, yet high potential for, benefit procedure, such as autologous bone marrow to patients who are at the highest risk of recurrent disease. As an example, in adult acute leukemia the morphologic cytogenetic studies contribute fundamentally to prognostic information about the disease. Patients with hematologic findings of acute leukemia and the Philadelphia chromosome abnormality are a group whose disease has a poor prognosis for remission induction and a poor duration of remission once in remission. Similarly, patients who have minus five and minus seven chromosome deletions often associated with antecedent hematologic disturbances or previous therapy for malignancy are a group whose disease has an extremely poor prognosis. Clearly, innovative procedures in these patients offer the maximum potential for benefit, particularly if they achieve cytogenetically normal or diploid complete hematologic remissions after remission induction therapy. In contrast, patients who have the 8-21 translocation or an inversion 16 abnormality are extremely favorable for both remission induction and duration of remission and, therefore, patients for which only well-established procedures need to be studied.

In summary, the use of autologous marrow as a modality of supportive treatment for systemic chemotherapy of malignancy is an important area for clinical research. While the usefulness remains to be clearly and objectively defined, there is no doubt that a great deal will be learned about the treatment of malignancy as a result of this important advance. At the same time, it is important to conduct such studies in a manner that maximizes the benefit/risk ratio for each patient who participates in the studies, and this requires extensive knowledge of the natural history of the diseases that we are treating with quantitative objective staging to allow the early quantitative studies (i.e., both phase I and phase II) to be conducted in a way that we pay attention to the potential for benefit, as well as the potential for risk. I certainly feel privileged to have heard all the important clinical research data that have been presented during these 2 days and I am confident that the second international symposium will reveal progress in this promising field.

Final Remarks

D. W. van Bekkum

I have the honor to be the last one to say a few words here. When Karel Dicke invited me to this meeting, I was not very enthusiastic because my personal interest is more in graft-versus-host disease (GVHD) following allogeneic bone marrow transplantation. This meeting was on autologous bone marrow transplantation (ABMT), which has the advantage of not causing GVHD. But there is always something to learn.

The revival of ABMT started about 7 or 8 years ago when Karel Dicke moved himself to Houston. We were at that time disappointed to find that all the fantastic tricks that we had developed to prevent GVHD in mice did not work as well in humans. Ten years ago we solved all the problems of allogeneic bone marrow transplantation in rodents. Few of these tricks worked in monkeys and really none of them in man. At least, that is what most people thought if they looked at the data from three, four, or, at best, 10 patients. However, as the data accumulated and series grew to, let us say, 50 or 60 patients, then it appeared gradually that most of the rules that emerged from work with rodents and monkeys did indeed apply to human beings as well. Man is just another animal species.

The reason that we began to promote ABMT was that something else has to be thought of when sufficient progress cannot be made. If GVHD cannot be beaten in allogeneic bone marrow transplantation, then autologous bone marrow might help somewhat since it does not cause that problem. ABMT had been tried, of course, many, many years before, but it had never been pursued so much because of the enthusiasm about the possibilities of allogeneic bone marrow transplantation. The autologous bone marrow boom that we have witnessed in the past few days at this meeting would probably not have developed if Karel Dicke had not found Holland too small a country for his activities. By moving to Houston, he found himself working under Dr. Emil J Freireich. Dr. Freireich would not allow, of course, treatments like allogeneic bone marrow transplantation. He believes in supportive care, as you have heard, and autologous bone marrow is just another form of supportive care. I am sure if all of these conditions had not prevailed and if the fantastic facilities at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston had not existed that this rapid progress in ABMT would not have taken place.

Much has been reported during this meeting on the subject of purging. All of you who are farmers on the side, as many people in Texas are, will know that the better the quality of the harvest is, the less purging is needed. Therefore, my suggestion to this meeting is the following: if you have access to the laboratory, try and grow pure pluripotent stem cells from autologous marrow. Already, 100% pure pluripotent stem cells can be isolated from mouse and rat bone marrow. Similar methodology should be developed for human marrow and for the growth factors required to produce multiplication. Those factors are in the process of being identified. By this approach, leukemic clonogenic cells will be automatically lost.

Having shown the way to avoid the difficult problems of purging, I find it is my pleasure to thank the organizers on behalf of all participants in this inspiring and rewarding meeting. We owe Karel Dicke, Gary Spitzer, and Axel Zander for generating the idea to have this meeting and for their efforts in making it such a pleasant and successful event. Obviously, these excellent scientists could not have achieved all this without the skilled assistance of Joanne Taylor and Debbie Rees. We thank you all for this remarkable and timely symposium.

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